
By

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Thesis submitted to the University of Limerick in fulfilment of the requirements of Doctoral of Philosophy
November 2011
Abstract

Antisense technology is a nucleic acid-based approach capable of down regulating the expression of specific genes. Several antisense drugs have or will shortly gain approval for medical use. Phosphorothioate oligonucleotides (PS oligonucleotides), in which one of the non-bridging oxygen atoms of the internucleotide phosphodiester linkage is replaced by a sulfur atom is one of the most extensively used backbone modifications of antisense oligonucleotides. Therefore, they are the focus of this study. Their unique manufacturing routes and bioactivity raises hitherto, unaddressed environmental issues. The manufacture and use of antisense oligonucleotides at industrial scale could result in its unintentional release to waste streams and hence into the environment. This research aims to determine the environmental issues associated with the production lifecycle of an antisense drug and to develop effective treatment processes to degrade/remove antisense drugs from specific manufacturing waste streams.

The composition of waste streams generated from each process step of the synthesis was identified and simulated using SuperPro Designer® 6.0 flow sheets. This study highlighted that large quantities of chemical wastes, most specifically, significant volumes of acetonitrile (2037 L) and toluene (1018 L) are produced during the synthesis of crude 20-mer PS oligonucleotide at 1.5 kg scale. It is predominantly during downstream processing, in particular purification, that there is a potential for loss of synthetic nucleic acid material during which it is estimated that upwards of 50% of synthetic oligonucleotide (750 g) is lost to waste streams.

In this study, the main strategies adapted for the degradation and removal of antisense oligonucleotides include physical heat treatment, acidic and basic pH at ambient room temperature and in combination with elevated temperature (Chapter Three), chemical treatment with soft metal ions (silver nitrate) and oxidising agents (iodine, potassium permanganate, potassium dichromate, sodium hypochlorite, peracetic acid, hydrogen peroxide and Virkon®) (Chapter Four) and enzymatic treatment with commercially available nucleases (Chapter Five). Analysis was undertaken both qualitatively and quantitatively using Polyacrylamide Gel Electrophoresis and Ion-Pair Reversed-Phase chromatography.

Based upon the concentration of reagents required, treatment duration, economic considerations and potential environmental impacts, six optimum treatments were chosen. These include treatment with low pH (HCl, 0.5 M) at 40°C, low pH (H₂SO₄, 0.5 M) at 40°C, iodine (25 mM), potassium dichromate (0.125 M), nuclease P1 (5 U/ml) and steam sterilisation for their efficacy in a simulated waste stream environment. Steam sterilisation was ineffective at degrading the PS oligonucleotides in the waste stream solution. Treatment with nuclease P1 was the most effective treatment process degrading the PS oligonucleotide by 96.9%. The next most effective treatment method was low pH with HCl at 40°C, low pH with H₂SO₄ at 40°C, acidic potassium dichromate and iodine degrading the PS oligonucleotide in simulated waste stream conditions by 92.9, 92.4, 88.3 and 86.7%, respectively. The application of low pH with HCl at 40°C or nuclease P1 presented as the most effective treatment methods of degrading the PS oligonucleotides in the simulated purification waste stream and demonstrated their suitability as the most technically feasible and eco-friendly methods of treatment. These methodologies may potentially be used to treat PS oligonucleotide containing waste streams, rendering them free of active drug product.
Declarations

I hereby declare that this work is the result of my own investigations and that this report has not been submitted in this form or any other form to this or any other university in candidature for a higher degree.

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Author: Carol Robinson

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Dated: November 2011
Acknowledgements

My sincere thanks and gratitude to my wonderful supervisors, Prof. Gary Walsh and Dr. Tom O’Dwyer for their time, continuous guidance, support and encouragement throughout this project.

I would like to thank the EPA, Ireland for funding this research. This work is funded by the EPA Doctoral Scholarship Program under the auspices of the National Development Plan (NDP).

I wish to express my gratitude to leading researchers in this field, Dr. Serge Beaucage (FDA), Dr. Rosel Kretschmer-Kazemi Far and Prof. Dr. Georg Sczakiel (Universität zu Lübeck), Jon Baker, Dr. Jamil Shanagar and Dr. Peter Guterstam (GE Healthcare), Dr Richard Hogrefe (TriLink Biotechnologies, Inc.), Dr, Anthony Scozzari and Dr. Daniel Capaldi (Isis Pharmaceuticals Inc.), Dr. Yogesh Sanghvi (Rasayan Inc.) and Dr. Nanda Sinha for their help and guidance in clarifying important details of our research.

I would also like to thank the staff of the CES department for their continuous help and assistance throughout this project.

I wish to express special gratitude to my friends and the members of our research group, Angela, Kevin, Finola, Jayne, Madlen, Mary and Michael for their continuous support, advice and encouragement.

To my family and friends for their loyal friendship and support throughout this project.

I would like to thank my Granny for her encouragement, phone calls, prayers and belief in me.

And finally I wish to express a special gratitude to my wonderful Mum (third supervisor), Dad and my sister Elaine, for their continuous support and encouragement. Thank you so much for all your help and patience especially towards the end. I really appreciate everything you do for me.
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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>AIDS</td>
<td>Acquired Immune Deficiency Syndrome</td>
</tr>
<tr>
<td>AP</td>
<td>Apurinic / apyrimidinic site</td>
</tr>
<tr>
<td>API</td>
<td>Active Pharmaceutical Ingredient</td>
</tr>
<tr>
<td>AX</td>
<td>Anion exchange chromatography</td>
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<tr>
<td>bp</td>
<td>Base Pair</td>
</tr>
<tr>
<td>CD</td>
<td>Circular Dichroism</td>
</tr>
<tr>
<td>CGE</td>
<td>Capillary Gel Electrophoresis</td>
</tr>
<tr>
<td>CMV</td>
<td>Cytomegalovirus</td>
</tr>
<tr>
<td>CpG</td>
<td>Cytidine-phospho-guanosine</td>
</tr>
<tr>
<td>cGMP</td>
<td>Current Good Manufacturing Practices</td>
</tr>
<tr>
<td>DCI</td>
<td>4,5-dicyanoimidazole</td>
</tr>
<tr>
<td>DMTr</td>
<td>4,4’-dimethoxytrityl</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>dsDNA</td>
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</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EPA</td>
<td>Environmental Protection Agency</td>
</tr>
<tr>
<td>ESI</td>
<td>Electrospray Ionisation</td>
</tr>
<tr>
<td>ES-MS</td>
<td>Electrospray Mass Spectrometry</td>
</tr>
<tr>
<td>FDA</td>
<td>Food and Drug Administration</td>
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<tr>
<td>GC</td>
<td>Gas Chromatography</td>
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<tr>
<td>HGT</td>
<td>Horizontal gene transfer</td>
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<tr>
<td>HIC</td>
<td>Hydrophobic interaction chromatography</td>
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<tr>
<td>HIV</td>
<td>Human immunodeficiency virus</td>
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<tr>
<td>HPLC</td>
<td>High performance liquid chromatography</td>
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<tr>
<td>ICH</td>
<td>The International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use</td>
</tr>
<tr>
<td>IPPC</td>
<td>Integrated Pollution Prevention and Control (IPPC)</td>
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<tr>
<td>IP-RP LC</td>
<td>Ion-Pair Reversed Phase Liquid Chromatography</td>
</tr>
<tr>
<td>LAL test</td>
<td>Limulus Amebocyte Lysate test</td>
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<tr>
<td>LNA</td>
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<tr>
<td>LOD</td>
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<tr>
<td>LOQ</td>
<td>Limit of Quantitation</td>
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<tr>
<td>MALDI/TOF</td>
<td>Matrix Assisted Laser Desorption Ionisation /Time of Flight</td>
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<tr>
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<td>NMR</td>
<td>Nuclear magnetic resonance</td>
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<tr>
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<td>Oligodeoxynucleotide</td>
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PAA  Polyacrylamide
PADS  Phenylacetyldisulfide
PAGE  Polyacrylamide gel electrophoresis
PNA  Peptide nucleic acid
PO  Phosphodiester
PS  Phosphorothioate
PS2  Phosphorodithioate
QA  Quality Assurance
QC  Quality Control
RNA  Ribonucleic acid
RNAi  Ribonucleic acid interference
RNase H  Ribonuclease H
RP  Reversed phase chromatography
RT  Room temperature
SAX  Strong Anion Exchange
siRNA  Small interfering ribonucleic acids
ss  Single stranded
SVPD  Snake venom phosphodiesterase
SSD  Sample Self Displacement
TCA  Trichloroacetic acid
TEA  Triethylamine
TEAA  Triethylammonium acetate
TFO  Triplex forming oligonucleotides
Tm  Melting temperature
tRNA  Transfer ribonucleic acid
UV-vis  Ultraviolet-visible
WHO  World Health Organisation

**Units**

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</tr>
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<td>ml</td>
<td>10⁻³ litres</td>
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<tr>
<td>μl</td>
<td>10⁻⁶ litres</td>
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<td>M</td>
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**Nitrogenous base**

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<tr>
<td>T</td>
<td>Thymine</td>
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<tr>
<td>U</td>
<td>Uracil</td>
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Chapter One: Introduction
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1.1 Introduction

Nucleic acid-based biopharmaceuticals have the potential to revolutionise the medical and pharmaceutical industry in the near future (Walsh, 2007). The sequencing of the human genome and the elucidation of numerous molecular pathways, that are central in disease, have provided unprecedented opportunities for the development of new therapeutics (Opalinska and Gewirtz, 2002). However, despite the widely acclaimed anticipation and excitement of these recent developments, only three nucleic acid-based products have gained worldwide approval to date. These include, one antisense-based product, Vitravene™, one gene therapy product, Gendicine (approved only in China), and one aptamer, Macugen®. Nevertheless, this is a relatively new era in biopharmaceuticals. The first nucleic acid-based drugs initial trials began over a decade ago (Walsh, 2007).

The application of synthetic antisense oligonucleotides, as therapeutic agents, represents a new paradigm in drug discovery and development (Srivatsa et al. 2000; Sanghvi, 2000). Throughout the past decade substantial work has been undertaken in the development of antisense technology (Crooke, 2004a). Antisense technology is now becoming more accepted and recognised and there is now a greater appreciation and understanding about the basic mechanism, the medical chemistry, the pharmacokinetics, pharmacological, and toxicological properties of antisense molecules. A variety of medical conditions are associated with the inappropriate production or over-production of gene products. Antisense technology is a nucleic acid-based approach capable of down regulating expression of specific disease-related genes, which offers the potential to be the cutting edge in biopharmaceuticals (Walsh, 2007). The intervention into disease states at the level of gene expression may potentially make drugs based on antisense techniques considerably more specific and efficient than alternative standard therapies (Walton et al. 2000).

This chapter summarises the literature review that was undertaken as part of the research for this thesis. The concept, application and development of antisense technology from its conception up to present day will be discussed. First generation phosphorothioate (PS) oligonucleotides will be focused on in greater detail and their chemical structure, biophysical properties, synthesis and applications in molecular biology discussed. The unique manufacturing routes and bioactivity of antisense oligonucleotides raises unanswered
environmental issues. The potential risks associated with waste streams generated from the large-scale production needs to be taken into consideration, most specifically issues pertaining to oligonucleotide related products of these wastes. This chapter will be concluded by outlining the potential environmental impacts associated with the large-scale production lifecycle of an antisense drug and technical methodologies to degrade/remove antisense drugs from waste streams to prevent the release of potentially active antisense product into the environment.

1.2 General introduction to nucleic acids

The nucleic acids, deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) are the molecular repositories and fundamental carriers of genetic information in all living organisms. They are necessary biopolymers in the transmission, expression and conservation of the genetic information. The structure of each protein, and ultimately of every biomolecule and cellular component, is a product of information encoded on the nucleotide sequence of a cell’s nucleic acids. A fragment of a DNA molecule that contains the information necessary for the synthesis of a functional biological product, whether protein or RNA, is referred to as a gene (Nelson and Cox, 2005). In order to maintain its function each cell must express thousands of genes simultaneously (Schlingensiepen and Schlingensiepen, 1997). DNA acts mainly as the carrier of genetic information in the nucleus while RNA transports the genetic information from the nucleus to the ribosome, where it is processed and translated into protein. As such, for expression of a gene to occur, the gene is transcribed from DNA into messenger RNA (mRNA), (one form of RNA, which functions as a working copy of the genetic material and transfers the information from DNA in the nucleus to the site of protein synthesis) (Brownie and Kernohan, 1999), and further translated into its corresponding protein. This flow of genetic information constitutes the central dogma of molecular biology, as outlined in figure 1.1, originally proposed by Francis Crick (Hecht, 1996).
The production of specific proteins, however, can be suppressed by means of antisense technology. Antisense technology is a nucleic acid-based approach capable of down-regulating the expression of specific disease-related genes (Walsh, 2007). This technology is based upon the principle of generating short, unmodified or chemically modified single-stranded stretches of nucleic acids (12 to 25 nucleotides in length) which may be either DNA or RNA based, (Walsh, 2007), complimentary, or antisense, to a target sequence (Hogrefe, 2009a; Walton et al. 2000; Bennett et al. 2004) designed to hybridize with specific mRNA strands, thus selectively inhibiting the production of specific gene products. This technology has been investigated to target expression of genes that are essential for malignant transformation and other pathogenetic mechanisms (Wei et al. 2008).
1.2.1 The composition and structure of a nucleic acid

The primary structure of nucleic acids (DNA and RNA) comprises of a linear array of monomers called nucleotides, which are covalently linked by a negatively charged phosphodiester backbone (figure 1.2).

Figure 1.2 Diagram illustrating the structure of DNA with the sequence AGTCG (i).

Also included is the structure of the 5 nitrogenous bases, Adenine (A), guanine (G), cytosine (C), thymine (T) and uracil (U) (ii), and the two pentose sugars, Deoxyribose (in DNA) and Ribose in (RNA) (iii).

Each nucleotide is composed of three components, a nitrogenous base (nucleobase), a five-membered pentose sugar, and a negatively charged phosphate group. The nitrogenous bases are derivatives of two parent compounds, purines and pyrimidines. Both DNA and RNA contain two major purine bases, adenine (A) and guanine (G), which have two fused heteroatom rings, and two major pyrimidines, cytosine (C) and thymine (T) in DNA and cytosine (C) and uracil (U) in RNA, which contain only one heteroatom six-member ring (figure 1.2). Nucleic acids have two forms of pentoses. A 2’-deoxy-β-D-ribose (in DNA) or β-D-ribose (in RNA) sugar ring attached to a heterocyclic aromatic nucleobase through an N-glycosidic bond. The successive nucleotides of both DNA and RNA are covalently linked via the two oxygens in the bridging positions of the phosphate group in which the 5’-hydroxyl group of one nucleotide unit is joined to the 3’-hydroxyl group of the consecutive
nucleotide resulting in a phosphodiester bond/linkage. Thus, the covalent backbone structure of nucleic acids comprise of alternating phosphate and pentose residues, with the nitrogenous bases joined to the backbone at regular intervals (figure 1.2) (Nelson and Cox, 2005; Hecht, 1996; Saenger, 1984; Jonas et al. 2000). Whereas the sugar moiety and the sugar-phosphate backbone of DNA/RNA predominantly play a structural role, the genetic information is determined by the sequence of nucleobase moieties. The resultant polynucleotide chain exhibits a primary structure of DNA and RNA with a high degree of similarity. The main difference between DNA and RNA is the absence of the 2’-hydroxyl in DNA (2’-β-D-deoxyribofuranosyl) which occurs in RNA (2’-β-D-ribofuranosyl). This subtle change in the sugar produces a noticeably diverse function of these two molecules (Saenger, 1984).

Within the cell, RNA generally exists as single strands whereas DNA predominantly exists in a double helix structure in which two DNA strands are held together by the hydrogen bonding between the nucleobases and by π-π stacking interactions that take place between the aromatic planes of the nucleobase units (Hecht, 1996; Saenger, 1984). The hydrogen bonding takes place between laterally opposite bases, ‘base pairs’ of the two strands of the DNA duplex in accordance with Watson-Crick base-pairing rules (A specifically binds to T and G specifically binds to C) (Watson and Crick, 1953) as presented in figure 1.3. In general GC- pairs, linked by three hydrogen bonds, are stronger than AT-pairs linked by two hydrogen bonds.
Figure 1.3 Base pairing interactions formed during Watson-Crick hybridization.
The hydrogen bonds formed in accordance with Watson-Crick base-pairing rules are illustrated on the left side of the figure (Bennett and Swayze, 2010).

1.3 Nucleic acid based drugs (NABDs)
The sequencing of the human genome and the elucidation of numerous molecular pathways, that are central in disease, have provided unprecedented opportunities for the development of new therapeutics (Opalinska and Gewirtz, 2002). An innovative and evolving class of molecules with potential therapeutic value is denoted by “nucleic acid based drugs” (NABDs). The high affinity and specificity of base pairing has made nucleic acids attractive agents for therapeutic and diagnostic applications. These molecules include antisense oligonucleotides, aptamers, ribozymes, DNAzymes, small interfering RNAs (siRNAs), decoy oligodeoxynucleotides, triplex forming oligonucleotides (TFOs) and immunostimulatory cytidine-phospho-guanosine (CpG) oligonucleotides (Aboul-fadl, 2006; Fattal and Bochot, 2006; Isaka et al. 2008; Buchini and Leumann, 2003; Opalinska and Gewirtz, 2002; Patil et al. 2005; Tachikawa and Briggs, 2006; Bouchard et al. 2010; Bhindi et al. 2007; Thiel and Giangrande, 2009; Robinson, 2004; Schlingensiepen and Schlingensiepen, 1997). The biological activity of NABDs is based on different mechanisms
of action but, all of them, have the ability to recognise, in a sequence-specific manner, a specific target that can either be a nucleic acid or a protein. Based on its mechanism of action, the NABDs can induce the digestion of an RNA, the transcriptional inhibition of a DNA sequence or the activity alteration of a target protein. Since NABDs can, in principle, be engineered to attack almost any cellular target, it is possible to specifically down-modulate, indirectly or directly, the functions of proteins which have undesired effects on the cell. The NABDs can therefore find application in numerous human diseases including cardiovascular diseases and cancer caused or exacerbated by the inappropriate expression of one or more genes. Among the aforementioned NABDs, it appears that antisense oligonucleotides are the most advanced clinically (Opalinska and Gewirtz, 2002) and hence form the sole focus of this thesis.

### 1.4 Antisense oligonucleotides as therapeutic agents

In 1977, Paterson and co-workers were the first to discover that exogenous, single-stranded nucleic acids inhibit translation of complementary RNA in cell-free systems (Paterson et al. 1977; Gewirtz, 2000; Aboul-fadl, 2006). In 1978, Zamecnik and Stephenson demonstrated the concept of an antisense drug by using a short 13-mer DNA to inhibit the viral replication of the Rous sarcoma virus in vitro (Zamecnik and Stephenson, 1978; Stephenson and Zamecnik, 1978; Gewirtz, 2000; Ahuja, 2000; Walton et al. 2000; Kurreck, 2008) which showed the antisense approach for gene silencing when antisense oligonucleotide acts at the mRNA level via Watson-Crick hybridization, thus preventing its translation into protein (Kurreck, 2003). Since then numerous studies have concentrated on synthesising oligonucleotides that are designed to specifically inhibit production of unwanted genetic material to alleviate bacterial, viral, and cancerous diseases (Uhlmann and Peyman, 1990; Micklefield, 2001).

The receptor for antisense-based drugs is RNA (Bennett and Swayze, 2010). The mRNA strand is the “sense” strand by definition, as its nucleotide sequence conveys the encoded blueprint which determines the subsequent amino acid sequence of a protein (Walsh, 2007). Any complementary sequence to this is said to be “antisense” which has led to this class of therapeutics being named antisense oligonucleotides. Antisense oligonucleotides are designed to bind/hybridize to a specific region of a target RNA via Watson-Crick base pairing, forming a duplex and this principle forms the fundamental basis in which antisense oligonucleotides specifically interact and bind to their target mRNA to bring about their
therapeutic effect (figure 1.4). The formation of this duplex modulates its function through a variety of post binding events resulting in inhibition of the expression of pathogenic genes (Kanazaki et al. 2000; Aboul-Fadl, 2006; Bennett and Swayze, 2010).

![Figure 1.4 Schematic diagram of how an antisense oligonucleotide can prevent the synthesis of a gene product by blocking translation.](image)

This process occurs in most instances in the nucleus of the cell (adapted from Walsh, 2007).

Antisense molecules can selectively inhibit the expression of one gene among the 30,000 that are present in a normal human cell (Walton et al. 2000; U.S. Department of Energy Genome Programs, 2008). Knowing segments of the nucleotide sequences of the mRNA inevitably makes it potentially feasible to synthesise an antisense oligonucleotide, (Walsh, 2007) designed to bind to a specific region of a target mRNA (Bennett and Swayze, 2010) and bring about its therapeutic effect.
Chapter One

1.4.1 Advantages of antisense oligonucleotides over traditional pharmaceuticals

Drug discovery attempts have traditionally focused on the search for compounds that modulate the protein products of genes. At present, the vast majority of drugs available either act at protein level, or the drugs themselves are in fact proteins. These compounds are generally agonists or antagonists of receptors, or they inhibit or stimulate enzymes or protein–protein interactions. Rather than targeting the protein itself, if one can target DNA or mRNA by an oligonucleotide complementary to the target sequence, it would be possible to stop the protein production (Scherer and Rossi, 2003). Since the discovery that oligonucleotides can down regulate gene expression, antisense technology has progressed as a new, powerful method of rational drug design (Aboul-fadl, 2006) with numerous advantages over traditional small molecule drugs (Table 1.1), which mainly target nongene disease mediators (Isis Pharmaceuticals, Inc., 2009).

Table 1.1 List of advantages of antisense therapeutics over traditional small molecule drugs.
Table compiled with information obtained from De Palma, 2002; Aboul-fadl, 2006; Schlingensiepen and Schlingensiepen, 1997 Isis Pharmaceuticals, Inc., 2009; Walsh, 2007; Corey, 2007; Murata et al. 2003.

<table>
<thead>
<tr>
<th>List of advantages of antisense therapeutics over traditional small molecule drugs</th>
</tr>
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<tbody>
<tr>
<td>▪ One of the most appealing qualities of antisense technology is its specificity. Antisense by definition, can target any given gene/gene product irrespective of the protein structure. Antisense therapy operates at the genetic level to prevent overactive or mutated genes from directing the synthesis of disease-related proteins. Conventional drugs, in contrast, operate based on the shape of proteins and charge interactions thus creating a greater opportunity for unfavourable interactions and often undesirable side effects.</td>
</tr>
<tr>
<td>▪ Antisense therapeutics target a disease at its genetic origin and modulate expression of the gene product while conventional pharmaceuticals simply counteract the symptoms of the disease by inhibiting the protein product of the malfunctioning gene.</td>
</tr>
<tr>
<td>▪ Antisense technology is broadly applicable. They can be aimed at a broader range of appreciable targets.</td>
</tr>
<tr>
<td>▪ Antisense technology offers a relatively straightforward process of drug design as opposed to traditional drugs, in that their chemistry is constant (homologus backbone and the four bases A, G, C and T), with only minor modifications of the drug’s nucleotides necessary to create a drug that is target specific. This allows developers to apply their knowledge about one drug to various other drugs. In contrast to this, the structure of each classical drug is unique, the shape, chemistry and size must be adapted. As a direct consequence testing an average of up to 10,000 substances producing one or two lead compounds for further development is necessary.</td>
</tr>
<tr>
<td>▪ Antisense drugs can be created at greater speeds, with fewer resources and at lower costs, “1 to 2 weeks for antisense compared with 6 to 24 months for small molecule drugs”.</td>
</tr>
<tr>
<td>▪ To date, reports from most clinical trials have confirmed relatively low toxicity and few significant side effects.</td>
</tr>
</tbody>
</table>

Consequently, nucleic acid based therapy presents a very simple and straight-forward means to down-regulate any gene of interest.
1.4.2 Advancements in antisense technology – clinical trials to date

Substantial development in antisense science and manufacturing has led to the FDA approval of the first antisense drug, Fomivirsen sodium (commercialised as Vitravene™), a 21 base pair phosphorothioate oligonucleotide, for the treatment of AIDS-related Cytomegalovirus (CMV) retinitis (Aboul-fadl, 2006; de Smet et al. 1999). To date, there are greater than 70 antisense oligonucleotides currently being evaluated in preclinical and clinical studies for a wide range of diseases including cancer, viral diseases, cardiovascular disease, inflammatory disorders, diabetes, respiratory problems and infectious diseases (Sepp-Lorenzino and Ruddy, 2008; Wraight and White, 2001; Wacheck and Zangemeister-Wittke, 2006; Xie et al. 2005; Pirollo et al. 2003; Wei et al. 2008; Jasti et al. 2000). Antisense products could also prove beneficial in the treatment of other disorders such as rheumatoid arthritis, restenosis and allergic disorders, for which blocking of gene expression would have a beneficial effect (Walsh, 2007). Cancer is the major target of on-going clinical trials followed by viral diseases such as hepatitis and AIDS (Tamm, 2006; Walsh, 2007). Moreover, greater than 30 pharmaceutical and biotechnology companies have declared an interest in, or have an active drug development programme under way in antisense therapeutics (Jain, 2000; Aboul-Fadl, 2006; Hogrefe, 2009a). Additionally, Glaser (2010) states that the number of oligonucleotide therapeutic programs has increased each year, continuing an upward trend from 2003, increasing to 231 in 2009, denoting a greater than 8% increase from 2008. A table detailing the majority of antisense oligonucleotides in clinical trials is presented in table 1.2.

Table 1.2 Examples of antisense oligonucleotide drugs marketed or in clinical trials

*2'-MOE chimera, 2'-methoxyethyl-DNA chimeric oligonucleotides with phosphorothioate linkages; 2'-O-Me chimera, 2'-O-methyl-DNA chimeric oligonucleotide with phosphorothioate linkages; LNA chimera, locked nucleic acid–DNA chimera with phosphorothioate linkages.

<table>
<thead>
<tr>
<th>Drug</th>
<th>Indication</th>
<th>Target</th>
<th>Mechanism</th>
<th>Chemistry*</th>
<th>Route</th>
<th>Status</th>
</tr>
</thead>
<tbody>
<tr>
<td>Formivirsen</td>
<td>Cytomegalovirus</td>
<td>IE2 gene</td>
<td>RNase H</td>
<td>PS ODN</td>
<td>Intravitreal</td>
<td>Approved</td>
</tr>
<tr>
<td>Oblimersen</td>
<td>Oncology</td>
<td>Bcl-2</td>
<td>RNase H</td>
<td>PS ODN</td>
<td>Systemic</td>
<td>Phase 3</td>
</tr>
<tr>
<td>Mipomersen</td>
<td>Cardiovascular</td>
<td>Apolipoprotein B</td>
<td>RNase H</td>
<td>2'-MOE chimera</td>
<td>Systemic</td>
<td>Phase 3</td>
</tr>
<tr>
<td>Trabedersen</td>
<td>Oncology-glioblastoma</td>
<td>Transforming growth factor-β</td>
<td>RNase H</td>
<td>PS ODN</td>
<td>Intratumoral</td>
<td>Phase 3</td>
</tr>
<tr>
<td>GS-101</td>
<td>Corneal neovascularization</td>
<td>Insulin receptor substrate-1</td>
<td>RNase H</td>
<td>PS ODN</td>
<td>Topical</td>
<td>Phase 3</td>
</tr>
<tr>
<td>LOR-2040</td>
<td>Oncology</td>
<td>Ribonucleotide reductase</td>
<td>RNase H</td>
<td>PS ODN</td>
<td>Systemic</td>
<td>Phase 2</td>
</tr>
<tr>
<td>Archexin</td>
<td>Arthritis</td>
<td>AKT-1</td>
<td>RNase H</td>
<td>PS ODN</td>
<td>Systemic</td>
<td>Phase 2</td>
</tr>
<tr>
<td>TPI ASM8</td>
<td>Asthma</td>
<td>CCR3 and IL-5 receptors (two oligos)</td>
<td>RNase H</td>
<td>PS ODN</td>
<td>Inhaled</td>
<td>Phase 2</td>
</tr>
<tr>
<td>Alicaforsen</td>
<td>Colitis</td>
<td>Intercellular adhesion molecule-1</td>
<td>RNase H</td>
<td>PS ODN</td>
<td>Enema</td>
<td>Phase 2</td>
</tr>
<tr>
<td>Custirsen</td>
<td>Oncology</td>
<td>Clusterin</td>
<td>RNase H</td>
<td>2'-MOE chimera</td>
<td>Systemic</td>
<td>Phase 2</td>
</tr>
<tr>
<td>Reference</td>
<td>Disease System</td>
<td>Gene/Protein</td>
<td>Technology</td>
<td>Phase</td>
<td></td>
<td></td>
</tr>
<tr>
<td>-----------</td>
<td>----------------</td>
<td>-------------</td>
<td>------------</td>
<td>-------</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LY2181308</td>
<td>Oncology</td>
<td>Survivin</td>
<td>RNase H</td>
<td>2'-MOE chimera</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AEG355156</td>
<td>Oncology</td>
<td>X-IAP</td>
<td>RNase H</td>
<td>2'-O-Me chimera</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TV/ATL1102</td>
<td>Multiple sclerosis</td>
<td>CD49D</td>
<td>RNase H</td>
<td>2'-MOE chimera</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ISIS 1113715</td>
<td>Multiple sclerosis</td>
<td>Protein tyrosine phosphatase-iB</td>
<td>RNase H</td>
<td>2'-MOE chimera</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Monarsen</td>
<td>Myasthenia gravis</td>
<td>Acetylcholine esterase</td>
<td>RNase H</td>
<td>2'-O-Me chimera</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ALNRSV-01</td>
<td>Respiratory syncytial virus</td>
<td>Nucleocapsid N gene</td>
<td>siRNA</td>
<td>dsRNA (modified)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PF-4523655</td>
<td>Age-related macular degeneration</td>
<td>DNA-damage-inducible transcript (REDD-1, RTP801)</td>
<td>siRNA</td>
<td>dsRNA (modified)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AVI-4126</td>
<td>Restenosis</td>
<td>c-Myc</td>
<td>Translation inhibition</td>
<td>Morpholino</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PRO-051</td>
<td>Duchene muscular dystrophy</td>
<td>Dystrophin</td>
<td>Splicing modulation</td>
<td>Drug-eluting stent</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GRN163L</td>
<td>Oncology</td>
<td>Telomerase</td>
<td>RNA binding</td>
<td>Lipid-conjugated phosphoramide</td>
<td></td>
<td></td>
</tr>
<tr>
<td>OGX-427</td>
<td>Oncology</td>
<td>Heat shock protein 27</td>
<td>RNase H</td>
<td>2'-MOE chimera</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LY2275796</td>
<td>Oncology</td>
<td>eIF-4E</td>
<td>RNase H</td>
<td>2'-MOE chimera</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AIR 645</td>
<td>Asthma</td>
<td>Interleukin receptor alpha</td>
<td>RNase H</td>
<td>2'-MOE chimera</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ISIS-CRPRx</td>
<td>Cardiovascular/ inflammation</td>
<td>C-reactive protein</td>
<td>RNase H</td>
<td>2'-MOE chimera</td>
<td></td>
<td></td>
</tr>
<tr>
<td>OMGP-GCGRRx</td>
<td>Diabetes</td>
<td>Glucagon receptor</td>
<td>RNase H</td>
<td>2'-MOE chimera</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ISIS-SGLT2Rx</td>
<td>Diabetes</td>
<td>Sodium-dependent glucose transporter 2</td>
<td>RNase H</td>
<td>2'-MOE chimera</td>
<td></td>
<td></td>
</tr>
<tr>
<td>iCO-007</td>
<td>Macular degeneration</td>
<td>C-Raf kinase</td>
<td>RNase H</td>
<td>2'-MOE chimera</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SPC2996</td>
<td>Oncology</td>
<td>Bcl-2</td>
<td>RNase H</td>
<td>LNA chimera</td>
<td></td>
<td></td>
</tr>
<tr>
<td>EZN2968</td>
<td>Oncology</td>
<td>Hypoxia inducing factor 1-α</td>
<td>RNase H</td>
<td>LNA chimera</td>
<td></td>
<td></td>
</tr>
<tr>
<td>EZN3042</td>
<td>Oncology</td>
<td>Survivin p53</td>
<td>RNase H</td>
<td>LNA chimera</td>
<td></td>
<td></td>
</tr>
<tr>
<td>QP1-1102</td>
<td>Acute kidney injury</td>
<td>Vascular endothelial growth factor</td>
<td>siRNA</td>
<td>dsRNA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ALN-VSP</td>
<td>Oncology</td>
<td>Vascular endothelial growth factor</td>
<td>siRNA</td>
<td>Modified dsRNA in liposome formulation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CALAA-01</td>
<td>Oncology</td>
<td>Ribonucleotide reductase</td>
<td>siRNA</td>
<td>dsRNA in nanoparticulate formulation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AVI-4658</td>
<td>Duchene muscular dystrophy</td>
<td>Dystrophin</td>
<td>Splicing</td>
<td>Morpholino</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SPC3649</td>
<td>Hepatitis C virus</td>
<td>microRNA 122</td>
<td>RNA blocking</td>
<td>LNA chimera</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

As can be seen from the table of selected antisense oligonucleotides the majority of the current drugs in clinical trials (table 1.2) incorporate the PS modification.
1.4.3 Mechanisms of action of antisense drugs

All antisense mechanisms demonstrate the binding of the oligonucleotide to the targeted RNA. What occurs after the oligonucleotide binds to the RNA is determined by the chemistry design of the oligonucleotide, where in the cell the oligonucleotide binds to the RNA, where on the RNA the oligonucleotide binds and auxiliary factors associated with RNA (Bennett and Swayze, 2010). When an antisense drug binds to its target mRNA, the mRNA is degraded and as a result is not translated into its functional protein (Isis Pharmaceuticals, Inc., 2009). However, they can inhibit mRNA function through one of a number of mechanisms of action, as illustrated in figure 1.5 up to four main mechanisms according to Schlingensiepen and Schlingensiepen (1997), or at least twelve mechanisms according to Isis Pharmaceuticals, Inc., 2009. Isis Pharmaceuticals, Inc., (2009) defines an antisense mechanism as “the process in which an antisense drug works after it binds (hybridises) to a target RNA to form a duplex”. These mechanisms include translational arrest by steric hindrance of ribosomal activity, interference with mRNA maturation by inhibiting splicing and destabilization of pre-mRNA which occurs in the nucleus and activation of RNase H (Chan et al. 2006).

**Figure 1.5 Mechanisms of action of antisense oligonucleotides** (Chan et al. 2006).

In the absence of antisense oligonucleotides, normal gene and protein expression occurs (1). The antisense oligonucleotide is taken up by cellular endocytosis and can hybridize with target mRNA in the cytoplasm. Formation of an antisense oligonucleotide–mRNA heteroduplex induces (2) activation of RNase H, resulting in selective degradation of bound mRNA or (3) steric interference of ribosomal assembly. These actions lead to target protein knockdown. Alternatively, the antisense oligonucleotide can enter the nucleus and regulate mRNA maturation by (4) inhibition of 5’ cap formation, (5) inhibition of mRNA splicing and (6) activation of RNase H. Theoretically, the antisense oligonucleotide can selectively knock down any target gene and protein expression bringing about its therapeutic effect.

ASO = Antisense Oligonucleotide
At present, it appears that the most used, important and validated mechanism by which antisense technology down-regulates gene expression is the use of naturally occurring endogenous RNase H enzymes by antisense oligonucleotides. Oligonucleotides that work through an RNase H–dependant cleavage mechanism are the best recognised class of antisense oligonucleotides, representing the majority of drugs in development to date (table 1.2) (Tamm, 2006; Behlke et al. 2005; Bennett and Swayne, 2010). On entering the cell nucleus the oligonucleotide binds to the target mRNA forming a sense-antisense duplex. The formation of this duplex initiates the recruitment of the endogenous nuclease, RNase H enzyme, which degrades the target mRNA resulting in the inhibition of target mRNA expression. RNase H, is a ubiquitous endonuclease enzyme that specifically degrades the RNA strand of an RNA-DNA duplex (Crooke, 2004a; Behlke et al. 2005). Antisense inhibition based on RNase H activation, therefore, offers a clear advantage with regards efficiency and dosage. This is because once the RNA target is cleaved by RNase H (permanent inactivation of the message) the antisense oligonucleotide is free to move on and can subsequently find other copies of the target. Therefore, using minimum amount of antisense oligonucleotide, maximum efficiency can be attained (Crooke, 2004b).

![RNase H ANTISENSE MECHANISM](Figure 1.6 RNase H antisense mechanism (Crooke, 2004a).)

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1.4.4 Design and target selection of antisense oligonucleotides

Successful binding of antisense oligonucleotides to its complementary mRNA strand does not solely rely on Watson and Crick base pairing. The stability and strength of interactions between antisense oligonucleotides and the complementary target mRNA is dependent on other influences such as the secondary structure of the target mRNA transcript, thermodynamic stability and the proximity of the hybridisation site to functional motifs on the designated transcript, such as the 5’ cap region or translational start site (Chan et al. 2006). Therefore, at least four parameters must be considered when designing antisense oligonucleotides so as to increase the ‘hit rate’. These include, (i) prediction of the secondary structure of the RNA, (ii) detection of favourable RNA secondary local structures, (iii) motifs searching and GC content calculation and finally (iv) binding energy ($\Delta G^\circ$) prediction (Chan et al. 2006).

The specificity of oligonucleotides is predominantly determined by the length (i.e. the number of bases) of the oligonucleotide. The length of an oligonucleotide is generally denoted by "mer" (from Greek *meros*, "part"), e.g. an oligonucleotide of 25 bases would be referred to as 25-mer. Theoretical and experimental information indicates that there is an optimum length at which specific inhibition of gene expression is maximised and nonspecific effects are minimised (Walton et al. 2000). The specificity of the antisense approach is based on the probability that any sequence longer than a minimal number of nucleotides of 13 for RNA and 17 for DNA occurs only once within the human genome (Tamm, 2006). Schlingensiepen and Schlingensiepen (1997) state that it has been calculated that the minimum length of an oligonucleotide has to be 12 bases to recognise a single specific sequence in the genome. Oligonucleotides that are too short do not inhibit gene expression, as they do not bind with adequate affinity to their substrates. In mammalian cells the shortest oligonucleotide documented to affect gene expression was 7 bases in length. An oligonucleotide that is too short is less likely to represent a unique sequence in a given cell’s genome and therefore more likely to bind to a non-targeted RNA and thus inhibit its expression (Walton et al. 2000). Therefore, 14 to 25-mer oligonucleotides offer optimal specific hybridisation properties, depending on the chemistry and base composition. Increasing the length of the oligonucleotide may bring about decreased specificity but increased toxicity by arresting different genes. Research has shown that antisense molecules greater than 30 bases trigger a genetic cascade of cellular antiviral response. Target selection
is also influenced by chemical modifications and base composition (Fraser and Wahlestedt, 1995; Schlingensiepen and Schlingensiepen, 1997; Walton et al. 2000).

### 1.4.5 Modifications of antisense oligonucleotides

Phosphodiester oligonucleotides were the first described antisense oligonucleotides (Vidal et al. 2005). Although these natural oligonucleotides are easy to synthesise, bind and activate RNase H, their therapeutic value is limited. They have many shortcomings such as, low bioavailability, instability against cellular nucleases and insufficient membrane penetration (Urban and Noe, 2003; Vidal et al. 2005), with a half-life of 15 minutes in serum (Walton et al. 2000; Monia et al. 1996; Walsh, 2007). All the above mentioned limitations and most specifically instability against cellular nucleases have warranted the chemical modification of oligonucleotides for the suitable use in therapeutic applications (Kandimalla et al. 1997; Cummins et al. 1995).

One approach to overcome these challenges is the development of chemically modified oligonucleotides to improve the efficacy of antisense therapeutics (Urban and Noe, 2003). Oligonucleotides as potential drugs have to fulfil certain criteria. Ideally, modified oligonucleotides should display increased stability and resistance to nucleases, activate RNase H activity, increase target affinity and biological potency, not disrupt normal Watson-Crick base pairing, control biodistribution (including intracellular uptake), not include any unanticipated sequence-independent biological effects, have favourable pharmacokinetic properties, not be physiologically toxic and facilitate ease of synthesis (Behlke et al. 2005; Wilson and Keefe, 2006). A dimer of an oligonucleotide displaying subunits that may be modified to enhance oligonucleotide drug properties is illustrated in figure 1.7.
Figure 1.7 The various approaches which can be taken to chemically modify an oligonucleotide, thus generating suitable and effective antisense molecules.

These modifications include subtle as well as not too subtle changes to either the oligonucleotide backbone, the sugar moiety and or the nitrogenous bases (adapted from Urban and Noe, 2003).

As stated previously, in naturally occurring nucleic acids, these subunits consist of nucleobases, carbohydrate sugars and phosphodiester-based linkages all of which can be chemically modified. Chemically modified oligonucleotides have proven to overcome limitations of the original native/phosphodiester oligonucleotide. This was effectively demonstrated by the development of the first and subsequent generations of antisense oligonucleotides that have significantly improved the properties of previous antisense oligonucleotides (Vidal et al. 2005). For the purpose of this study first generation backbone modifications (PS oligonucleotides) will be outlined in detail. Examples of additional significant modifications, beyond the scope of this thesis, are detailed in table 1.3.
Table 1.3 Structural and chemical properties of various candidate compounds for potential use as antisense oligonucleotides.
Table compiled with information obtained from Chan et al. 2006; Opalinska and Gewirtz, 2002; Tamm, 2006; Crooke, 2004a; Urban and Noe, 2003; Vidal et al. 2005; Malik and Roy, 2008; Walton et al. 2000; Kurreck et al. 2002; Kurreck, 2008.

<table>
<thead>
<tr>
<th>Antisense oligonucleotide modification</th>
<th>Structure / group modified</th>
<th>Nuclease resistance</th>
<th>RNase H Activation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phosphodiester (PO)</td>
<td>Unmodified natural phosphodiester linkage (Natural DNA)</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>Phosphorothioate (PS)</td>
<td>Replacement of one of the non-bridging oxygen atoms in the phosphate group with a sulfur.</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Methylphosphonate (MP)</td>
<td>Replacement of one of the non-bridging oxygen atoms in the phosphate group with a methyl group.</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>N3’–P5’ Phosphoramidite</td>
<td>Replacement of the 3’-oxygen in the deoxyribose ring with a 3’-amino group.</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>Alkyl-modified oligonucleotides</td>
<td>2’modifications on the ribose ring</td>
<td>Yes</td>
<td>No</td>
</tr>
</tbody>
</table>
PNAs (peptide nucleic acids)
Phosphodiester backbone is replaced with a flexible pseudopeptide polymer (N-(2-aminoethyl)glycine) and nucleobases are attached to the backbone by methylene carbonyl linkage.

LNAs (locked nucleic acids)
A conformationally restricted nucleotide which contains a 2′-O, 4′-C-methylene bridge in the β-D-ribofuranosyl configuration.

PMOs (Phosphoroamidate morpholino oligomer)
Ribose sugar is substituted by a six-membered morpholino ring and the phosphodiester bond is substituted by a phosphoroamidate linkage.

1.4.5.1 Backbone modified antisense oligonucleotides (First generation)
Due to the inherent instability of the phosphodiester linkage to nucleases, the oligonucleotide backbone (where the nucleolytic attack occurs) poses an obvious first target for improvement which prompted the development of first generation oligonucleotides which are chemical
derivatives of the phosphodiester backbone, with particular reference on the phosphorus atom (Walton et al. 2000; Cook and Glenn, 1997; Uhlmann and Peyman, 1990). They are produced by replacing one of the non-bridging oxygen atoms in the phosphate group with either a sulphur (phosphorothioates) or methyl group (methylphosphonates) (Opalinska and Gewirtz, 2002; Tamm, 2006). Additional endeavours to modify the backbone have been attempted. Substitution of the phosphodiester with groups such as acetals (Jones et al. 1993; Zhang et al. 1999), amides (De Mesmaeker et al. 1994) and hydroxylamines (Sanghvi et al. 1997) has resulted in replacing the phosphorous atom and maintaining or increasing binding affinity. However, advancement over the phosphorothioate backbone has not, to date, been demonstrated (Bennett and Swayze, 2010). As such, the phosphorothioate modification, first synthesised in 1969, is the best known of this class and will be focused on in greater detail.

1.5 Phosphorothioate oligonucleotides
PS oligonucleotides in which one of the non-bridging phosphate oxygen atoms of the internucleotide phosphodiester linkage is replaced by a sulphur atom, (figure 1.8), were one of the earliest and remain one of the most extensively used, and successful backbone modifications of antisense oligonucleotides to date (Hogrefe, 2009a; Bennett and Swayze, 2010; Ito et al. 2003; Golden et al. 2002; Bennett et al. 2004; Morita et al. 2002, Kurreck, 2008). PS oligonucleotides were initially synthesised by Stec and colleagues, (Stein, 1996), with the purpose of increasing the lifetime of antisense oligonucleotides under physiological conditions by increasing their stability towards hydrolytic degradation, mediated by enzymes such as 3’- or 5’-exonucleases and other phosphodiesterases, and also to enhance their specificity towards the target sequence (De Mesmaeker et al. 1995). The substitution of sulphur for oxygen in the phosphate ester bestows numerous advantageous properties onto oligonucleotides that are pivotal for their use as systemic antisense drugs. They are the most widely used modified oligonucleotides for both in vitro and in vivo applications as they are capable of combining the best ratio of stability versus effectiveness with the ability to activate RNase H (Morita et al. 2003), in comparison to other modified oligonucleotides (Schiavone et al. 2004). Furthermore, the PS modification has a substantial pharmacokinetic benefit by increasing the binding to plasma proteins. This prevents rapid renal excretion and enables binding to other acceptor sites that facilitate uptake to tissues (Bennett and Swayze, 2010). In addition to possessing appropriate physicochemical and biological properties, and unlike most other chemically modified oligonucleotides, PS oligonucleotides can be synthesised inexpensively.
However, it has been reported that the affinity of PS oligonucleotides for complementary RNA is not as high as that observed with PO oligonucleotides (Stein et al. 1988; Walton et al. 2000; Morita et al. 2002) which may hinder their sequence accessibility. Some have also displayed significant non-specific interactions with proteins which renders the correct interpretation of the antisense effect brought about by these oligonucleotides as doubtful and problematic (Kurreck, 2003). In contrast to PO oligonucleotides, PS oligonucleotides have one to three orders of magnitude higher binding affinity for various cellular proteins, particularly the heparin binding proteins and several cellular receptors (Lebedeva, and Stein, 2001). These non-antisense mediated pathways are understood to be responsible for most, if not all, of the toxicities. Vidal et al. (2005), states that on the whole, despite initial concerns regarding non-specific effects, antisense oligonucleotides have satisfactory toxicity profiles. In contrast to first generation antisense oligonucleotides, second generation oligonucleotides and subsequent generations are much less likely to produce these side effects (Cooper et al. 1999; Vidal et al. 2005).

PS oligonucleotides were the first antisense agents to enter clinical trials and, to date, they have become the most comprehensively studied and best characterised antisense agents in cell culture, animals and man (Schlingensiepen and Schlingensiepen, 1997). Furthermore, PS oligonucleotides have advanced through various stages of clinical trials and represent the only commercially available antisense oligonucleotide (Vitravene™) to date. Additionally, the vast majority of the current antisense drugs in clinical trials (table 1.2) incorporate PS modifications (Hogrefe, 2009a; Walsh, 2007; Pirollo et al. 2003). As such they will most
likely represent the major antisense oligonucleotide chemistry entering clinical trials in the future (Corey, 2007; Bennett and Swayze, 2010).

1.5.1 Chemistry of phosphorothioate oligonucleotides

As sulphur displays similar properties to oxygen, (same group in periodic table), it acts as an ideal substitution as it enhances some properties of the unmodified oligonucleotides whilst still maintaining structural integrity and biocompatibility (Whitley, 2000). Such a modification is a conservative modification as the negative charge of the phosphate group, (Eckstein, 2002), is maintained (preserving the anionic character) and the size of the sulphur atom is only slightly larger than the oxygen atom (Verma and Eckstein, 1998; Thorogood et al. 1996). The overall net charge of the phosphate is not affected, due to the fact that sulphur has only a slightly larger van de Waals radius than oxygen. Additionally, the length of the phosphorus-sulphur bond is essentially the same as that of phosphorus-oxygen bond (Heidenreich et al. 1993). As PS oligonucleotides are mesomeric, the negative charge may be localised on either oxygen or sulphur or distributed between both atoms. This may be dependent on the environmental conditions, such as, in an enzyme pocket, and on the nature of the counter ion (Eckstein, 2002). However, current evidence supports negative charge localization on sulphur (Thorogood et al. 1996). Sulphur and oxygen vary in polarisability and electronegativity (Heidenreich et al. 1993). Due to the fact that the electronegativities of sulphur and oxygen vary quite considerably 2.5 versus 3.5, thiosubstitutions affect the distribution of the electrons on phosphate and its substitutions. In addition to this the sulphur atom is also more easily polarised and more hydrophobic than oxygen (Loverix et al. 1998).

The phosphorus atom in a phosphodiester linkage of an oligonucleotide may be described as being ‘pro chiral’ or ‘non-chiral’ (Corradini et al. 2007; Cook, 2004). As can be seen in figure 1.9, the pro-R (non-bridging) oxygen atom is orientated towards the major groove, within the double helix, whilst the pro-S oxygen atom is more exposed and directed towards the minor groove. Increasing attention has been dedicated to the varying spectroscopic and chemical properties of the two pro chiral non-bridging oxygen atoms, as they can display a different role in the interaction of DNA with other species (Corradini et al. 2007).
Substitution of one of the two non-bridging oxygen atoms of the internucleotide phosphate with another atom generates an asymmetric centre on the phosphorus atom (Eckstein, 2002; Hartmann et al. 1999). Given that a nucleotide unit already contains a first asymmetrical centre within its sugar moiety, further asymmetry at the phosphorous atom of the nucleotide produces a diasymmetric nucleotide (Cook, 2004). The diastereotopicity of these oxygens gives rise to a pair of diastereomeric phosphorothioates which are designated as having either the Rp or Sp diastereomer orientation (figure 1.10) (Almer et al. 1996; Cook, 2004). The sulfurisation methods generate a mixture of isomers, at each incorporation site which results in racemic mixtures having $2^n$ isomers, where $n$ is equal to the number of phosphorothioate inter sugar linkages in the oligonucleotide. For example a 21-mer PS oligonucleotide containing 20 asymmetric centres has $2^{20}$ or 1,048,576 diastereomers (Cook and Glenn, 1997). As a consequence of this, such an oligonucleotide results in the averaging of the many characteristics which are noticeably different in stereoregular ‘all Rp’ and ‘all Sp’ oligomers (Wilk and Stec, 1995).
The large number of diastereomers generated for each modified oligomer was a major issue of concern with reference to phosphorothioates (De Mesmaeker et al. 1995). The automated synthesis of all PS DNA is not stereospecific, and cannot be rendered so (Stein et al. 1988). From previous studies, Yu et al. (2000) observed that enzymatically synthesised stereo-regular Rp PS oligonucleotides displayed stereochemistry-dependant biophysical and biochemical properties in comparison to a stereo-random PS oligonucleotide. These properties are detailed in Table 1.4.

Table 1.4 Comparison of properties of ‘all Rp’ diastereomers and ‘all Sp’ diastereomers.

Table compiled with information obtained from Yu et al. 2000; Agrawal, 1996.

<table>
<thead>
<tr>
<th>Properties of ‘all Rp’ diastereomers (In contrast to stereo-random and Sp PS oligonucleotides)</th>
<th>Properties of ‘all Sp’ diastereomers (In contrast to stereo-random and Rp PS oligonucleotides).</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Rp diastereomers</strong></td>
<td><strong>Sp diastereomers</strong></td>
</tr>
<tr>
<td>Higher binding affinity to the target RNA</td>
<td>Lower binding affinity to the target RNA</td>
</tr>
<tr>
<td>Greater RNase H activation</td>
<td>Lower RNase H activation,</td>
</tr>
<tr>
<td>Higher Tm (melting point)</td>
<td>Lower Tm (melting point)</td>
</tr>
<tr>
<td>Less resistant to nuclease degradation</td>
<td>More resistant to nucleases.</td>
</tr>
</tbody>
</table>

1.5.2 Physicochemical properties of phosphorothioate oligonucleotides

PS oligonucleotides have many similar biochemical and physicochemical properties as phosphodiester polymers (Stein, 1996). However, Gilar et al. (1998a) states that despite the fact that PS oligonucleotide modification is one of the most conservative, the chemical properties of a PS molecule differ from its phosphodiester counterpart (Koziolkiewicz et al. 2002).
The PS modification confers several biophysical and biochemical properties, as summarised in figure 1.11, that are essential for their use as systemic antisense drugs.

**Properties of PS oligonucleotides**

Properties essential in an oligonucleotide for antisense activity

- Nuclease stability
- Specificity for target RNA
- Hybridization via Watson Crick base pairing
- Ease of synthesis
- Cellular uptake
- RNase H activation

Physicochemical properties of a 21-mer PS oligonucleotide

- Polyanionic backbone
- Hydrophilic
- High molecular weight (21-mers - 6682)
- Highly negatively charged (20 charges)
- Chirality in backbone, presence of two diastereomers
- Biological and chemical instability (nucleases, pH depurination, etc).
- **Solid state:** they are amorphous, electrostatic, hydroscopic solids with low-bulk densities, possessing very high surface areas, and poorly defined melting points”.
- Good solution stability and solubility
- Have a lower pKa and are more hydrophobic than phosphodiesters.
- Increase in lipophilicity

**Figure 1.11 Chemical, biochemical and biophysical properties of first generation oligonucleotides.**

Figure compiled with information obtained from Pirollo et al. 2003; Cook, 2001; Gewirtz et al. 1998; Gilar et al. 1997; Gilar et al. 1998a; Gilar et al. 1998b; Raoof et al. 2004; Morvan et al. 1993; Sepp-Lorenzino and Ruddy, 2008; Wyrzykiewicz, 1997; Vidal et al. 2005; Bennett et al. 2004; Kurreck, 2008.

It can be concluded that the replacement of the oxygen atom with a sulphur atom is quite conservative. However, it is the disparity between these groups in some properties that have proven to be most interesting such as

- the presence of two diastereomers,
- an increase in stability against degradation by nucleases,
- its nucleophilic character,
- the ease of cleavage by iodine, and
- the favoured association with soft metal ions (Eckstein, 2002).
1.5.3 Additional uses of phosphorothioate oligonucleotides

The importance of chemically synthesised oligonucleotides is primarily due to the extensive range of application to which oligonucleotides can be directed (Reddy et al. 1996; Gilar et al. 2002). Chemically modified nucleic acids and oligonucleotides are widely used as model systems for native DNA and RNA and an overview of some of the potential applications of synthetic oligonucleotides can be seen in figure 1.12.

![Diagram representing the wide variety of applications of synthetic oligonucleotides](Egli and Pallan, 2007)

Nucleotide analogues have become crucial and routine tools for probing structural and mechanistic aspects of nucleic acid biochemistry (Beevers et al. 1999; Ryder and Strobel, 1999). The chirality of the phosphorus centre allows the determination of stereospecificity and the stereochemical course of reactions that occur at phosphorus (Verma and Eckstein, 1998).

1.6 Production and synthesis of antisense oligonucleotides

Upon approval of an antisense drug, for a systemic treatment, hundreds of kilograms or perhaps even tonnes of that specific sequence will most likely be required (Reese and Yan, 2002; Deshmukh et al. 2000a). The large-scale manufacture of antisense oligonucleotides is a multi-step process that comprises of two main stages: solid-phase synthesis and downstream processing. In the initial procedure, the desired oligonucleotide sequence is assembled by a computer-controlled solid-phase synthesizer (Capaldi and Scozzari, 2007). Automated synthesis of PS oligonucleotides is generally carried out on a solid support (derivatized CPG, crosslinked polystyrene), using commercially available phosphoramidites as the starting materials (Capaldi et al. 2004) (four phosphoramidites corresponding to the
four nucleosides A, C, T, and G) (Deshmukh et al. 2001). Most current large-scale manufacturing efforts utilise the four-step solid-phase phosphoramidite approach which comprises of four chemical reactions (detritylation, coupling, sulfurization, capping) that are subsequently separated by rinsing steps designed to remove excess reagents (Capaldi and Scozzari, 2007).

Repetition of this synthesis cycle permits the assembly of PS oligonucleotides on scales ranging from a few nanomoles to several hundreds of millimoles. Upon completion of the chain-assembly steps, the oligonucleotide is cleaved and separated from the solid-support matrix and then released from the support and deprotected by treatment with ammonium hydroxide (Capaldi et al. 2003). Purification, isolation and drying of the final drug product completes the synthesis cycle (downstream processing) (Capaldi et al. 2004; Capaldi and Scozzari, 2007). At large scale the current standard purification techniques to manufacture antisense oligonucleotides under current Good Manufacturing Practices (cGMP) conditions are reversed-phase chromatography and anion exchange chromatography (Lajmi et al. 2004; Deshmukh et al. 2000a). The quality of the oligonucleotide is then generally determined by a combination of analytical techniques including strong anion-exchange (SAX) chromatography, capillary gel electrophoresis (CGE), $^{31}$P NMR spectroscopy, and electrospray mass spectrometry (ES-MS) (Capaldi et al. 2003). A comprehensive review of the manufacture of phosphorothioate oligonucleotides is detailed in Chapter Two.

PS oligonucleotides can be synthesized and obtained for moderately reasonable prices in kilogram scales. Antisense companies still pledge a great deal of their development funding to improving the synthesis of these compounds. They clearly expect a continual stream of phosphorothioate oligonucleotides to enter clinical trials, as well as to obtain FDA approval (Aboul-fadl, 2006).

1.7 **General environmental implications of antisense based therapeutic production**

An assessment of the environmental impact of a production process should be a necessary element of the process design. Operations that generate significant quantities of hazardous materials should be avoided, especially at industrial scale. Biopharmaceutical industries produce significant environmental impacts not alone with the chemical waste produced from such facilities but the potential release of nucleic acids waste also. The wastes generated
from the overall synthesis of antisense oligonucleotides include both chemical and oligonucleotide related wastes. Chemical reagents released as waste from the manufacture of antisense oligonucleotides could have deleterious impacts upon the environment. Significant quantities of organic solvents, such as acetonitrile and toluene, are required in the automated solid-phase synthesis of oligonucleotides and can considerably contribute to the polluting capacity of the wastewaters. A more detailed assessment of the chemical wastes produced is discussed in Chapter Two.

With progressively more stringent limits and an increasing list of controlled parameters, the potential risks associated with waste streams generated from the large-scale production ought to be taken into consideration, most specifically issues pertaining to oligonucleotide related products of these wastes. It is predominantly during downstream processing, in particular purification, that there is a potential for loss of synthetic nucleic acid material during which it is estimated that upwards of 50% of synthetic oligonucleotide is lost to waste streams (Hogrefe, 2009b; Deshmukh et al. 2001). The majority of these process related impurities are almost complete antisense oligonucleotides, shorter oligonucleotides (termed failed sequences), (Gilar et al. 2002), which ought to be treated and suitably disposed of. There is little information published pertaining to the nature of these industrial effluents and/or of their effects on receiving waters. Oligonucleotides are novel compounds, and to date there appears to be no regulatory guidelines in place for specifically inactivating/treating active antisense therapeutic drugs. At present, no provisions or regulatory guidelines have been generated in the Integrated Pollution Prevention and Control (IPPC) licensing system (EPA, 2009). Therefore, it may be desirable to develop specific methodologies to degrade/remove antisense drugs from waste streams as a ‘safety net’ to prevent and circumvent the accidental release of active antisense product into the environment.

1.7.1 The environmental impact and fate of naked/free nucleic acids

At present, biotechnological processes are generating an increasing variety of naked/free nucleic acids that are released unregulated into the environment. ‘Naked’ nucleic acids are DNA/RNA produced in the laboratory which range from oligonucleotides of less than 20 nucleotides to artificial constructs thousands and millions of base pairs in length, frequently containing heterogeneous combinations of genes from pathogenic bacteria, viruses and other genetic parasites which belong to every kingdom of living organisms (Ho et al. 2001). The majority have never existed in nature, or if they have, not to such a large extent. They can be defined as, xenobiotics, substances unfamiliar to nature, having the potential to cause harm.
Ho et al. (2001), states that the naked/free nucleic acids produced by genetic engineering biotechnology are potentially the most harmful xenobiotics to pollute our environment. Nucleic acids, unlike chemical pollutants that dilute out and degrade over time, can be taken up by all cells to multiply, mutate and recombine indefinitely. It is important that appropriate measures are established and implemented to prevent the release of any of these naked/free nucleic acids into the environment. Assessments on the potential risk of DNA vary significantly from one source to another. It appears that no clear definite answer has been agreed.

1.7.2 Persistence of DNA in the environment

In environmental biotechnology/microbiology, there is limited research on both the persistence of nucleic acids in soil, water and sediment and their potential environmental impact in these environments (Trevors, 1996). DNA is abundant in the environment (Fink and Moran, 2005; Trevors, 1996), and can constitute over 10% of extractable P (phosphorus) in soil (Turner and Newman, 2005; Levy-Booth et al. 2007). Naked/free DNA is known to persist in all natural environments. High concentrations of DNA are found in the soil, marine and freshwater sediments and also in the air–water interface (Ho et al. 2001; Dale et al. 2002; Lorenz and Wackernagel, 1994). Several studies have indicated that the released DNA is functional and that it can persist from several hours to years in soil depending on the soil type (Dale et al. 2002; Gebhard and Smalla, 1999; Kay et al. 2002; Paget et al. 1998; Wackernagel, 1996; Doblhoff-Dier et al. 2000; Romanowski et al. 1992; Romanowski et al. 1993b). A schematic of the possible fates of DNA, when released into the soil, is presented in figure 1.13.

![Figure 1.13 Fate of eukaryotic DNA release into the soil environment](adapted from Fink and Moran, 2005).
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The fate of DNA in the soil environment is influenced by the biological, physical and chemical properties of both soil and DNA. DNA adsorption to the soil medium is predominantly determined by soil characteristics such as cation concentration, soil mineralogy, concentration of humic substances and soil pH (Levy-Booth et al. 2007). Upon release into the environment, extracellular DNA may: (a) persist by cation binding onto soil minerals (sand and clay), humic substances and organo-mineral complexes (Lorenz and Wackernagel, 1987; Crecchio and Stotzky, 1998), (b) be enzymatically degraded by microbial nucleases and consumed as a nutrient for microbial and plant growth (Paget et al. 1992; Romanowski et al. 1991; Ceccherini et al. 2003) and/or (c) enter the microbial DNA cycle via natural transformation of competent bacteria (bacterial cells that are able to take up exogenous DNA) (de Vries et al. 2001; Gebhard and Smalla, 1999; Lorenz and Wackernagel, 1994).

The adsorption of DNA to soil minerals and humic substances protects it from extracellular, microbial nucleases and DNases, which degrade unbound DNA in the soil solution (Paget et al. 1992; Crecchio and Stotzky, 1998; Blum et al. 1997; Paget et al. 1998; Lorenz and Wackernagel, 1987). Binding sites of the soil medium can enhance DNA persistence, (a) by protecting bound DNA from enzymatic nuclease mediated degradation (Crecchio and Stotzky, 1998) and (b) by adsorbing nucleases and DNases, thus decreasing the probability of enzymatic DNA restriction (Blum et al. 1997). This degree of protection was also observed in waste water treatment plants by nucleic acids adsorbed to sludge particles (Bauda et al. 1995; Aardema et al. 1983).

In the environment, DNA is continuously under attack by chemical modifications, physical shearing forces, and microbially secreted nucleases (Doblhoff-Dier et al. 2000). As stated previously, unlike their unmodified counterparts, modified PS oligonucleotides, by design, display increased chemical and nuclease stability (Wyrzykiewicz and Cole, 1994) and hence would likely persist for longer in the environment if released. In addition to this, adsorption of DNA onto sand or clay minerals in soils or aquatic systems protects DNA from natural DNase digestion, increasing the probability of uptake (Trevors, 1996; Lonenz and Wackernagel, 1994; Romanowski et al. 1993a; England et al. 1998; Bauer et al. 2004). Many bacteria can take up DNA fragments from the environment, a genetically specified feature known as natural competence. Cells can use the nucleotides that are released by degradation of incoming DNA and also any strands displaced by its recombination as a
source of nutrients and as a mechanism to underpin genetic evolution (Maughan et al. 2010; Maughan and Redfield, 2009).

Consequently, if isolated DNA can persist in various environmental media, then it is possible for nucleic acids to be taken up by the digestive system of humans or animals with the food or drinking water. It has been reported that fragments of DNA, ranging from complete genomes or chromosomes down to individual nucleotides, are readily taken up by higher organisms through direct contact, ingestion and inhalation (Doblhoff-Dier et al. 2000). In animals, the most common site for natural exposure to foreign DNA is the gastrointestinal tract, by the ingestion of nutrients (Doblhoff-Dier et al. 2000). It is reported that DNA can also persist in the digestive tract, where sizable fragments may be taken up and incorporated by resident microbes and cells of the mammalian host (Ho et al. 2001; Schubbert et al. 1994). Unmodified nucleic acids, which are found extensively in food, have not been linked with toxic or pathogenic effects on humans or animals. None of the components of nucleic acids are understood to be acute toxicants, but like proteins and other constituents of food, may indirectly cause unfavourable metabolic effects if ingested exclusively at high doses for a prolonged period of time in the absence of a balanced diet (EPA, 1997).

The assumptions that DNA cannot be taken up through unbroken skin, surface wounds, or the intestinal tract, or that it would be rapidly destroyed if taken up have now been overtaken by empirical findings (Ho et al. 2001). Recent research and clinical trials in antisense technology have demonstrated how readily these modified nucleic acids can enter practically every type of human cell. However, it is important to note that this technology does not involve homologous recombination and it is unlikely that it would be integrated into the host’s genome and therefore passed on to successive generations. It is theoretically not possible that the RNA-oligodeoxyribonucleotide complex would participate in homologous recombination and the results compiled in genotoxicity assays, to date, demonstrate this (Levin, 1999).

1.7.3 Potential environmental impacts of antisense oligonucleotides

The unregulated release of active antisense product into the environment in small or large quantities may give rise to unaddressed/unanswered issues of concern. Inevitably, the manufacture and use of antisense oligonucleotides at industrial scale could result in its unintentional release to waste streams and inevitably into the environment. Thus, the possible release of active antisense product from the biopharmaceutical industry to the
ecosystem via accidental leakage, spillage, or intentional release of waste streams containing product/product variants may present hazardous ecological effects as outlined in table 1.5. The risk of accidental release of such products would be inappropriate and may also have possible impacts on animal and human health. Additionally, from both a public perception and acceptance of manufacturing activity, consideration and regulations should be in place to prevent the release of such active product.

Table 1.5 Potential hazards of antisense oligonucleotides.
Table compiled with information obtained from Ho et al. 2001; Bennett et al. 2004; Chan et al. 2006; Vidal et al. 2005.

<table>
<thead>
<tr>
<th>Potential environmental and ecological hazards of antisense oligonucleotides</th>
</tr>
</thead>
<tbody>
<tr>
<td>- Non-target interference with gene function from antisense DNA (Adverse biological effects either in blocking the function of homologous genes or genes with homologous domains)</td>
</tr>
<tr>
<td>- Side effects related to the chemical backbone of the oligonucleotide</td>
</tr>
<tr>
<td>- Severe immune reactions</td>
</tr>
<tr>
<td>- Non-specific interactions</td>
</tr>
<tr>
<td>- Autoimmune reactions</td>
</tr>
<tr>
<td>- Insertion mutagenesis</td>
</tr>
<tr>
<td>- Insertion oncogenesis</td>
</tr>
<tr>
<td>- Additional environmental implications:</td>
</tr>
<tr>
<td>- Eutrophication (The composition of DNA is composed of a phosphodiester back with a phosphate backbone and nitrogenous bases each of which could potentially lead the unnecessary release of increased levels of nitrogen and phosphorous in to the aquatic environment).</td>
</tr>
<tr>
<td>- Increased levels of sulphur in the environment.</td>
</tr>
</tbody>
</table>

Antisense oligonucleotides are modified, and as such, are not naturally occurring compounds. EPA (1997) states that analogues used as therapeutic agents often have significant toxicity associated with their application. Like all other xenobiotics, antisense therapeutic agents, possess their own inherent toxicities that derive from the physical or chemical characteristics of the compounds. The class related toxicities are brought about through mechanisms other than hybridization, for example oligonucleotide-protein interactions. The majority, if not all toxicities detected in preclinical studies are class-related toxicities (Levin, 1999).

As stated previously, the PS linkage significantly increases stability to nucleolytic degradation, and as such, would likely persist for longer in the environment. If ingested or taken up by humans or animals it has been extensively demonstrated that PS oligonucleotides display sufficient stability in plasma, tissues and cells to avoid metabolism before reaching the target RNA thereby persisting in the body for longer periods of time. Several studies have demonstrated that, once taken up, PS oligonucleotides distribute in most
cells (Crooke, 1998). Furthermore, it has been reported that PS modification has a substantial pharmacokinetic benefit by increasing the binding to plasma proteins which prevents rapid renal excretion and enables binding to other acceptor sites that facilitate uptake to tissues (Bennett and Swayze, 2010). Additionally, PS oligonucleotides can efficiently cause RNase H cleavage to the target RNA. Heteroduplexes as short as five nucleotides in length can activate RNase H mediated degradation of the RNA component (Warfield et al. 2006). Similarly, Lima and Crooke, (1997) established that only a four deoxynucleotide stretch in an antisense oligonucleotide/RNA hybrid is required for the cleavage by activation of RNase H activity. This outlines the significance of degrading the antisense oligonucleotide to almost monomeric level prior to release into the waste water stream to prevent unwarranted uptake in cells and potential deleterious effects on entry. An additional cause for concern relates to binding of oligonucleotides to the DNA fibre which may lead to triplex formation and ultimately induction of site-specific mutations. The probability of formation of triple helices of the antisense product with the DNA fibre and the potential resulting outcomes should be taken into consideration (EMEA, 2005).

1.7.4 Methods of nucleic acid degradation

Due to the uncertainty surrounding the environmental fate and impacts of PS modified oligonucleotides, it seems appropriate that the release of such products/product variants into the environment ought to be controlled in an appropriate manner and that treatment systems should be developed to inactivate/degrade such products to environmental release. Phosphate diester is significantly more kinetically stable than other biological functional groups such as esters or amides (Williams, 2004; Cheng et al. 2007). However, DNA is not impervious to degradation. Damage can take place at the bases, the sugars, and the phosphodiester internucleotide linkage of DNA (figure 1.14), compromising its strand continuity and information content. The degradation of small synthetic DNA and RNA derivatives and their analogues mainly involves chemical instability, which is described as any process entailing modification of the molecule by bond formation or cleavage, thus producing a new chemical entity (Pogocki and Schöneich, 2000). Degradation of DNA entails the cleavage of covalent bonds (Jelen et al. 1997). Nucleic acid–derived drugs can be degraded by numerous pathways. Hydrolysis and oxidation are the most prominent pathways (figure 1.14) (Pogocki and Schöneich, 2000; Bauer et al. 2003).
Two key features of DNA make it susceptible to chemical modification, the glycosidic bond between the deoxyribose sugar and the nucleobase, a bond that is extremely susceptible to hydrolysis, and the presence of a large sum of oxygen and nitrogen atoms in the nucleobases (figure 1.14) (Suzuki et al. 1994; Roberts et al. 2006). Cleavage of the N-glycosyl bond between a nucleobase and its 2’-deoxyribose moiety in DNA yields an apurinic/apyrimidinic (abasic or AP) site. This process is called depurination or depyrimidination and occurs spontaneously under physiologic conditions. Depyrimidination is reported to occur at a rate of 5% of that for depurination (Willerslev et al. 2004; Lindahl and Karlström, 1973). Abasic sites may be produced chemically at random sites in DNA by heat or acid treatment or by irradiation (Ravikumar et al. 2003) which results in biologically inactive DNA/RNA. In DNA, the cleavage of the N-glycosidic bond is followed by rupture of the sugar-phosphate backbone at apurinic sites (Pogocki and Schöneich, 2000; Lindahl, 1996). In contrast to RNA, the lack of the 2’-hydroxyl group in DNA increases the strength of the phosphodiester
bonds of the sugar backbone, but weakens the glycosidic bond that joins the bases to the sugars. Consequently, RNA has a slower rate of depurination than DNA, but direct cleavage of its phosphodiester bonds occurs more rapidly (Willerslev et al. 2004; Pogocki and Schöneich, 2000). Unlike RNA, where the phosphodiester internucleotide linkage is sensitive to base-catalysed hydrolysis in aqueous solution, DNA is resistant to alkaline hydrolysis. This is primarily due to the presence of the 2’-hydroxyl group which can participate in an intramolecular attack on the phosphate group, leading to hydrolysis of the phosphate internucleotide bond (Jelen et al. 1997; Miller, 1990). The influence of physicochemical (elevated temperature, steam sterilisation, acidic and basic pHs) methods of degrading PS oligonucleotides are outlined in Chapter Three.

An additional pathway of degradation involves deamination which is the hydrolytic cleavage of amino groups. The miscoding bases hypoxanthine, uracil, thymine and xanthine can be produced by the hydrolytic deamination of adenine, cytosine, 5-methylcytosine and guanine, respectively. Cytosine is most susceptible to this reaction (Willerslev et al. 2004; Pogocki and Schöneich, 2000; Miller, 1990). Hydrolysis of the sugar-phosphate backbones of DNA and RNA can also be catalysed by metal ions such as Fe(III), Mg(II), Ca(II), Zn(II), Ni(II), Pb(II), or trivalent lanthanides (Pogocki and Schöneich, 2000). Nucleic acids are also susceptible to oxidative modifications resulting in the production of oxidized bases and adducts and strand breaks. Free radicals, such as peroxide (-O₂) and hydroxy (-OH), along with hydrogen peroxide (H₂O₂), can cause oxidative damage to DNA and RNA (Willerslev et al. 2004; Lindahl, 1996; Evans et al. 2000; Duez et al. 2000). A more comprehensive review of the effects of metal ions and oxidising agents on nucleic acids and PS oligonucleotides is outlined in Chapter Four.

The application of nucleases offers an additional potential method for the removal of PS oligonucleotides from waste streams. All of the above mentioned methods entail the incorporation of additional chemical solutions to the production process thereby adding to environmental effects of the waste streams. Nucleases potentially present as a more environmentally friendly and potentially energy saving alternative. The focus of Chapter Five is on the enzymatic degradation of the nuclease resistant PS oligonucleotide to find a nuclease(s) that will hydrolyse PS oligonucleotides at a reasonable rate (60 minutes) and thereby investigating their application to degrade oligonucleotides in waste product streams.
Nucleic acid–derived drugs display both physical and chemical instability (Pogocki and Schöneich, 2000). Instability of PS oligonucleotides has been largely attributed to two degradation mechanisms, oxidation and acid-catalysed hydrolysis (Bennett et al. 2004; Hardee et al. 2001). Therefore, based on the biophysicochemical properties of PS oligonucleotides (section 1.5.2) and the susceptibility of natural DNA to degradation, specific degradation methods were chosen for the suitable treatment of PS oligonucleotide containing waste streams. In conclusion, this information suggests that depurination/b-elimination, oxidation and hydrolysis of the backbone by enzymatic means present the three major pathways of PS oligonucleotide degradation.

1.8 Analytical methods of quantification of oligonucleotides

Antisense oligonucleotides can be detected and quantified by various analytical methods (Kumar et al. 2006). To date UV-Spectroscopy (Schweitzer and Engels, 1997; Manz et al. 2004), chromatography (Leeds and Cummins, 2001; Gilar et al. 2002; Gilar, 2001; McFarland and Borer, 1979; Andrus and Kuimelis, 2000a; Thompson and Wells, 1988; Westman et al. 1987; McLaughlin, 1989; Huber and Oberacher, 2001), electrophoresis (Schweitzer and Engels, 1997; Aynié et al. 1996; Andrus and Kuimelis, 2000b), or 31P NMR spectroscopy (James, 2000; Macdonald et al. 1996; Jaroszewski et al. 1996), have been used. At present, the most commonly employed method of detection and quantification is mass spectrometry (MS): liquid chromatography–mass spectrometry (LC-MS), electrospray ionization (ESI) and Matrix-assisted laser desorption (MALDI) in combination with various mass separation and detection techniques (Elliott, 2005; Kumar et al. 2006; Polo and Limbach, 2000; Castleberry et al. 2008; Alazard and Russell, 2005, Gilar et al. 2003; Bothner et al. 1995; Fountain et al. 2003; Wei et al. 2006). Confirmation of oligonucleotide structure, including molecular mass determination, presence of protecting groups, detection of oxidative and other degradative processes such as hydrolysis by nucleases, detection of base modifications such as deamination, depurinated sequences etc., can be achieved by specific mass spectrometric detection and analysis (van Breemen et al. 1998; Kumar et al. 2006; Apffel et al. 1997; Smirnov et al. 1996). For the purpose of this study, Polycrylamide Gel Electrophoresis (PAGE) and Ion-Pair Reversed-Phase Liquid Chromatography (IP-RP LC) were the two main analytical methods employed for the analysis of PS oligonucleotides and their subsequent degradation analysis. Both methodologies will be discussed in greater detail in Chapter Three.
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1.9 Project Aims and Thesis Overview

Antisense therapeutics is a relatively new area in the biopharmaceutical industry. Due to this, little consideration has been given to the potential environmental impacts associated with such a facility. The unique manufacturing routes of antisense oligonucleotides and bioactivity raises unaddressed environmental issues. With the inevitable development of antisense technology, and the increasing number of products processing through clinical trials, the manufacture and use of antisense oligonucleotides at industrial scale could result in its unintentional release to waste streams and hence into the environment. This research aims to establish the environmental impacts associated with the product lifecycle of an antisense therapy and to develop technical methodologies to treat specific manufacturing waste streams rendering them free of active antisense product.

The initial part of the research described in the thesis entailed an evaluation of the current large-scale production of antisense oligonucleotides (Chapter Two). This study entailed a comprehensive assessment of the raw material inputs and the possible chemical and biological waste outputs arising at each stage of the synthesis process and an assessment of the resulting environmental impacts. The main objective of this section is to provide essential background information which is central to this research and to establish appropriate boundaries for the research being undertaken. A comprehensive model of the manufacturing process of antisense therapeutics, recommended for industrial scale, along with the distinctive chemical and oligonucleotide related wastes, generated from each stage of the synthesis process, was simulated using SuperPro Designer ® 6.0. This initial assessment, of the manufacturing process, permitted the identification of pollutant “hot spots” which provided an opportunity to suggest potential recommendations for suitable alterations that would ameliorate such issues.

The second stage of this study entailed the optimisation of two analytical methodologies, PAGE and IP-RP LC to detect and quantify antisense oligonucleotides and their degradation products (Chapter Three-Five). In this study, the stability of PS oligonucleotides was investigated to characterise the degradation pathways and to develop effective treatment processes to adequately and effectively treat waste streams from biotechnological laboratories and industrial processes. Physicochemical (elevated temperature, steam sterilisation, acidic and basic pHs) methods of degrading PS oligonucleotides were identified and systematically analysed both qualitatively and quantitatively in Chapter Three. Chemical (soft metal ions and oxidising agents) and enzymatic (five commercially available nucleases)
means were also assessed under similar conditions for their efficacy at degrading PS oligonucleotides as described in Chapter Four and Five respectively.

The stability of PS oligonucleotides in simulated anion exchange (AX) waste stream, generated from a typical antisense oligonucleotide purification, was assessed (Chapter Six). Six optimum treatment methodologies were chosen and compared to establish the most effective method in simulated AX waste stream solutions to assess if the waste stream components would hinder the efficacy of the treatment methodology. This approach was implemented and a wide range of laboratory experiments were carried out to determine the most effective method for the removal of PS oligonucleotide waste to suitable levels for the subsequent disposal of these wastes which can, therefore, be passed with less risk into the waste water treatment plant. The most effective method was chosen based upon the concentration of reagent required, treatment duration, effectiveness in simulated waste stream conditions, feasibility and practicality of the process, cost implications and potential environmental impact. At the end of the study, the most effective and feasible treatment method will be identified which could potentially be effective on an industrial scale when used to pre-treat a waste treatment influent stream, thus rendering it free of active drug product.
Chapter Two: The Manufacture of Antisense Therapeutics – Modelling and Assessment of the Synthesis Process and Purification
Chapter Two: The Manufacture of Antisense Therapeutics – Modelling and Assessment of the Synthesis Process and Purification

2.1 Introduction

The successes and encouraging clinical trial results obtained thus far suggest that the widespread use of antisense therapeutics will require their synthesis on an industrial scale (Capaldi and Scozzari, 2007). Microgram to gram quantities of antisense oligonucleotides is generally adequate for in vitro and pharmacological screening purposes. For application of antisense oligonucleotides in clinical trials kilograms are required, whilst upon approval of an oligonucleotide drug for a systemic treatment, hundreds of kilograms or perhaps even tonnes of that specific sequence will most probably be required (figure 2.1) (Reese and Yan, 2002; Deshmukh et al. 2000a; Deshmukh et al. 2000b; Lajmi et al. 2004; Gonzalez et al. 1998; Sinha, 1997). It is estimated that for a systemic dosing for treatment of a chronic disease between 700-900 kilograms of drug per year would be required to supply the current demand (Cheruvallath et al. 2003a).

![Increasing quantity of oligonucleotide needed with each stage of development](image)

*Figure 2.1 A schematic diagram of increasing quantity of oligonucleotide required at each stage of development* (adapted from Trilink Biotechnologies, 2008).

Over the last 10 years, the synthesis and scale-up of modified oligonucleotides has undergone significant progress. The availability of raw materials and the latest advancements in automated solid-phase synthesis and purification methods, in addition to analytical techniques, have facilitated the large-scale manufacture of high quality antisense drugs at relative ease (Zhang and Tang, 1998; Sanghvi et al. 1997; Sinha and Michaud, 2007; Behlke and Devor, 2005). With the rising success of antisense drugs in various stages
of clinical trials, emphasis on the development of safe, efficient and cost-effective processes for oligonucleotide synthesis has become paramount (Sanghvi, 2000). With new developments in chemical syntheses and improvements in the manufacture of GMP oligonucleotides, evaluation on the use of hazardous substances, handling of toxic materials, requirement of specialised disposal of hazardous waste and the overall environmental implications and issues associated with such procedures ought to be seriously considered for the sustainable success of the large-scale synthesis of antisense therapeutics.

The first part of this chapter outlines the manufacture of antisense oligonucleotides from initial solid-phase synthesis, through to the final active pharmaceutical ingredient (API) as it is imperative to obtain a detailed understanding of the future production process. The focus of this chapter is on the production of clinical and commercial supplies of phosphorothioate oligonucleotides, so only large-scale manufacture will be discussed in detail. This chapter as with subsequent chapters will focus on phosphorothioate modified DNA antisense oligonucleotide drugs due to their successive progress through clinical trials to the NDA (new drug application) and approval for distribution and sale (Deshmukh et al. 2000a).

The second part of this chapter focuses on the environmental analysis of the synthesis process. Distinctive chemical and biological wastes arising from each stage of the synthesis process are highlighted, identified and diagrammatically represented using the process modelling software SuperPro Designer® 6.0. The raw material and chemical reagent inputs and waste outputs of the process are determined and assessed in detail while factors such as energy, water usage etc. are excluded. By simulating the process this can aid in crucial decision making during process development and this may assist in process design most specifically in terms of reducing environmental impact. This may help in creating more environmentally friendly and efficient processes generating smaller quantities of less harmful wastes (Linninger et al. 1995).

Significant progress has been made in the area of reagent and chemical consumption and the use of alternative environmentally more friendly reagents used in the synthesis of oligonucleotides (Cheruvallath et al. 2003b; Krotz et al. 1999a) with the emphasis on both cost effectiveness and environmentally friendly chemicals, thus reducing waste disposal costs (Krotz et al. 1999b; Sanghvi et al. 2001). Various methods of optimization have been described and continue to be developed. However, oligonucleotide waste generated
throughout the synthesis process has gone unreported in these investigations. The aim of this chapter is to highlight the process waste streams that are of significant environmental concern with regards chemical and most specifically oligonucleotide wastes and to identify the waste stream that would likely contain the majority of the synthetic nucleic acid wastes.

A review of the available literature published on the relevant area of the study was undertaken. As there is a vast commercial interest in these technologies, details of industrial-scale manufacturing and purification processes for oligonucleotide production are not commonly reported in detail in the academic literature (Srivatsa et al. 2000; Sanghvi and Schulte, 2004; Sanghvi, 2010 personal communication). Moreover, to date there is little information in the scientific literature pertaining to quantitative information relating to the inputs and waste outputs of the manufacturing process at large-scale. More specifically there are no reports on the nucleic acid waste generated during the synthesis process. A case study was carried out and facilitated by personal communication with personnel from GE Healthcare, leading distributors of oligonucleotide synthesisers, to provide a clear concept of the process. The discussion with relevant personnel from various companies including Isis Pharmaceuticals Inc. (leading developer of antisense products) provided pivotal information on the related subject matter.

The main aims of this chapter are to:

- Present a comprehensive overview of the manufacturing of antisense oligonucleotides with particular emphasis on PS oligonucleotides from solid-phase synthesis, cleavage and deprotection and purification to obtain the final active pharmaceutical ingredient (API).
- Design/illustrate a comprehensive model of the manufacturing process of antisense therapeutics using SuperPro Designer® 6.0 detailing quantities (mass/volumes) similar to quantities utilised in the large-scale synthesis of antisense therapeutics.
- Highlight the inputs and outputs (chemical and oligonucleotide waste streams) generated at each stage of the synthesis process.
- Highlight recent developments and suggest further recommendations for reagents and processes that allow for the use of safe and more environmentally-friendly chemicals and to minimize toxic waste generation.
Assess and compare the current methodology for the large-scale purification of antisense therapeutics and review the most suitable method of purification based on performance (yield), economic viability and environmental impact.

### 2.1.1 Large-scale synthesis of antisense oligonucleotides

The large-scale manufacture of antisense oligonucleotides is a multi-step process that comprises of two main stages: solid-phase synthesis (figure 2.3 and 2.4) and downstream processing (figure 2.5 and 2.6). In the initial procedure, the desired oligonucleotide sequence is assembled by a computer-controlled solid-phase synthesiser. Subsequent downstream processing consists of deprotection steps, purification, isolation and drying to yield the final drug substance (Capaldi and Scozzari, 2007).

### 2.1.2 Solid-phase synthesis

Three synthetic approaches, phosphotriester, phosphoramidite and H-phosphonate, have been demonstrated to be effective in the preparation of oligonucleotide analogs. The phosphotriester approach has primarily been used in solution-phase synthesis, while the phosphoramidite and H-phosphonate approaches have been predominantly used in solid-phase synthesis (Reese, 2002; Eritja, 2007). To date, the solid-phase synthesis, based on the phosphoramidite approach, has dominated large-scale oligonucleotide synthesis due to its superior coupling efficiency in addition to its capability to control the state of each linkage in a site-specific manner (Zhang and Tang, 1999; Sanghvi, 2000; Capaldi et al. 1999). The majority of large-scale manufacturing of oligonucleotides employ the four step solid-phase phosphoramidite approach first described over 25 years ago by Beaucage and Caruthers (Beaucage and Caruthers, 1981; Capaldi and Scozzari, 2007). Moreover, oligonucleotide synthesis, irrespective of the scale or type (DNA, RNA, aptamer etc.) is performed using β-cyanoethyl-phosphoramidite chemistry (Sinha et al. 2006). In fact, the fundamental synthetic methodologies for the preparation of oligonucleotides with phosphorothioate linkages (first generation oligonucleotides) and the introduction of modified nucleosides (second and third generation oligonucleotides) have remained largely unchanged from the original phosphoramidite chemistry (Sinha and Michaud, 2007). Slight modifications to the original procedure allow the phosphorothioate linkage to be introduced whereby the iodine-water solution used in the oxidation step of the synthetic cycle is replaced with a sulfur transfer reagent (sulfurization step). Additionally, slight variations to the synthetic method are also required for the synthesis of morpholino oligomers, while PNA oligomers are prepared using standard peptide synthesis chemistry (Sinha and Michaud, 2007).
2.1.2.1 Solid-phase synthesisers

The assembly of oligonucleotides is performed using solid-phase synthesisers. As the emphasis of this chapter is on large-scale oligonucleotide synthesis, only synthesisers of this scale will be focused on and discussed in detail. Numerous automated DNA synthesizers are available ranging in different synthesis scales (GE Healthcare, 2010; Zhang and Tang, 1998; Zhang and Tang, 1999; Sanghvi et al., 1999; Capaldi and Scozzari, 2007). Significant scale-up improvements were achieved by Pharmacia Biotech (now GE Healthcare) and Hybridon (now Idera) with the introduction of flow-through reactor-based technology, which is probably the single most significant advance in large-scale oligonucleotide synthesis of the past 15 years (Sinha and Michaud, 2007; Capaldi and Scozzari, 2007).

Currently the OligoProcess™ synthesiser has been extensively used for the large-scale GMP manufacture of oligonucleotides. It has been reported that the OligoProcess™ synthesiser has the capacity to produce more than four kilograms (600 millimole scale) of high quality crude 20-mer oligonucleotide over a period of approximately 5.3 hours which would equate to the production of a metric ton of crude oligonucleotide per annum (Business wire, 2003). At its most basic level, the OligoProcess™ synthesiser (figure 2.2) is a device for delivering exact quantities of solvents and reagents to a steel reactor packed with an appropriate solid support (Capaldi and Scozzari, 2007).

![Figure 2.2 OligoProcess™ - Production Scale Oligonucleotide Synthesiser](GE Healthcare, 2010)

To exploit the potential of the packed-bed approach, a suitable solid support with sufficient compressibility resistance that can be packed evenly into a synthesis column is necessary. Synthetic scale is regulated by adjusting the amount of solid support in the reactor, for example, at a support loading of 200 µmol/g, a 600-mmol synthesis needs 3 kg of support.
2.1.2.2 The four stages of solid-phase synthesis

As a prelude to synthesis, the synthesis column is packed with the calculated quantity of solid support, loaded with either the 3’-terminal nucleoside, or more commonly now, “Unylinker”. Primer support is packed as a slurry in acetonitrile. The desired sequence is chosen from a list or a pre-programmed option by the operator, who then makes the required modifications based on scale, and initiates the synthesis. After a series of initial steps during which the reagent lines are primed and the support is washed with acetonitrile to remove residual moisture, synthesis begins by allowing reagents and solvents to pass through in a predetermined manner (Capaldi and Scozzari, 2007; Sanghvi et al. 1999). Attachment of each nucleotide residue to the growing chain support, from the 3’ end toward the 5’ end, in a linear sequential manner, entails four chemical reactions, detritylation, coupling, sulfurization and capping. The chemistry utilised and steps involved in the synthesis of oligonucleotides are represented in figure 2.3 and 2.4.

![Figure 2.3 Phosphorothioate oligonucleotide solid-phase synthesis](Zhang and Tang, 1998).

**Step 1 (Detritylation)**

The synthesis cycle begins with detritylation, the removal of the 4,4’-dimethoxytrityl protecting group (DMT) from the support-bound nucleoside or Unylinker molecule, to generate the corresponding 5’-hydroxyl derivative (figure 2.4 (a)). This is achieved by
pumping a solution of dichloroacetic acid in dichloromethane/toluene through the column. Currently, detritylation is performed using a 3/10% v/v solution of DCA in toluene. Following detritylation, the support is subsequently washed with dry acetonitrile in preparation for coupling (Amersham Bioscience, 2002; Capaldi and Scozzari, 2007; Srivatsa et al. 2000).

**Step 2 (Coupling)**
The second reaction entails coupling the specific phosphoramidite monomer (A, C, G, or T) mixed with the activator tetrazole (figure 2.4 (b)). Tetrazole converts the inert phosphoramidite monomer to a highly reactive intermediate (tetrazolide) that readily reacts with the 5’-hydroxyl groups on the support. At Isis Pharmaceuticals Inc., both 1H-tetrazole and 4,5-dicyanoimidazole (DCI) have been applied in this step (Capaldi and Scozzari, 2007; Amersham Biosciences, 2002; Srivatsa et al. 2000). After the coupling step, the support is washed again with acetonitrile to remove excess reagents and by-products.

**Step 3 (Sulfurization)**
The newly formed P(III) phosphate triester is converted to the corresponding P(V) phosphorothioate triester in the sulfurization step (figure 2.4 (c)). There is a wide range of reagents available for this process step. However, the reagent of choice, at present, appears to be phenylacetyldisulfide (PADS), made up in equal volumes of acetonitrile and 3-picoline (Srivatsa et al. 2000; Capaldi and Scozzari, 2007). Following sulfurization, the column is washed with acetonitrile.

**Step 4 (Capping)**
The final step in the synthesis cycle is capping unreacted 5’-hydroxyl groups (figure 2.4 (d)). Capping is assumed to acylate any 5’-hydroxyl groups that failed to react with phosphoramidite during the preceding coupling step and thus prevent them from reacting in any successive cycle. This is carried out by simultaneous treatment with N-methylimidazole, pyridine and acetonitrile (Cap A), and acetic anhydride and acetonitrile (Cap B). The support is subsequently thoroughly washed with acetonitrile prior to initiating the next cycle (Srivatsa et al. 2000; Amersham Biosciences, 2002; Sanghvi et al. 1999; Capaldi and Scozzari, 2007).
This four-step cycle (figure 2.3 and 2.4) is repeated until the desired sequence is assembled. On completion of automated synthesis, the solid support-bound oligonucleotide is then deprotected and liberated from the solid support in a cleavage and deprotection procedure achieved by treatment with concentrated aqueous ammonium hydroxide/ammonia to give rise to the crude oligonucleotide material which becomes the starting material for purification (Capaldi and Scozzari, 2007; Deshmukh et al. 2000a; Deshmukh et al. 2000b; Capaldi et al. 2003; Sanghvi et al. 1999). It contains various classes of impurities in addition to the desired full length oligonucleotide (Gonzalez et al. 1998; Sanghvi et al. 1999; Sanghvi and Schulte, 2004). The nomenclature of various classes of impurities is outlined in table 2.1.
Table 2.1 The typical profile of a crude oligonucleotide following solid-phase synthesis (Sanghvi and Schulte, 2004). The majority of impurities are incomplete/modified versions of the product itself.

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Type of oligonucleotide or impurity</th>
</tr>
</thead>
<tbody>
<tr>
<td>n-mer</td>
<td>A full-length oligonucleotide product with the correct sequence</td>
</tr>
<tr>
<td>n-1</td>
<td>A deletion sequence short by one base unit</td>
</tr>
<tr>
<td>n-2</td>
<td>A deletion sequence short by two base units</td>
</tr>
<tr>
<td>n-X</td>
<td>Shorter sequences with more than two deleted base units</td>
</tr>
<tr>
<td>DMT-off n-mer</td>
<td>A full-length product without a 5'-O-DMT group</td>
</tr>
<tr>
<td>DMT-on n-X mer</td>
<td>Shorter sequences with a 5'-O-DMT group</td>
</tr>
<tr>
<td>(P=O)₁</td>
<td>A phosphorothioate oligonucleotide containing one phosphodiester linkage</td>
</tr>
<tr>
<td>(P=O)₂</td>
<td>A phosphorothioate oligonucleotide containing two phosphodiester linkages</td>
</tr>
<tr>
<td>(P=O)ₙ</td>
<td>A phosphorothioate oligonucleotide containing more than two phosphodiester linkages</td>
</tr>
<tr>
<td>n+1</td>
<td>An oligonucleotide product that is one unit longer than the correct length</td>
</tr>
<tr>
<td>n+X</td>
<td>An oligonucleotide product with more than one unit added to the sequence</td>
</tr>
<tr>
<td>CE-adduct</td>
<td>An oligonucleotide with the addition of an acrylonitrile group</td>
</tr>
<tr>
<td>Terminal PS</td>
<td>An oligonucleotide with a 3'-terminal phosphorothioate unit</td>
</tr>
<tr>
<td>DMT-OH</td>
<td>A DMT alcohol</td>
</tr>
</tbody>
</table>

Since the assembly of oligonucleotides is carried out by the addition of one nucleotide unit at a time to produce the desired length of the final product, the most significant process-related impurities are deletion sequences, such as n-1, n-2 and n-xmers, where n represents the desired full-length product and x represents the number of deletions. These arise from chain failure to elongate. Deletions occur at random locations along the oligonucleotide chain. Consequently, for a 21-mer oligonucleotide without adjacent same-base nucleotides, 20 unique (n-1) deletion sequences are possible. Crude synthetic oligonucleotides also contain partial phosphodiester (P=O)ₙ impurities generated by incomplete sulfurization, from oxidation of the intermediates or following ammonia deprotection steps of the synthesis cycle. Among various phosphodiester impurities, the most predominant type is the monophosphodiester (P=O)₁, which consists of a single phosphate linkage. Other minor impurities include impurities of longer products (n+x) produced due to the double coupling promoted by the acidic nature of the activator used throughout the coupling steps. Hence, the crude product obtained from current solid-support synthesis is contaminated with highly homologous failure sequences generated from incomplete detritylation, coupling, sulfurization, or capping during synthesis (Sanghvi and Schulte, 2004; Sanghvi et al. 1999; Sanghvi, 2000; Deshmukh et al. 2001; Deshmukh et al. 2000a; Gilar et al. 2002; Capaldi, et al. 2003; Deshmukh et al. 2000c). Despite the large sum of structurally similar impurities present in the crude oligonucleotides, a number of separation methods have been effectively used to eliminate or minimise the level of impurities, previously outlined, before they can be considered for clinical use (Sanghvi and Schulte, 2004, Sanghvi, 2000).
2.1.3 Downstream processing – Purification and post purification steps

Synthetic oligonucleotides are large and complex molecules that possess several unique properties which can be utilised for their purification (Sanghvi and Schulte, 2004; Deshmukh et al. 2000a). Some of the key methods are summarised in table 2.2 (Deshmukh et al. 2000a). However, the majority of these synthetic oligonucleotide purification strategies are generally only applied at small-scale.

Table 2.2 Application and properties of several chromatographic techniques for the purification of nucleic acids and oligonucleotides (adapted from Deshmukh et al. 2000a).

<table>
<thead>
<tr>
<th>Technique</th>
<th>Property used</th>
<th>Scale</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Small-scale</td>
</tr>
<tr>
<td>Ion exchange</td>
<td>Charge on phosphate backbone</td>
<td>Yes</td>
</tr>
<tr>
<td>Reversed phase</td>
<td>Hydrophobicity of bases on DMT protecting group</td>
<td>Yes</td>
</tr>
<tr>
<td>Hydrophobic</td>
<td>Hydrophobicity of bases on DMT protecting group</td>
<td>Yes</td>
</tr>
<tr>
<td>interaction</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Affinity</td>
<td>Watson-Crick base pairing</td>
<td>Yes</td>
</tr>
<tr>
<td>Gel permeation</td>
<td>Size of the molecule</td>
<td>Yes</td>
</tr>
</tbody>
</table>

Mixed Mode

|                               |                                                    |           |            |
|                               |                                                    | Yes       | No         |
| 1. Ion-paired (RP)            | Hydrophobicity of bases and charge interaction     |           |            |
| 2. Hydroxyapatite            | Charge and phosphate groups                        | Yes       | No         |
| 3. Slalom chromatography     | Size and hydrophobicity of large nucleic acids     | Yes       | No         |
| RPC-5 media                  | Charge interaction and hydrophobicity              | Yes       | No         |

New emerging purification methods

|                               |                                                    |           |            |
| Displacement chromatography  | A component with higher binding strength than the sample is used to displace the sample from the column. | Yes       | No         |
| Sample Self displacement (SSD) Chromatography | A stronger binding component within a sample displaces a weaker binding component of the same sample | Yes       | No         |
| Membrane Chromatography      | Same as AX using membrane instead of AX bead media. | Yes       | Yes        |

Selection of the purification method depends on the purity requirement of the final compound, the target application and the quantity required. High purity oligonucleotides are required for diagnostic probes and antisense drug applications, while lower purity oligonucleotides may be suitable as oligonucleotide primers, used for in vitro applications (Deshmukh et al. 2000b). For therapeutic use, the oligonucleotide purity is generally required to be greater than 95%. For special applications such as X-ray crystallography and NMR spectroscopy, it should be above 99%. In general, greater than 95% purity for oligonucleotides can be achieved in a single-step IEX-HPLC purification, whereas a two-
step, orthogonal IEX and RP HPLC can achieve greater than 99% purity (Sproat and Rupp, 2004).

As opposed to synthesis, there are three distinct chromatographic approaches that can be implemented for purification of synthetic oligonucleotides (Sinha et al. 2006). To date, only Reversed Phase Chromatography (RP-HPLC), Hydrophobic Interaction Chromatography (HIC), and Anion Exchange (AX) Chromatography have demonstrated their suitability for GMP (Good Manufacturing Practice) use, as documented in published reports, demonstrating the feasibility of these methods at hundred-gram levels (Deshmukh et al. 2000a; Deshmukh et al. 2000b).

Once the desired sequence has been assembled on the solid support, on completion of automated synthesis, the terminal 5’-DMT group may be retained or removed, depending on choice of purification method. Crude trityl-on (DMT-on) solid-phase synthesis products are generally purified via RP-HPLC or HIC chromatography. The acid-labile hydrophobic 5’-DMT protecting group serves as a powerful handle during separation with reversed-phase (RP) purification. After purification, the DMT group is removed under acidic conditions and the resultant oligonucleotide product is then precipitated from solution, isolated and lyophilized to obtain the dry active pharmaceutical ingredient (Gilar et al. 2002; Deshmukh et al. 2000a). The fundamental framework for a rapid and reliable RP purification method is illustrated in figure 2.5 (a) as a schematic of the current classic RP purification protocol and its associated post purification procedures (Deshmukh et al. 2000c; Deshmukh et al. 2000b).
Figure 2.5 Schematic flow diagram of purification strategies for solid-phase synthesised Phosphorothioate oligonucleotides by RP-HPLC (a) and HIC (b). (adapted from Deshmukh et al. 2000c; Deshmukh et al. 2000b; Zhang and Tang, 1998; Zhang and Tang, 1999).

HIC has also been employed and demonstrated, at large-scale, (100 g scale) for separation of DMT-on crude oligonucleotides from DMT-off failure sequences. A significant advantage of this methodology is that the ammonical oligonucleotide cleavage solution can be loaded directly on the chromatographic column without prior ammonia stripping. HIC purification product pooling generally follows protocols comparable with those utilized for RP purified oligonucleotides whereby the DMT-on product is eluted followed by detritylation and subsequent purification by anion-exchange chromatography as illustrated in figure 2.5 (b). While both HIC and RP media achieve comparable results, limited published research suggests that cost advantages and capacity may give preference to RP media (Deshmukh et al. 2000a). Despite the advantages of HIC, to date only Puma (1996), has described this technique for the large-scale purification of antisense oligonucleotides (Deshmukh et al. 2000a; Sanghvi and Schulte, 2004; Deshmukh et al. 2000b; Zhang and Tang, 1998).
As oligonucleotides have multiple negative charge, AX chromatography is a logical purification and separation method. This method is dependent on the electrostatic attraction between the phosphate groups of oligonucleotides and the positively charged groups of the AX media. For AX purification, the oligonucleotide is normally detritylated before loading on the column. The DMT group can be removed on the synthesis support (DMT-off) and the resultant crude solution is purified by anion-exchange (AX) chromatography. The post purification pool is then desalted and lyophilized to dryness to produce the finished active pharmaceutical ingredient. A schematic for this approach is illustrated in figure 2.6 (a) (Deshmukh et al. 2000a; Sanghvi and Schulte, 2004; Gilar et al. 2002).

![Automated Solid-Phase Synthesis](image)

Figure 2.6 Schematic flow diagram of purification strategies for solid-phase synthesised Phosphorothioate oligonucleotides by AX-HPLC (a) and Orthogonal RP and AX (b). (adapted from Deshmukh et al. 2000c; Deshmukh et al. 2000b; Sinha, 1997).
In some instances, orthogonal RP and AX methods have been employed to produce high purity oligonucleotides as presented in figure 2.6 (b). The crude DMT-on oligonucleotide is initially purified on a RP column, as previously described, to generate purified DMT-on oligonucleotide. Following detritylation of the product the oligonucleotide is further purified by AX chromatography to produce high-length purity and low phosphodiester content. This process, a double purification method, is currently used for the manufacture of Vitravene™ (Sanghvi et al. 1999). While this method produces highly purified oligonucleotides, it also poses limitations, such as reduced overall yield, required optimization on various equipment and prolonged handling of materials and associated potential for loss (Sanghvi et al. 1999; Deshmukh et al. 2000a; Sanghvi and Schulte, 2004; Deshmukh et al. 2000b; Gilar et al. 2002).

A significant challenge in the development of large-scale production of oligonucleotides is the design of a process that meets current good manufacturing practices (cGMPs). Due to the highly regulated nature of therapeutic agents, their manufacturing processes must be robust, scalable, validated, and in agreement with cGMPs. Compliance with cGMP is not only necessary for FDA requirements, but it is also essential for cost-efficient production. The establishment of control strategy is dependent on the development of analytical methodologies that are capable of measuring those essential product characteristics, either of starting materials, or drug substance, which may have an impact on efficacy and safety of the formulated drug product (Capaldi and Scozzari, 2007; Zhang and Tang, 1998; Gonzalez et al. 1998).

2.1.4 Analytical methods for therapeutic oligonucleotides

During production, numerous in-process tests are carried out to monitor vital steps. The yield for the synthesis is assessed prior to purification by ascertaining the oligonucleotide concentration from UV absorbance and preliminary quality is monitored by electrophoretic and chromatographic methods. On completion of production, the purified oligonucleotide is subjected to thorough testing to confirm its identity, purity, quality and strength (Gonzalez et al. 1998). Table 2.3 outlines a list of analytical tests for oligonucleotide pharmaceutical substance or product. Many of these tests were developed specially for antisense oligonucleotides for use as therapeutic agents. The aim of analytical processes is to identify and quantify impurities, to detect potentially active metabolites, and to characterise the pharmacokinetic properties of the drug (Glaser, 2010). The quality and identity of the oligonucleotide is generally determined by a combination of analytical techniques including
strong anion-exchange (SAX) chromatography, ion pair reverse-phase HPLC, capillary gel electrophoresis (CGE), $^{31}$P NMR spectroscopy, and electrospray mass spectrometry (ES-MS). Supplementary analytical techniques may be employed to completely characterise oligonucleotide products and their impurities (Capaldi et al. 2003; Deshmukh et al. 2000b; Behlke and Devor, 2005; Bahr et al. 2009; Gonzalez et al. 1998; Srivatsa et al. 2000; Sinha, 1997).

Table 2.3 Typical analytical techniques and tests required for antisense oligonucleotide drug control

Table compiled with information obtained from Sanghvi et al. 1999; Gonzalez et al. 1998.

<table>
<thead>
<tr>
<th>Purpose</th>
<th>Method/Equipment</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Identity Tests</strong></td>
<td></td>
</tr>
<tr>
<td>Appearance</td>
<td>Colour and morphology</td>
</tr>
<tr>
<td>MW determination</td>
<td>ES/MS or MALDI-TOF MS</td>
</tr>
<tr>
<td>Retention time</td>
<td>IEX HPLC</td>
</tr>
<tr>
<td>P=S vs. P=O ratio</td>
<td>$^{31}$P NMR</td>
</tr>
<tr>
<td>Measure duplex $T_m$</td>
<td>UV</td>
</tr>
<tr>
<td>Sequencing</td>
<td>MALDI-TOF MS</td>
</tr>
<tr>
<td>Assay vs. external reference</td>
<td>CGE</td>
</tr>
<tr>
<td><strong>Purity Tests</strong></td>
<td></td>
</tr>
<tr>
<td>Area - % impurity profile</td>
<td>IEX-HPLC</td>
</tr>
<tr>
<td>Electrophoretic purity</td>
<td>CGE</td>
</tr>
<tr>
<td>Moisture content</td>
<td>Karl Fischer coulometric titration</td>
</tr>
<tr>
<td>Organic volatile impurities</td>
<td>Capillary GC</td>
</tr>
<tr>
<td>Heavy metals</td>
<td>ICP-MS or specific atomic absorption</td>
</tr>
<tr>
<td>Residual buffer salts</td>
<td>CZE</td>
</tr>
<tr>
<td><strong>Quality Tests (Current USP (test method identification))</strong></td>
<td></td>
</tr>
<tr>
<td>Endotoxin level</td>
<td>LAL and USP (85)</td>
</tr>
<tr>
<td>Pyrogen</td>
<td>USP (151)</td>
</tr>
<tr>
<td>Particulate matter</td>
<td>USP (1)</td>
</tr>
<tr>
<td>Sterility</td>
<td>USP (71)</td>
</tr>
<tr>
<td>Content Uniformity</td>
<td>USP (905)</td>
</tr>
<tr>
<td><strong>Strength Tests</strong></td>
<td></td>
</tr>
<tr>
<td>Assay</td>
<td>UV absorbance with theoretical extinction coefficient</td>
</tr>
<tr>
<td>Assay</td>
<td>Ion-exchange HPLC with authenticated reference standard</td>
</tr>
<tr>
<td>Assay</td>
<td>CGE with authenticated reference standard</td>
</tr>
</tbody>
</table>
2.2 Materials and Methods

2.2.1 Review of the literature

A comprehensive review of the relevant literature was undertaken including books, journal papers, patents, vendor company profiles to review the current up to date methods and protocols for the large-scale synthesis of PS oligonucleotides. Moreover, case studies and personal communications and interviews with personnel from GE Healthcare and Isis Pharmaceuticals Inc. provided additional information on existing production processes. Peter Guterstam (PhD, Oligonucleotide Synthesis Specialist GE Healthcare) provided quantitative data on the current solid-phase synthesis process. Isis Pharmaceuticals Inc. are the leading company in antisense technology and as such they hold the monopoly and majority of patents for methodology in the synthesis of antisense therapeutics. Most of information within the literature is predominantly described by researchers at Isis Pharmaceuticals Inc. and therefore this chapter is largely based on the publications emanating from this company.

2.2.2 Environmental modelling of the production of phosphorothioate oligonucleotides

The raw materials, used in addition to the wastes, emissions and by-products generated from the production of PS oligonucleotides were determined via analysis of relevant material and methods and discussion sections of selected literature sources. Flow diagrams detailing the manufacturing process were constructed using Microsoft Word 2010. The unique chemical and biological wastes produced from the manufacture of PS oligonucleotides were diagrammatically represented using the process modelling software SuperPro Designer® 6.0. (Intelligen, Europe Inc., Thessaloniki, Greece).
2.3 Results and discussion

2.3.1 Simulation of phosphorothioate oligonucleotide production process

Figures 2.7 and 2.8 illustrate the production process in addition to the potential aqueous and solid waste generated following each step of the synthesis process. It was constructed using the process modelling software SuperPro® Designer 6.0 as outlined in section 2.2.2. It is divided into three main sections, solid-phase synthesis, downstream processing - purification and post purification as highlighted by the differential colour scheme. The chemical waste streams from the process are outlined with a green box while the synthetic oligonucleotide waste from the process is outlined with a purple box. Based on the information obtained from the literature and the quantitative data received from GE Healthcare, two case studies were chosen. RP-HPLC (figure 2.7) and AX-HPLC (figure 2.8) are currently the two most commonly employed methods of purification and thus form the basis of these two case studies. The solid-phase synthesis in both process diagrams is the same. SuperPro Designer® 6.0 has been utilised for the simulation of a variety of processes (Kuhn et al. 2010; Biwer et al. 2005a; Biwer et al. 2005b; Ramírez et al. 2009). However, to date, this software has not been specifically used for the simulation of the production process of antisense therapeutics.
Figure 2.7 Flow sheet generated using SuperPro Designer® 6.0 illustrating PS oligonucleotide production process steps (1.5 Kg production scale) incorporating RP purification based on information obtained by GE Healthcare, 2010; Capaldi and Scozzari, 2007. The chemical waste streams from the process are outlined with a green box while the synthetic oligonucleotide waste from the process is outlined with a purple box.
Chapter Two

Figure 2.8 Flow sheet generated using SuperPro Designer® 6.0 illustrating PS oligonucleotide production process steps (1.5 Kg production scale) incorporating AX purification based on information obtained by GE Healthcare, 2010; Amersham Biosciences, 2002; Deshmukh et al. 2001. The chemical waste streams from the process are outlined with a green box while the synthetic oligonucleotide waste from the process is outlined with a purple box.
2.3.2 Material mass balance and waste streams generated from the solid-phase synthesis of antisense phosphorothioate oligonucleotides

The raw materials used, wastes generated and by-products arising from the manufacture of PS oligonucleotides were identified as illustrated in figures 2.7 and 2.8 using either RP purification or AX purification respectively. From figure 2.7 and 2.8 it was determined that the first waste stream (WS 1) emitted from the manufacturing process was from the solid-phase synthesis of the synthetic oligonucleotides. Table 2.4 outlines the process steps, chemical reagents required, input volumes and likely waste output (kg/L) following each stage of the solid-phase synthesis process. The data presented in this study was drawn from discussions with and data from personal communications with GE Healthcare, (Peter Guterstam, Oligonucleotide Synthesis Specialist) which is generally not documented in scientific literature at this scale. The calculations are based on a phosphorothioate DNA 20-mer sequence to produce a modest scale of 1.5 kg crude PS oligonucleotide. This represents a model of the solid-phase synthesis of PS oligonucleotides. The exact percentages and molarity of some of the components may differ depending on the manufacturing company. Moreover, figure 2.9 represents the various waste components that may arise following the chemical synthesis of PS oligonucleotides as determined by Sanghvi et al. 2001.
Table 2.4 List of the process steps, chemical reagents required and likely waste outputs for the synthesis of 1.5 kg crude 20-mer phosphorothioate oligonucleotide

Table compiled with information obtained from personal communication GE Healthcare, 2010).
* = Total volume (L) of acetonitrile required in the various process steps
UD = Undetermined

<table>
<thead>
<tr>
<th>Process step</th>
<th>Chemical and reagents required</th>
<th>Input volumes (L)</th>
<th>Likely Waste output</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Chemical</td>
</tr>
<tr>
<td>Wash</td>
<td>Acetonitrile</td>
<td>1750</td>
<td>Acetonitrile</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>2037.3 L*</td>
</tr>
<tr>
<td>Detritylation</td>
<td>3% Dichloroacetic acid in toluene</td>
<td>1050</td>
<td>Dichloroacetic acid</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>31.5 L</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Toluene</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>1018.5 L</td>
</tr>
<tr>
<td>Coupling</td>
<td>0.3 M 5-Benzylthio-1-H-tetrazole in acetonitrile</td>
<td>75</td>
<td>5-Benzylthio-1-H-tetrazole</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>4.3 Kg</td>
</tr>
<tr>
<td></td>
<td>DNA – amidites (0.2M in acetonitrile)</td>
<td>50</td>
<td>DNA amidites</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>UD</td>
</tr>
<tr>
<td>Sulfurization</td>
<td>0.2 M Phenylacethyldisulfide in acetonitrile/picoline (1:1 v:v)</td>
<td>175</td>
<td>Phenylacethyldisulfide</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>UD</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Picoline</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>87.5</td>
</tr>
<tr>
<td>Capping</td>
<td>Cap A (20% N-methylimidazole in acetonitrile)</td>
<td>44</td>
<td>N-methylimidazole</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>8.8 L</td>
</tr>
<tr>
<td></td>
<td>Cap B (20% Acetic anhydride, 30% sym-collidine in acetonitrile)</td>
<td>44</td>
<td>Acetic anhydride,</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>sym-collidine</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>8.8 L</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>13.2 L</td>
</tr>
<tr>
<td>Cleavage and deprotection</td>
<td>20% Diethylamine in acetonitrile</td>
<td>22</td>
<td>Diethylamine</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>4.4 L</td>
</tr>
<tr>
<td></td>
<td>25% ammonia in water</td>
<td>40</td>
<td>25% ammonia in water</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>40 L</td>
</tr>
<tr>
<td>Solid-phase support</td>
<td>Solid-phase (polystyrene-based particles [Primer Support 200])</td>
<td>1.75 kg</td>
<td>UA</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>UD</td>
</tr>
</tbody>
</table>

2.3.3 Material mass balance and waste streams generated from the complete manufacture of antisense phosphorothioate oligonucleotides

Table 2.5 and 2.6 represent the wastes generated from the complete manufacture of antisense therapeutics using either RP purification or AX purification respectively and some of the chemical and biological components of these wastes. Due to the limited information within the literature and the proprietary nature of this information, only the components (i.e. qualitative rather than quantitative) generated in each waste stream, could be readily identified.
Table 2.5 Summary of the various steps and waste constituents present in aqueous and in solid wastes following the manufacture of PS oligonucleotides using RP purification.

<table>
<thead>
<tr>
<th>Step</th>
<th>Location and equipment</th>
<th>Waste stream</th>
<th>Chemical Waste</th>
<th>Synthetic oligonucleotide waste</th>
</tr>
</thead>
<tbody>
<tr>
<td>Solid-phase synthesis</td>
<td>Solid-phase synthesiser</td>
<td>WS 1</td>
<td>Table 2.4</td>
<td>No</td>
</tr>
<tr>
<td>Cleavage and deprotection</td>
<td>Solid-phase synthesiser</td>
<td>WS 2</td>
<td>Cyanoethyl cleavage (20% Diethylamine in Acetonitrile) 25% ammonia in water Acrylonitrile liberated</td>
<td>Cyanoethyl groups Small quantities</td>
</tr>
<tr>
<td>Filtration</td>
<td>Solid-phase synthesiser</td>
<td>SW 1</td>
<td>Solid support</td>
<td>Small quantities</td>
</tr>
<tr>
<td>Removal of ammonia</td>
<td>Stripping Tank</td>
<td>WS 3</td>
<td>Ammonia</td>
<td>Unlikely</td>
</tr>
<tr>
<td>RP-HPLC purification</td>
<td>RP-HPLC</td>
<td>WS 4</td>
<td>Sodium acetate, Methanol</td>
<td>Yes 50% oligonucleotide waste related products</td>
</tr>
<tr>
<td>Precipitation</td>
<td>Precipitation tank</td>
<td>WS 5</td>
<td>Ethanol</td>
<td>Yes</td>
</tr>
<tr>
<td>Detritylation</td>
<td>Detritylation tank</td>
<td>WS 6</td>
<td>0.01 M Sodium acetate Acetic acid</td>
<td>Yes (DMT group)</td>
</tr>
<tr>
<td>Precipitation (x2)</td>
<td>Precipitation tank</td>
<td>WS 7 and 8</td>
<td>Sodium acetate, Ethanol</td>
<td>Yes</td>
</tr>
<tr>
<td>Filtration</td>
<td>NA</td>
<td>WS 9</td>
<td>NA</td>
<td>Yes</td>
</tr>
</tbody>
</table>

At Isis Pharmaceuticals Inc. RP-HPLC purification has been scaled up under cGMP conditions for the manufacture of clinical trial and marketed antisense oligonucleotides (Deshmukh et al. 2000b; Sanghvi et al. 1999; Sanghvi and Schulte, 2004). The fundamental framework for a rapid and reliable purification method is illustrated in figure 2.7 as a schematic of the current classic RP purification protocol and its associated post purification procedures. In general, an aqueous solution of crude DMT-on oligonucleotide is loaded onto the column at low mobile-phase organic content. The organic content (methanol) of the mobile phase is subsequently increased to elute the DMT-off failure sequences and protect group debris, e.g., benzamide, prior to being stepped up a second time to elute the DMT-on material (Sanghvi et al. 1999; Deshmukh et al. 2000a; Deshmukh et al. 2000b; Sanghvi and Schulte, 2004; Capaldi and Scozzari, 2007). The distinctive product pool arising from the RP chromatographic purification of DMT-on synthetic oligonucleotides generally contains alcohol and low levels residual buffer salt. This material is detritylated in organic acid, such as, acetic acid or some milder acid in aqueous solution, to remove the DMT group. By the addition of cold ethanol, the detritylated oligonucleotide is precipitated from solution, a
process optimised for large-scale application. The product is then subsequently lyophilized to dryness to yield the final API (Deshmukh et al. 2000a; Deshmukh et al. 2000b).

Table 2.6 Summary of the various steps and waste constituents present in aqueous and in solid wastes following the manufacture of PS oligonucleotides using AX purification.

<table>
<thead>
<tr>
<th>Step</th>
<th>Location and equipment</th>
<th>Waste stream</th>
<th>Chemical Waste</th>
<th>Synthetic oligonucleotide waste</th>
</tr>
</thead>
<tbody>
<tr>
<td>Solid-phase synthesis</td>
<td>Solid-phase synthesiser</td>
<td>WS 1</td>
<td>Table 2.4</td>
<td>No</td>
</tr>
<tr>
<td>Cleavage and Deprotection</td>
<td>Solid-phase synthesiser</td>
<td>WS 2</td>
<td>Cyanoethyl cleavage (20% Diethylamine in Acetonitrile) 25% ammonia in water</td>
<td>Small quantities</td>
</tr>
<tr>
<td>Filtration</td>
<td>Solid-phase synthesiser</td>
<td>SW 1</td>
<td>Solid support</td>
<td>Small quantities</td>
</tr>
<tr>
<td>Purification</td>
<td>AX-HPLC</td>
<td>WS 3</td>
<td>20 mM Sodium hydroxide, 2.5 M Sodium chloride, Phosphoric acid (rejuvenation)</td>
<td>Yes 50% oligonucleotide waste related products</td>
</tr>
<tr>
<td>Desalting and Concentration</td>
<td>Ultrafiltration/ Gel filtration on size exclusion column (Sephadex™ G-25)</td>
<td>WS 4</td>
<td>Water Sodium chloride</td>
<td>Yes</td>
</tr>
</tbody>
</table>

At industrial scale, several modes of AX chromatography have been employed for the purification of synthetic oligonucleotides (Deshmukh et al. 2000a). A schematic for this downstream processing procedure is illustrated in figure 2.8. DMT-off crude, following cleavage and deprotection is chromatographed on an AX column. Standard purifications include the use of strong anion exchange resins, which are eluted with an increasing gradient of sodium chloride at high pH. Deshmukh et al. (2001) demonstrated the large-scale purification (75 g) of a crude 20-mer PS oligonucleotide using AX-HPLC. This AX purification of ISIS-2302 (Alicaforsen; Isis Pharmaceuticals Inc.) is the largest scale protocol thus far described in the literature (Capaldi and Scozzari, 2007; Sanghvi and Schulte, 2004) and thus forms the basis of the purification methodology outlined in figure 2.8. This was scaled up ten-fold to coincide with the quantitative information that was given for the solid-phase synthesis of 1.5 kg crude oligonucleotide material.

In contrast to RP purification, PS oligonucleotides purified via AX chromatography contain significant levels of HPLC buffer salts. Numerous techniques are available for the removal of salts introduced during small-scale AX purification however, for large-scale purifications,
ultrafiltration and gel permeation are most commonly performed. Ultrafiltration is most likely the best method for desalting on large-scale as it is extensively employed in industry and thus large-scale equipment is readily available and the product is isolated as an aqueous solution. Additionally, ultrafiltration can be used to reduce endotoxin levels, and also to deliver final concentrations of approximately 50 mg/ml (Gonzalez et al. 1998). The last two process steps in this flow diagram, ultra filtration and lyophilisation are standard methods of operation, are easily scalable and do not pose any health or safety issues at large-scales (Sinha et al. 2006).

2.3.4 Nucleic acid waste generated from the manufacture of antisense phosphorothioate oligonucleotides

It is predominantly during downstream processing, in particular purification, that there is a potential for loss of synthetic nucleic acid material (table 2.5 and 2.6). It is estimated that upwards of 50% of synthetic oligonucleotide is lost to waste streams, during oligonucleotide purification (Hogrefe, 2009b). As illustrated in figure 2.8, based on a study carried out by Deshmukh et al. (2001), 150 g crude 20-mer PS oligonucleotide was loaded on a column. Purification and pooling of the appropriate fractions resulted in 75 g of product, with a purity of > 95%, generating a 50% loss in oligonucleotide related material. When this was scaled up ten-fold this resulted in the production of 750 g product variants that could be released to waste and hence into the environment. This resultant material would likely contain deletion sequences \((n-1)\), \((n-2)\) etc., (where \(n\) represents the desired full length product), longmers \((n+x)\) and partial phosphodiester components, \((P=O)_1\), \((P=O)_2\) etc. A variety of additional smaller impurities generated as by-products during solid-phase synthesis and subsequent work-up steps may also be present (Deshmukh et al. 2000a; Srivatsa et al. 2000). Additionally, post purification steps including detritylation, precipitation (RP-HPLC) and desalting and concentration (AX-HPLC) could also result in the release of small quantities of oligonucleotide waste. The majority of these process related impurities are almost complete antisense oligonucleotides. Moreover, it has been reported that, the impurities resulting from phosphorothioate synthesis are toxic in tissue culture as well as in in vivo applications (Devor and Behlke, 2005). The possible accidental release of such active antisense products into the environment would be inappropriate. This finding thereby sets the rational, for the subsequent chapters, for the investigation of specific methodologies to degrade/remove antisense drugs from waste streams as a ‘safety net’ to prevent and circumvent the accidental release of active antisense product into the environment.
2.3.5 Developments and recommendations for solvent consumption in solid-phase synthesis and its impact on the waste stream and environment

In recent years, the emphasis on waste management has moved to waste minimisation. This entails systematically reducing or eliminating waste at the source (New et al. 2000). This section aims to highlight recent developments and suggest further recommendations for reagents and processes that allow for the use of safe and more environmentally-friendly chemicals and to minimize toxic waste generation so that antisense oligonucleotides can be sustainable drug products. For the synthesis of a 20-mer oligonucleotide more than 80 steps of sequential reactions are required using over 20 raw materials and reagents (table 2.4) (Zhang and Tang, 1999). Sinha et al. (2006) states that “the chemical reactions in solid support mediated synthesis are almost quantitative, driven by the use of excess reagents”. On smaller scales of synthesis, the usage of excess amount of chemicals, reagents and solvents may not have a considerable impact on environmental safety, overhead costs, and availability of raw materials. Conversely, at larger scales, all of the above named issues become significant obstacles for the development of oligonucleotides (Sinha et al. 2006).

From an environmental perspective and an economic point of view, it is imperative to contemplate how many starting material atoms are present in drugs and how many go to waste. The majority of the atoms that make up the oligonucleotide drug are supplied by the nucleoside phosphoramidites. Up to eight different nucleoside phosphoramidites may be necessary depending on the sequence and chemistry (Sanghvi, 2000; Sanghvi et al. 2001; Capaldi and Scozzari, 2007). Whilst phosphoramidite chemistry operates at very high coupling efficiency (~99%), Sanghvi et al. (2001) identified that it has the shortcoming of generating two high-molecular-weight waste products, as illustrated in figure 2.9 (2 and 6). The DMT group, utilised in 5'-hydroxyl protection, consists of 35% of total nucleoside phosphoramidite weight and is subsequently released as cation 2 (figure 2.9) in the waste stream. It has been reported that the DMT group has toxicological effects (Srivatsa et al. 2000).
The solid-phase synthesis of antisense oligonucleotides is inherently wasteful given that a large excess of nucleosidic phosphoramidite synthons is required for optimum coupling efficiency to drive the reaction to completion (Scremin et al. 1994; Griffey et al. 2002; Zhang and Tang, 1998). Amidites (figure 2.9 (6)) which are also present in the waste stream are used in a 0.5- to 0.7-fold molar excess (Sanghvi et al. 2001). Conversely, Sanghvi et al. (1999) reported the use of as little as 1.5 molar excess of the amidite on OligoProcess synthesisers (Sanghvi et al. 1999). However, current practice at Isis Pharmaceuticals Inc. is to use 1.75 equivalents of phosphoramidite per coupling (Capaldi and Scozzari, 2007; Srivatsa et al. 2000). Due to the considerable quantities of oligonucleotide syntheses
performed for research purposes and for large-scale manufacture for clinical trials (figure 2.1), the waste of expensive nucleoside phosphoramidites is a serious economic and ecological problem (Griffey et al. 2002). Consequently, to reduce the loss of expensive phosphoramidite synthons it would be advantageous to develop a simple procedure for the efficient regeneration of these phosphoramidites (Scremin et al. 1994).

To remedy this, Brill, (1994) reported a method to recover the nucleosides from solid-phase synthesis whilst Beaucage et al. detailed a straightforward procedure for the regeneration of the phosphoramidites (Scremin et al. 1994). Scremin et al. (1994) have also developed an effective process for the capture and reuse of the excess amidite which functions without cleaving the costly 3'-oxygen-phosphorus bond. Guo et al. (1998) have developed a straightforward procedure for the complete capture and recovery of the DMT group. Following this two-step process the regenerated DMT chloride is suitable for reuse in the preparation of 5'-O-protected nucleosides.

A phosphoramidite monomer is usually coupled to the 5'-hydroxy of the growing chain on a solid support in an automated solid-phase synthesis. A block coupling strategy can be employed to reduce synthesis cycle time by half, increase product purity, reduce the use of chemical reagents and solvents whilst doubling the oligonucleotide plant synthetic throughput capacity and increasing crude yield and quality. The application of dinucleotide or trinucleotide blocks makes purification of the final product simpler. It has been verified that contents of (n-1)-mers and phosphodiester linkages can be significantly decreased using dimers or a combination of dimers and trimers in the synthesis of PS oligonucleotides. Therefore, a cost-efficient process for dinucleotide or trinucleotide synthesis ought to be developed to successfully use these blocks in large-scale synthesis (Zhang and Tang, 1999; Gonzalez et al. 1998; Krotz et al. 1997).

Even though it is apparent that solvent volumes are significant, following solid-phase synthesis, significant strides have been undertaken to minimise solvent consumption. The first principle of green chemistry stipulates that it is more beneficial to prevent waste production than having to treat waste or remedy the situation after it has been created (EPA, 2011). A decade ago, for the synthesis in a stirred-bed Milligen 8800 reactor significant solvent volume was required to produce mass ratios, in the ratio of 17 L per gram of crude oligonucleotide. Due to the improvements in synthesiser design, packed-bed synthesis, where solvents flow through the support in a chromatographic-like manner, a significant
reduction in solvent consumption was observed. OligoProcess™ was most efficient in reducing solvent consumption. This fixed-bed design requires only 2.7 L of solvent to produce 1 g of crude oligonucleotide, thereby achieving a noteworthy six-fold reduction in reaction solvent consumption. Moreover, over a period of ten years, the amount of solvent consumed has been reduced, whereby, in 2006 a large-scale synthesis consumed only 50% of the acetonitrile it did in 1996 (Sanghvi et al. 2001; Capaldi and Scozzari, 2007; Zhang and Tang, 1998; Sanghvi et al. 1999; Capaldi et al. 1999).

In spite of the aforementioned advances significant quantities of acetonitrile, toluene and ammonia are required for the synthesis of 1.5 kg crude PS oligonucleotide (table 2.4) and thus represent the greatest volume of chemical waste from this process. Table 2.7 list the aforementioned chemical reagents, that are present in the waste stream (WS 1), their R phrase(s), likely hazardous decomposition products and health rating (Fisher Scientific, 2011). R-phrases (short for Risk Phrases) are defined in Annex III of European Union (EU) Commission Directive 67/548/EEC: Nature of Special Risks Attributed to Dangerous Substances and Preparations. The list was subsequently republished in Commission Directive 2001/59/EC. The R phrase stipulates the specific danger of a material (Yen and Chen, 2009). Also outlined in table 2.7 is the potential application of the recovery and reuse of each chemical reagent.
Table 2.7 List of selected chemical reagents that present in the waste stream (WS 1), their R phrase(s), likely hazardous decomposition products, health rating and the application of recovery and reuse.

NFPA = National Fire Protection Association
Table compiled with information from Fisher Scientific, 2011; SRM Limited, 2011; Krotz et al. 1999b; Veolia Environmental Services, 2011; Acosta-Esquijarosa et al. 2006; ThermoEnergy Corporation, 2011.

<table>
<thead>
<tr>
<th>Chemical reagent</th>
<th>R-phrase(s) and Risk Combination Phrases</th>
<th>Hazardous Decomposition Products</th>
<th>Health Rating (NFPA 704)</th>
<th>Recovery/Reuse</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetonitrile</td>
<td>R11 - Highly flammable R36 - Irritating to eyes R20/21/22 - Harmful by inhalation, in contact with skin and if swallowed</td>
<td>Hydrogen cyanide (hydrocyanic acid). Nitrogen oxides (NOx). Carbon monoxide (CO). Carbon dioxide (CO2).</td>
<td>2</td>
<td>Yes (Distillation)</td>
</tr>
<tr>
<td>Toluene</td>
<td>R11 - Highly flammable R38 - Irritating to skin R63 - Possible risk of harm to the unborn child R65 - Harmful: may cause lung damage if swallowed R67 - Vapours may cause drowsiness and dizziness R48/20 - Harmful: danger of serious damage to health by prolonged exposure through inhalation</td>
<td>Carbon monoxide (CO). Carbon dioxide (CO2).</td>
<td>2</td>
<td>Yes (Distillation)</td>
</tr>
<tr>
<td>Ammonia</td>
<td>R34 - Causes burns R50 - Very toxic to aquatic organisms</td>
<td>Nitrogen oxides (NOx).</td>
<td>3</td>
<td>Yes (Flash vacuum distillation with ion exchange)</td>
</tr>
<tr>
<td>Acetone</td>
<td>R11 - Highly flammable R36 - Irritating to eyes R66 - Repeated exposure may cause skin dryness or cracking R67-Vapors may cause drowsiness and dizziness</td>
<td>Carbon monoxide (CO). Carbon dioxide (CO2). Formaldehyde. Methanol.</td>
<td>1</td>
<td>Yes (Distillation)</td>
</tr>
</tbody>
</table>

An area of significant concern is the consumption of anhydrous acetonitrile which, since the publication of the phosphoramidite method, has been used as the universal solvent to prepare phosphoramidite, sulphur transfer reagent, tetrazole, and capping solutions. It is also used in large quantities as the process “wash solvent” (figure 2.4) following each step of the four-step solid-phase synthesis cycle. This wash step may appear somewhat environmentally insignificant at small-scale but this is not the case at large-scale (Capaldi et al. 1999; Capaldi and Scozzari, 2007). Capaldi et al. (1999) noted that following a single 150 mmol synthesis of a 20-mer PS oligonucleotide approximately 1000 L of acetonitrile was consumed, 90% of which is used in the washing steps. In this case study, it was determined that a cumulative volume (wash steps and reaction steps) of 2037.3 L acetonitrile is generated following the synthesis of 1.5 kg crude 20-mer PS oligonucleotide material. It has been documented that the use of anhydrous acetonitrile is unnecessary for wash steps during synthesis and thus has
been substituted by the more cost effective, commercial grade acetonitrile. Capaldi et al. (1999) established that acetonitrile containing up to at least 200 ppm water can be used as the wash solvent and to prepare the sulphur transfer reagent and capping solutions employed at large-scale for the solid-phase synthesis of PS oligonucleotides (Zhang and Tang, 1999; Capaldi et al. 1999). The application of commercial grade acetonitrile, for selected steps in the synthesis cycle, would be a notable step towards minimising the cost of oligonucleotide chemical synthesis.

Alternatively, Gaytán, (2009) has proposed the application of acetone as the primary washing solvent during automated synthesis to reduce acetonitrile usage. This study came about following the global acetonitrile shortage at the end of 2008. HPLC-grade acetone was successfully used as a replacement for the majority of the acetonitrile lost in the washing steps. Acetone is widely used in laboratories to carry out clean-up of glassware as it is a non-toxic, inexpensive solvent that readily dissolves several hydrophobic as well as hydrophilic materials. In contrast to acetonitrile which has a NFPA health rating of 2, acetone has a NFPA health rating of 1 (table 2.7). However, the water content of HPLC-grade acetone is, on average, 0.3%. This is rather high for dissolution of the monomer-phosphoramidites and activating reagent but nevertheless suitable for the wash steps. It is likely that an anhydrous form [such as the extra-dry acetone available from Fisher Scientific (Waltham, MA, USA)] could replace acetonitrile for the entire process (Gaytán, 2009).

Additionally, in the detritylation step, a large excess DCA is required for the removal of 5’-terminal dimethoxytrityl group. This could equate to 1000 to 1500 L of 3% DCA solution in dichloromethane (now toluene) per synthesis at scales greater than 100 mmoles. The annual consumption of dichloromethane could be greater than 200,000 L (Sinha et al. 2006). In this case study, for the synthesis of 1.5 kg crude 20-mer PS oligonucleotide, 1050 L of 3% DCA in toluene (1018.5 L) (Table 2.4) was used. This step was traditionally performed in dichloromethane. Toxicity, carcinogenicity, volatility and cost disposal issues have limited the use of dichloromethane in the chemical industry resulting in stringent regulation, for example, by the Occupational Safety and Health Administration. Toluene was identified as a green substitute for dichloromethane and has not compromised the quality and yield of product or introduced any new impurities. Moreover, toluene is a widely accepted industrial solvent owing to its lower vapour pressure and well-established environmental fate thus eliminating the production of significant quantities of chlorinated waste in oligonucleotide
synthesis (Sanghvi, 2000; Sinha and Michaud, 2007; Zhang and Tang, 1999; Krotz et al. 2000; Sanghvi et al. 2001). Whilst the replacement of dichloromethane with the more environmentally friendly solvent toluene has reduced the potential health and safety hazards and generation of large volumes of hazardous waste consumption, generation of vast volumes of DCA in toluene are quite significant per annum. As recycling solvents may become an issue for ton-scale production of oligonucleotides, Krotz et al. (1999b) collected the deblock solutions following each DMTr removal. It was established that a straightforward distillation permitted the recovery of high purity toluene that was reused as a deblock solvent in a successive synthesis. This presents a beneficial methodology by which toluene waste could be minimised and recycled for reuse (table 2.7). The application of simple distillation could also be applied to the recovery of high purity acetonitrile suitable for reuse most specifically in the wash steps (SRM Limited, 2011; Veolia Environmental Services, 2011; Acosta-Esquijarosa et al. 2006). On-site recycling minimises waste and reduces both disposal and transportation costs for the company (Veolia Environmental Services, 2011).

In early oligodeoxynucleotide syntheses a large molar excess of tetrazole, a mildly acidic compound, was extensively utilised as an activator for the coupling step (step Two). 5-Ethylthio-1H-tetrazole has presented as a more effective activator, delivering improved yields. Since regulatory authorities in Europe and the USA have classed solid tetrazole and its derivatives as an explosive, significant research has been carried out to search for alternative activator(s) to eliminate the use of 1H-tetrazole and thus replace it with a less reactive reagent, thereby creating a safer process for future manufacture of oligonucleotides (Sanghvi, 2000; Sanghvi et al. 2001; Eleuteri et al. 2000). Some novel activators suggested include, 5-(ethylthio)-, 5-(4-nitrophenyl)- and 5-(3-nitrophenyl)-1H- tetrazole, 2,3-dicyanoimidazole and saccharin-N-methylimidazolium salt (SMI), Pyridinium chloride and pyridine-TFA salt (Sinha and Michaud, 2007; Zhang and Tang, 1999; Sinha et al. 2006). Despite the fact that numerous compounds have been assessed on a small scale, only 1H-tetrazole and 4,5-dicyanoimidazole (DCI) have been reported for use in the large-scale production of PS oligonucleotides. Initial results suggest that DCI may be a viable activator if tetrazole handling or tetrazole-contaminated solvent recovery present as safety issues. At Isis Pharmaceuticals Inc., DCI has recently replaced 1H-tetrazole for the synthesis of all new MOE gapmer oligonucleotides due to the fact that unlike 1H-tetrazole, DCI is not classified
explosive, is a safer reagent, has eliminated the safety and supply issues and is considerably more stable in acetonitrile (Capaldi and Scozzari, 2007; Sanghvi et al. 1999).

Additionally alternative synthesis reagents have been reviewed for their efficacy in various steps of the synthesis process to minimize the formation of variant impurities and to reduce the overall cost of solid-phase synthesis (Sinha et al. 2006). For example, the sulfur transfer reagent 3-H-1,2-benzodithiol-3-one-1,1-dioxide (Beaucage reagent) contributes a large percentage of this cost. PADS has now replaced the aforementioned reagent and it is estimated that it is roughly 20 times cheaper than Beaucage reagent per gram basis (Sanghvi, 2000; Zhang and Tang, 1998; Capaldi and Scozzari, 2007; Kumar et al. 2007; Krotz et al. 2004; Cheruvallath et al. 2000).

Whilst significant strides have been undertaken to minimize solvent consumption, due to the improvements in synthesiser design, additional steps should be taken to further reduce the cost and environmental burden of such processes. The more cost effective, commercial grade acetonitrile, containing up to at least 200 ppm water, could be used as the wash solvent and to prepare the sulphur transfer reagent and capping solutions which would be a notable step towards minimising the cost of oligonucleotide synthesis. Alternatively, acetone could be used as the primary washing solvent to reduce acetonitrile usage. Additionally, the application of simple distillation could also be applied to the recovery of toluene and high purity acetonitrile suitable for reuse most specifically in the wash steps. Moreover, the capture and recovery of expensive nucleoside phosphoramidites should also be employed. All of these methods would have a strong impact on the economic, environmental and ecological considerations stemming from large-scale oligonucleotide synthesis.

2.3.6 Evaluation of the most suitable method for the large-scale purification of PS oligonucleotides

The process used for purification of oligonucleotides can also pose a significant impact on cost, throughput, and environmental safety and waste generation (Sinha et al. 2006). As stated previously, at large-scale, the current standard purification techniques to manufacture antisense oligonucleotides under cGMP conditions are reversed-phase chromatography and anion exchange chromatography (Lajmi et al. 2004; Deshmukh et al. 2000a). Sanghvi and Schulte, (2004) and Sanghvi et al. (1999) state that AX is a superior choice for large-scale separation of desired synthetic oligonucleotide products for numerous reasons as summarised in table 2.8.
Table 2.8 Advantages and limitations of AX chromatography (Sanghvi and Schulte, 2004).

<table>
<thead>
<tr>
<th>Advantages</th>
<th>Limitations</th>
</tr>
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<tbody>
<tr>
<td>AX chromatography is the most widely used method in the industry for biomolecules</td>
<td>There are few reports in the literature for application on oligonucleotides.</td>
</tr>
<tr>
<td>The system utilises aqueous buffers and avoids organic solvents.</td>
<td>The system produces large, aqueous fractions with low oligonucleotide concentration.</td>
</tr>
<tr>
<td>The equipment, i.e. column and media, are cheaper than that for RP chromatography</td>
<td>The equipment has a larger foot-print and provides larger fractions than RP chromatography.</td>
</tr>
<tr>
<td>Using the DMT-off feed avoids the labourious post-purification detritylation step, which is essential for RP chromatography.</td>
<td>An additional desalting step is required.</td>
</tr>
<tr>
<td>Relatively higher loading is required compared to RP chromatography.</td>
<td>Reprocessing of the side fractions may be required.</td>
</tr>
<tr>
<td>The system eliminates DMT-OH contamination in the final product.</td>
<td></td>
</tr>
<tr>
<td>The removal of the phosphodiester impurity is more effective than for RP chromatography.</td>
<td></td>
</tr>
<tr>
<td>A higher purity product is produced compared to RP chromatography.</td>
<td></td>
</tr>
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</table>

Firstly, from an environmental standpoint, AX chromatography avoids use of large amounts of organic solvents commonly used in RP purification, raising safety concerns (Sanghvi et al. 1999; Sinha et al. 2006; Deshmukh et al. 2000c). Three commonly employed buffers for RP-HPLC purification of antisense oligonucleotides consist of two volatile buffer systems: 100 mM TEAA/ACN and 100 mM ammonium acetate/methanol and a nonvolatile buffer system of 200 mM sodium acetate/methanol. At large-scale, weakly buffered sodium acetate buffer systems are generally preferred and used as they provide highly useful combination of selectivity and capacity. In addition, the use of sodium acetate allows isolation of the desired sodium salt of the drug substance without a successive salt-exchange step. For therapeutic antisense oligonucleotides, sodium is a favored countercation due to physiological reasons, and it also furnishes products of manageable hygroscopicity and good photostability. Methanol and acetonitrile are commonly used as the organic eluent component. Methanol is often preferred at production scale for economic reasons. For strong anion exchange chromatography most of the eluting buffers are 100% aqueous salt solutions with either neutral or high pH for example, Buffer A: 20 mM NaOH and Buffer B: 20 mM NaOH + 2.5 M NaCl. The application of aqueous buffers reduces safety issues and hazardous waste generation and is also more cost effective (Sinha et al. 2006). As AX chromatography accepts crude DMT-off oligonucleotide as feed it thereby avoids post purification detritylation and concomitant oligonucleotide precipitation steps. It is clearly evident from the two process diagrams (figure 2.7 and 2.8) that AX purification requires less process steps thereby reducing the cost, labour, and additional requirement of potentially hazardous chemical reagents such as acetic acid and ethanol.
Additionally, purification by AX HPLC uses less expensive equipment, separates n-1, n-2, n+1-mers effectively, resulting in high product purity in a single step and permits very high loading of crude antisense oligonucleotide (Sanghvi et al. 1999). The loading capacity of strong anion exchange media is considerably higher than reverse phase C-18 or equivalent media and hydrophobic interaction media or weak anion exchange media. This therefore, increases the throughput in purification and decreases the quantity of chromatography resin needed (Sinha et al. 2006). Moreover, the purification process, using strong anion exchange media, does not necessitate the use of high-pressure liquid chromatography instrumentation. Explosive-proof high-pressure liquid chromatography instrument is necessary for large-scale purification with reverse phase media. The application of low-pressure liquid chromatography, using conventional anion-exchange hardware typical of industrial bioseparations, (Deshmukh et al. 2000a; Deshmukh et al. 2000c) thereby minimises potential safety issues (Deshmukh et al. 2000b; Sinha et al. 2006).

One major disadvantage associated with AX-HPLC is the need for post-chromatography desalting step and the requirement to concentrate large volume aqueous fractions (Sanghvi et al. 1999). Another additional limitation as outlined by Capaldi and Scozzari, (2007) is that the recovery of full-length product is noticeably less than 100%. Furthermore Sanghvi and Schulte, (2004) state that a more extensive method development effort is usually needed for large-scale purification of antisense oligonucleotides using AX mode (Sanghvi and Schulte, 2004; Deshmukh et al. 2000b). It is for the first two reasons that Isis pharmaceuticals state their preference to purify MOE gapmers by RP-HPLC (Capaldi and Scozzari, 2007). However, following personal communication with Sanghvi, (2010) a further reason why Isis Pharmaceuticals Inc., choose RP HPLC purification is due to the size of their manufacturing facility which is not designed to accommodate and cater for the larger AX chromatography hardware.

To further improve the AX purification method the salt concentration used at large-scale could be optimised to lower the high salt content that would be released to waste. For example Shanagar, (2005) demonstrated the purification of synthetic oligonucleotides by anion exchange using a buffering gradient system of Buffer A: 0.01 M NaOH, pH 12 and Buffer B: 0.01M NaOH, pH 12 containing 2 M NaCl, a decrease in both sodium hydroxide and sodium chloride concentration than that used in the case study in figure 2.8. Additionally, membrane chromatography offers some additional beneficial properties for its
application for the large-scale purification of PS oligonucleotides. Purification of antisense oligonucleotides by membrane chromatography has been documented to yield a purity that is equal to the conventional AX media. A significant advantage of pre-packed reusable membrane columns is that it offers fast, high-resolution separations without column packing and column packing validation (Sanghvi and Schulte, 2004). Lajmi et al. (2004); Lajmi, (2005) gave an account of a successful large-scale application of AX membrane chromatography under an SSD (sample self displacement) mode for the purification of a 20-mer PS oligonucleotide. The high throughput, robustness, and increased dynamic capacity presented by membrane chromatography could make it a possible alternative to bead-based chromatography. Furthermore, the elimination of column packing and its validation, for membrane-based purification, may demonstrate to be beneficial from an economic perspective (Sanghvi and Schulte, 2004).

Based on the evidence put forward in this section of the chapter, it appears that AX offers a robust and economical process for the purification of antisense oligonucleotides, and it is likely that it may become the method of choice for commercial-scale production in the future and thus forms the basis of the waste stream analysis studies discussed in Chapter Six.

Based on personal communication (Shanagar, 2010 (GE Healthcare)) it appears that AX is the preferred purification method of choice by both the FDA and commercial companies. Sanghvi, (2010 personal communication) states that less than 10% of companies use RP HPLC whilst the majority use AX. Additionally, AX presents a more environmentally friendly method to scale up to ton-scale production on slightly less expensive equipment with reduced organic solvent requirement (Deshmukh et al. 2000b).
2.4 Concluding remarks

It is anticipated that market demand for antisense products for the future treatment of cancers or inflammation may require manufacture of PS oligonucleotide drugs at the metric ton level (Sanghvi et al., 1999). Therefore, a thorough environmental assessment of such facilities is required. Stringent constraints in terms of environmental and performance requirements present a significant challenge at large-scale.

This chapter summarises the literature survey that was undertaken as part of this research in the context of oligonucleotide manufacture. Large-scale oligonucleotide synthesis is a continuously evolving and progressing process. Research in this area has produced developments in synthetic approaches, building blocks, purification techniques and analytical procedures, all which have contributed to making the synthesis of oligonucleotides readily available. In one synthetic run, multikilogram quantities of high quality oligonucleotides can be synthesised using β-cyanoethylphosphoramidite chemistry on large-scale automated syntheisers. While no significant change has occurred, advances in synthesiser design, and the development and implementation of optimised reagents, solid supports, and synthesis, purification, and isolation technologies have transformed the art so that the synthesis of multi-hundred-kilogram quantities of modified DNA and RNA can be achieved at a reasonable cost with improved yield and purity (Capaldi and Scozzari, 2007). Significant achievements have been made to eliminate or reduce the use or generation of toxic materials, solvents, and reagents. Additionally, methodologies that allow the reuse of valuable materials such as solid supports, amidites, and protecting groups have also being investigated to improve the atom economy and cost-efficiency of oligonucleotide production in addition to the use of safer reagents during oligonucleotide synthesis (Sanghvi et al., 2001).

Biopharmaceutical industries produce significant environmental impacts not only with the chemical waste produced, from such facilities, but also the potential release of nucleic acid waste. The distinctive chemical and oligonucleotide related waste streams generated from each process step of the synthesis was identified and simulated using SuperPro Designer® 6.0 flow sheets (figure 2.7 and 2.8). While the lack of comprehensive manufacturing detail available in the literature prevents fully quantitative estimation of all waste stream constituents, this study highlighted that large quantities of chemical wastes, most
specifically, significant volumes of acetonitrile (2037 L) and toluene (1018 L) are produced during the synthesis of crude 20-mer PS oligonucleotide at 1.5 kg scale.

A comparison of the two most commonly employed purification methodologies (RP or AX chromatography) for antisense therapeutics was also undertaken to determine the most cost effective, environmentally favourable and efficient method. AX presents a more environmentally friendly method to scale up to ton-scale production on slightly less expensive equipment with reduced organic solvent requirement.

The manufacture of antisense oligonucleotides at industrial scale will carry with it the risk of accidental release into the environment. It is predominantly during downstream processing, in particular purification, that there is a potential for loss of synthetic nucleic acid material during which it is estimated that upwards of 50% of synthetic oligonucleotide (750 g) is lost to waste streams. The release of active antisense products could potentially pose an environmental threat and as a result in house regulations should be established to minimise such an event. As DNA synthesis technology and its commercialisation are experiencing rapid development and advancement, it is important to develop a governance framework policy. Furthermore, it may be desirable to develop specific methodologies to degrade/remove antisense drugs (marketed) from waste streams as a ‘safety net’ to prevent and circumvent the accidental release of active antisense product into the environment (Chapters Three to Chapter Six).
Chapter Three: Detection and Quantification of Phosphorothioate Oligonucleotides and their Physicochemical Stability
Chapter Three: Detection and Quantification of Phosphorothioate Oligonucleotides and their Physicochemical Stability.

3.1 Introduction
To date, very little information is available with regards to remediation options for the suitable treatment of antisense oligonucleotide containing process waste. A core aim of this project is the development of treatment methodologies capable of degrading or modifying PS oligonucleotides in such waste streams. This chapter outlines the methodologies employed in the study of the detection and quantification of PS oligonucleotides, as well as analysis of their physicochemical stability to temperature, steam sterilisation and pH.

3.1.1 Nucleic acid degradation
Modification of nucleic acids (DNA and RNA) can take place at the bases, the sugars, and the phosphodiester internucleotide linkage, compromising strand continuity and information content. Degradation of DNA entails the cleavage of covalent bonds (Jelen et al. 1997). Numerous environmental factors, for example, high temperature, oxidative conditions, reactive chemical agents, both man-made and naturally occurring, and ionizing radiation can all lead to alteration and degradation of DNA (Sheppard et al. 2000; Miller, 1990).

3.1.2 The influence of temperature on the stability of nucleic acids
Much research has been undertaken on the structure of the DNA molecule and the effects of various physical and chemical agents on that structure. Specifically, the effect of heat on the properties, structure and biological activity has been the focus of several investigations (Ginoza and Zimm, 1961; Roger and Hotchkiss, 1961; Ginoza and Guild, 1961). Heat is particularly interesting as it leads to extensive physical changes in the state of the molecule in a critical narrow temperature range (Ginoza and Zimm, 1961). On heating, the reported behaviour of DNA differs from that of RNA. The different behaviour is presumed to reflect the great disparity in their configurations, DNA possessing in its native state a rigid, secondarily bonded, double-stranded helical structure, while, the secondary structure in RNA is certainly less extensive (Ginoza and Zimm, 1961).
On exposure of neutral DNA solutions to elevated temperatures, two main types of structural changes occur (Lindahl and Nyberg, 1972) – denaturation and degradation. Denaturation entails the rupture of hydrogen bonds between paired bases, the disturbance of stacking interactions which leads to unwinding of the double helix, to generate two single strands when all hydrogen bonds have been broken. Denaturation does not involve any breakage of covalent bonds (Doty et al. 1960; Ginoza and Zimm, 1961; Jelen et al. 1997; Nelson and Cox, 2005). The rate at which DNA denatures (referred to as its melting temperature ($T_m$)) (typically > 80-85°C) (Nelson and Cox, 2005; McDonnell, 2007) depends on numerous factors such as, the topological condition of the DNA, the length of the DNA sequence, the base composition of the DNA, and the composition of the buffer (Nelson and Cox, 2005). The melting temperature ($T_m$) for multiple copies of DNA molecules is defined “as the temperature at which half of the DNA strands are in the double-helical state and half are in the "random-coil" states” (SantaLucia, 1998).

Another, slower inactivation process also occurs, which is due to heat-induced degradation of the primary structure and can be detected as an irreversible loss of biological activity of transforming DNA (Ginoza and Zimm, 1961; Roger and Hotchkiss, 1961; Alberts and Doty, 1968). Degradation of DNA, by contrast to denaturation, entails the rupture of covalent bonds (Jelen et al. 1997). The reaction occurs both above and below the $T_m$ and is accelerated at low pH (Roger and Hotchkiss, 1961). For prolonged periods at temperatures of 85°C or higher, the polynucleotides strands are damaged further by specific breaks in the phosphodiester linkages between nucleotides in the sugar-phosphate backbone leading to nucleic acid fragmentation (McDonnell, 2007). The chemical effects of elevated temperatures on DNA are considered to be hydrolytic (Peak et al. 1995). This irreversible inactivation is primarily due to depurination of the DNA (Tamm et al. 1953; Greer and Zamenhof, 1962), and/or to the formation of chain breaks (Tamm et al. 1953) and cross-links occurring as a result of depurination (Marguet and Forterre, 1998). Additional types of heat-induced DNA degradation include base deamination predominantly at the 4 position of cytosine (Peak et al. 1995; Shapiro and Klein, 1966), hydrolytic cleavage of pyrimidine glycosyl bonds (Shapiro and Danzig, 1972) and destruction of deoxyribose residues.
3.1.3 The influence of pH on the stability of nucleic acids

It is well documented that DNA is susceptible to acid hydrolysis and the PS modification does not constrict this (Bahr et al. 2009). Hardee et al. 2001 states that PS oligonucleotide degradation can be generally achieved via two main mechanisms, acid-catalysed hydrolysis and desulfurization. Many procedures have been described in the literature for the complete acid hydrolysis of DNA (Fisher and Giese, 1988; Eick et al. 1983; Swarts et al. 1996). However, in all cases formic acid was employed under a variety of conditions in which the concentration of the acid ranged from 60 to 98%, the temperature from 130 to 180°C, and the time from 0.5 to 3.0 hours (Shimelis and Giese, 2006). In contrast to acid hydrolysis, DNA is resistant to alkaline hydrolysis. However, alkaline pH values are regularly used for gentle denaturation of DNA (Jelen et al. 1997).

Two sites are present in nucleotides, which are susceptible to hydrolysis, the glycosidic bond between the deoxyribose sugar and the nucleobase (red arrow) and the phosphodiester bonds (blue arrow) (figure 3.1) (Bahr et al. 2009). Degradation of DNA in acidic conditions occurs by initial cleavage of glycosyl bonds at purine residues (depurination), followed by chain rupture at the weakened abasic sites, rather than by direct cleavage of the phosphodiester bonds between two intact nucleotides (Lindahl, 1996; Sheppard et al. 2000; Jonas et al. 2000; Jelen et al. 1997).
Chapter Three

Figure 3.1 Covalent structure of DNA and sites of hydrolytic attack.
A short segment of one strand of the DNA helix is shown with the four common bases. Arrows show the major sites of hydrolytic attack (adapted from Lindahl, 1996).

In general, low acidic pH leads to depurination of nucleic acids. Depurination is the loss of the purine bases from nucleosidyl units through cleavage of the glycosidic bond between the base and the sugar of DNA under acidic conditions (figure 3.2) (Simon et al. 1999) rendering the nucleic acid functionally inactive. Hydrolytic cleavage of the N-glycosidic bond between a base and its associated deoxyribose group results in the formation of an apurinic/apyrimidinic (AP) site (Weinfield et al. 1989; Lhomme et al. 1999; Neto et al. 1992).
Although base loss occurs spontaneously at neutral pH, the process is greatly enhanced following treatments with physical or chemical agents such as alkylating agents, by ionizing radiation, as well as simply treating with acid or base (Sugiyama et al. 1994). Glycosyl bond cleavage is therefore highly pH dependent, and the rates of cleavage are higher for deoxypurine than for deoxypyrimidine nucleosides, with deoxyguanosine being the most labile nucleoside (Lhomme et al. 1999). The loss of purines occurs at a twenty times higher rate than pyrimidines (Malvy et al. 2000). While this reaction proceeds faster in acid than in neutral solution for all deoxyribonucleosides, the rates of hydrolysis of pyrimidine derivatives are much less pH-dependent in comparison to purine derivatives (Ravikumar et al. 2003). In addition to this it has been observed that thymine is not protonated even at pH values lower than pH 1.0 (Li et al. 2007). Studies for depurination rates have been performed intensively in the literature and results and observations can be summarised under the following four points:

- At low pH and elevated temperature the rate of depurination is accelerated.
- Guanine is released somewhat more rapidly than adenine.
- The depurination rate is accelerated by 106 fold by alkylation
- Depurination rate of single-stranded DNA is four-fold greater than native double-stranded DNA (Suzuki et al. 1994).

A two-step reaction generates cleavage of the phosphodiester backbone. As stated previously, the first step of this reaction is hydrolysis of the N-glycosidic bond. The abasic sites formed in DNA, which are otherwise stable at neutral pH, can subsequently be cleaved via the β-elimination reaction catalysed by acid, alkali, or amines (Dallas et al. 2004), such as piperidine (Amosova et al. 2006) which results in the cleavage of the sugar phosphate bond (Weinfield et al. 1989). In the context of this study, functional inactivation and/or degradation of PS oligonucleotides, in process waste streams, could potentially be achieved by heat and or acid treatment.
3.1.4 Detection and quantification of oligonucleotides

Antisense oligonucleotides can be detected and quantified using a variety of analytical techniques previously outlined in section 1.8 and 2.1.4. For the purpose of this study, two analytical methodologies, Ion Pair–Reversed Phase Liquid Chromatography (IP-RP LC) and Polyacrylamide Gel Electrophoresis (PAGE) were chosen and optimised to detect and quantify PS oligonucleotides to provide quantitative and semi-quantitative determination of the extent of degradation following physicochemical treatment. These methods were chosen as they are easily accessible, commonly employed in most laboratories, generate both accurate qualitative and quantitative data and require small quantities/volumes of test sample (oligonucleotide). Initial analysis was carried out using an isocratic HPLC method but this approach was found to have insufficient resolving power (intact vs. degraded PS oligonucleotides), and hence this approach was discontinued.

3.1.5 Ion-pair reversed-phase liquid chromatography (IP-RP LC)

Oligonucleotides are fundamentally polymeric anions (polyphosphates) which contain hydrophobic moieties and can be separated by numerous chromatographic modes including ion pair reversed-phase HPLC, anion-exchange chromatography, and mixed-mode HPLC which have been described previously in the literature for the quantification of both modified and unmodified oligonucleotides (Djordjevic et al. 1998; Leeds and Cummins, 2001; Gilar et al. 2002; Gilar, 2001; McFarland and Borer, 1979; Andrus and Kuimelis, 2000a; Thompson and Wells, 1988; Westman et al. 1987; McLaughlin, 1989; Huber and Oberacher, 2001). IP-RP LC has traditionally been used for oligonucleotide analysis. The most frequently used mobile phase is an aqueous triethylammonium acetate (TEAA) buffer solution which acts as the ion-pairing agent with acetonitrile as the organic modifier. Gradient elution is generally performed for the separation of oligonucleotides (Djordjevic et al. 1998 Gilar et al. 2007; Gilar et al. 2002).

The ion-pairing additive in the mobile phase is adsorbed on a hydrophobic sorbent and thus provides for charge-to-charge interactions with the negative charges contained on the oligonucleotide backbone (phosphate groups) to attain a regular retention of oligonucleotides according to their chain length (figure 3.3) (Waters Corporation, 2009; Gilar et al. 2002). The introduction of ion-pairing buffers also improves separation selectivity to allow longer (10-30 mer) oligonucleotides to be adequately resolved (Gilar et al. 2002).
Figure 3.3 Proposed mechanism of IP-RP LC for synthetic oligonucleotide separations (adapted from Waters Corporation, 2009).

XBridge™ OST C18 column is the reverse phase column used in this study.
TEA – Triethylammonium ion (ion pairing agent).

The degree of electrostatic interaction, and thus retention, is determined by a number of factors, including hydrophobicity of the column packing, charge, hydrophobicity and concentration of the pairing ion, ionic strength, concentration of organic modifier, temperature and dielectric constant of the mobile phase, and charge and size of the nucleic acid molecule. Elution of the adsorbed oligonucleotides is achieved by a decrease in the surface potential due to desorption of the amphiphilic ions from the stationary phase with a gradient of increasing organic modifier concentration (Huber and Oberacher, 2001). Separation selectivity and resolution decreases with increasing oligonucleotide length making the separation of long oligonucleotides challenging. Modified oligonucleotides such as phosphorothioate and 2-O alkyl modified species are also more difficult to analyse.

Additional solvophobic interactions between hydrophobic regions of the solutes – the nucleobases and the hydrophobic surface of the stationary phase can also occur in IP-RP LC (Huber and Oberacher, 2001). The residual interaction of nucleobases has an impact on overall retention and separation selectivity especially when using TEAA ion-pairing mobile phases. Separation of N and (N–1) mers may be either enhanced or suppressed by the sequence contribution (Waters Corporation, 2009; Schweitzer and Engels, 1997; Gilar et al. 2002). The series of polarity and consequently elution is G>C>A>T (Schweitzer and Engels, 1997) or C<G<A<T (Gilar et al. 2002).
3.1.6 Polyacrylamide Gel Electrophoresis (PAGE)

Electrophoresis is one of the most commonly employed techniques for the separation of nucleic acids (Schweitzer and Engels, 1997; Aynié et al. 1996). Electrophoresis, which was initially described by Tiselius in the 1930’s, can be defined as “the migration of analytes within an electrolyte solution under the influence of an electrical field” (Bogh, 2005). For nucleic acids, the direction of migration, from negative to positive electrodes, is a result of the naturally occurring negative charge of the sugar phosphate backbone, which migrates towards the positively charged anode (figure 3.4). Molecules are separated based on their ratio of mass to charge during electrophoresis (Schweitzer and Engels, 1997) with smaller oligodeoxyribonucleic acids travelling faster than larger strands (Leeds and Cummins 2001). Cross linked polyacrylamide (PAA), which forms a tighter matrix, is generally utilized as a supporting gel for antisense oligonucleotides whilst agarose gels are more suitable for larger molecules as it forms a more open matrix (Schweitzer and Engels, 1997; Voet and Voet, 1995). The concentration of PAA required is based on the length of the oligonucleotide being analysed. Generally, a high concentration of PAA (20%) is required for short oligonucleotides whilst lower concentrations (3%) are applicable for the separation of oligonucleotides up to 1000 base pairs (bp). PAGE encompasses the possibility of running a number of samples on one gel with high sensitivity, particularly in combination with radioactively labeled oligonucleotides. Electrophoresis is generally performed in the presence of urea which acts as a denaturing agent. Under these conditions the oligonucleotides are separated based on their length and sequence specific effects are reduced (Schweitzer and Engels, 1997).

![Figure 3.4 Electrophoretic separation of oligonucleotides.](image)
Depending on the nature and quantity of oligonucleotide being used, a variety of methods for the visualisation of these small biomolecules may be carried out. The most basic method for the visualization of the oligonucleotides is UV-shadowing (Schweitzer and Engels, 1997). Oligonucleotides may also be detected by staining the gel. A variety of different staining techniques are commonly employed which include “Stains-all”, silver staining (Schweitzer and Engels, 1997), methylene blue, SYBR Green (Andrus and Kuimelis, 2000b), and SYBR Gold (Molecular Probes, Inc., 2009; Williams, 2001). In order to achieve greater sensitivity, radioactive samples may be utilized, which can subsequently be identified by autoradiography or fluorography (Chambers and Rickwood, 1993).

3.1.6.1 Silver Staining
Silver staining, one of the most sensitive methods of detection, was established more than two decades ago as a sensitive procedure to identify trace amounts of proteins in polyacrylamide gels. Since then, silver staining has been improved and developed to be applied to the visualisation of other biological molecules such as nucleic acids with high sensitivity and is one of the methods of staining used in this study (Caetano-Anollés and Gresshoff, 1994; Bassam and Caetano-Anollés, 1993). This staining technique operates due to the exchange of counterions of the oligonucleotides with silver ions. These are subsequently reduced to metallic silver. Dark bands are formed by the deposited silver grains while the polyacrylamide stays colourless (Schweitzer and Engels, 1997). Silver staining presents similar sensitivity to autoradiography, avoiding the radioactive handling and waste disposal issues (Bassam and Gresshoff, 2007; Ji et al. 2007). Caetano-Anollés and Gresshoff, (1994) report that nucleic acids, separated on polyester-backed polyacrylamide gels, can be detected at the picogram level by using a simple acidic silver stain.

3.1.6.2 Stains-all
A second method of staining was also utilised in this study, Stains-all. Stains-all, a cationic carbocyanine dye, is a sensitive stain commonly used for the differential staining of nucleic acids and proteins following electrophoretic separation (Dahlberg et al. 1969; Chrambach et al. 1967). The application of differential staining of nucleic acids and proteins acts as follows; DNA (blue, $\lambda$ 675 nm), RNA (bluish purple) and proteins (red, $\lambda$ about 510 nm). It has been established that the level of sensitivity detected on a polyacrylamide gel is as little as 90 ng tRNA and 3 ng (123 BP) fragment) of pBR322/Hae III DNA (Sigma-Aldrich Co., 2008).
3.2 Materials and Methods

3.2.1 Source of chemical reagents

The chemical reagents used in this study were analytical grade and were sourced from Sigma-Aldrich Chemical Co., Dublin, Ireland. Other suppliers include Fluka – a subsidiary of Sigma, British Drug House Laboratory Supplies, Pooles, Dorset, U.K. (BDH); May & Baker Laboratory Chemicals Ltd, Dagenham, U.K. (M&B), Merck, Darmstadt, Germany (Merck) & Oxoid, Basinstoke, U.K. (Oxoid) and Lennox, Dublin, Ireland. Water used to prepare reagent solutions and subsequent dilutions was obtained from a Mili-Q water purification system (Milipore).

3.2.2 Oligonucleotides

A 21–mer all phosphodiester (PO) and all phosphorothioate (PS) oligonucleotide comprising of the sequence of Vitravene™, (5’-GCG TTT GCT CTT CTT CTT GCG-3’) was obtained from Eurofins MWG Operon (London, UK). Stock solutions were prepared by reconstituting each pellet in autoclaved filter sterilized purified water (Mili Q) to a final concentration of 100 μM or 1000μM and from this further diluted 1:10 to a working concentration of 100 μM and stored in aliquots of 100 μl at -20°C. Final sample concentrations were verified using a Nanodrop™ ND-1000 Spectrophotometer.

3.2.3 Method development of IP-RP LC

3.2.3.1 Mobile phase composition

1M stock solution Triethylammonium acetate (TEAA) was prepared according to Schweitzer and Engels, (1997) by mixing on ice 140 ml triethylamine and 58 ml acetic acid. The pH was adjusted to 7.0 using either triethylamine or acetic acid and made up to a volume of 1 L. To make a working solution of 0.1 M TEAA (buffer A), 1 M TEAA was diluted with a nine fold volume of purified water (Mili Q). Buffer B consisted of a combination of both 0.1 M TEAA and chromatography grade acetonitrile (Romil (190 SPS Far UV/gradient quality), Lennox, Dublin). Various mobile phase compositions of buffer B Acetonitrile / 0.1 M TEAA were investigated to ascertain the most suitable ratio premix for buffer B. Four premix blends of aqueous and organic solvents (Mobile Phase B: Acetonitrile / 0.1M TEAA were investigated.

1. Mobile phase A: 0.1 M TEAA and Mobile phase B Acetonitrile only
2. Mobile Phase A: 0.1 M TEAA and Mobile B: Acetonitrile / 0.1 M TEAA, 20/80, v/v.
3. Mobile Phase A: 0.1 M TEAA and Mobile B: Acetonitrile / 0.1 M TEAA, 80/20, v/v.
4. Mobile Phase A: 0.1 M TEAA and Mobile B: Acetonitrile / 0.1 M TEAA, 50/50, v/v.
All solutions were filtered through a 0.45 µM membrane filter and degassed, by sonication, using the ultra sonic bath for 15 minutes prior to use.

3.2.4 Analytical IP-RP LC conditions

Chromatographic separation was performed on a Perkin Elmer liquid chromatographic system equipped with a Series 200 LC binary pump delivery system and a UV Spectrophotometric detector (Shimadzu SPD-6A). An X Bridge™ OST (Oligonucleotide Separation Technology) C\textsubscript{18} column (Waters, Dublin, Ireland), 2.5 µm (4.6 x 50mm) was used as the stationary phase for separation. A column guard (Phenomenex) was attached to the head of the analytical column. Elution of the oligonucleotides was carried out using a gradient of 0.1M triethylammonium acetate (TEAA), (pH 7.0), (A) and buffer solution (B) containing 0.1M TEAA and acetonitrile (50/50 V/V) using the following elution gradient: 10% to 90% (B) over 25 minutes, followed by washing at 90% (B) for 3 minutes, back to 10% (B) for 5 minutes and column re-equilibration at 10% (B) for 10 min based on a modified version described by Schweitzer and Engels, (1997). The mobile phase was delivered at a flow rate of 1.0 ml/min. The injection volume was 20 µl for all HPLC runs. Analysis was performed at ambient temperature and detection was carried out at a wavelength of 260 nm.

3.2.5 Method validation

The analytical parameters employed for validation of the proposed gradient HPLC method were, precision, specificity and identification, linearity, limit of detection (LOD) and limit of quantitation (LOQ). The method was validated according to ICH Q2B guideline 1996 (ICH, 1996).

3.2.5.1 Linearity

The linearity of the response of peak area to concentration was determined at 13 concentration levels, (each in triplicate), ranging from 0.25 µM to 200 µM for both the unmodified PO and modified PS oligonucleotides. A calibration curve was constructed by plotting the peak area calculated against the concentration of the oligonucleotides. The limit of detection (LOD) and limit of quantitation (LOQ) were derived from linearity studies.
3.2.6 Electrophoretic detection and semi-quantification of PS and PO oligonucleotides

Electrophoretic detection and semi-quantification of both unmodified and modified oligonucleotides was carried out using 20%/25% denaturing PAGE containing 7M urea (Schweitzer and Engels, 1997). Twelve concentration levels, 0.25, 0.5, 1, 2.5, 5, 10, 25, 50, 75, 100, 150 and 200 µM were prepared. Equal volumes of each concentration were mixed with 10 µl tracking dye. The samples were heated at 90°C for 2 minutes followed by cooling on ice. From this mixture 10 µl of sample was loaded on each lane and allowed to electrophorese at 250 V (or a constant current of 20 mA). The resultant gels were subsequently stained by silver staining (Schweitzer and Engels, 1997) and Stains-all. The gels were stained with Stains-all overnight, in the dark, at room temperature with freshly prepared staining solution. Destaining was achieved by exposing the stained gel to light until sufficient bands were evident. The gels were then subsequently photographed and scanned.

3.2.7 IP-RP LC analysis of thermal stability of PO and PS oligonucleotides

Equal volumes of 50 µM PS oligonucleotide solution (section 3.3.2) was added to TE buffer (10mM Tris pH 8, containing 1mM EDTA) and distributed into 6 micro tubes corresponding to each analysis time point, 0, 0.5, 1, 2, 4 and 8 hours. Samples were incubated in a water bath or heating block at 40, 60, 80 or 100ºC respectively. After incubation, samples were immediately placed on ice, briefly vortexed and subsequently injected onto the HPLC column (section 3.2.4). Each sample was analysed in triplicate and the mean and standard deviation was calculated for each parameter studied.

3.2.8 Electrophoretic analysis of thermal stability of PO and PS oligonucleotides

The PO and PS oligonucleotides were incubated at ambient room temperature or elevated temperatures as described in section 3.2.7 followed by PAGE analysis (section 3.2.6). Each sample was carried out in triplicate.

3.2.9 IP-RP LC analysis of the effect of steam sterilisation on PO and PS oligonucleotides

Sealed micro tubes containing 70µl 25 µM PO or PS oligonucleotide were autoclaved (121ºC and 15 psi for 15 minutes). The micro tubes were immediately placed on ice and injected onto the HPLC column (triplicate analysis).
3.2.10 Electrophoretic analysis of the effect of steam sterilisation of PO and PS oligonucleotides.

Similar to the method outlined in section 3.2.9 PO and PS oligonucleotides (25 µM) were subjected to steam sterilisation and subsequently analysed by PAGE followed by staining with silver staining and Stains-all.

3.2.11 IP-RP LC analysis of the pH stability of PO and PS oligonucleotides

Stock solutions of 1M NaOH and 1M HCl were made up to a volume of 100 and 250ml respectively with purified water (Mili Q). From these, serial dilutions were made up and their respective pH values were recorded. The concentration used in the analysis of the stability of PO and PS oligonucleotides over a range of pH values are outlined in table 3.1.

<table>
<thead>
<tr>
<th>Chemical reagent</th>
<th>Concentration (M) (Final reaction conc.)</th>
<th>Length of incubation (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium hydroxide (NaOH)</td>
<td>0.005</td>
<td>1, 30 and 60</td>
</tr>
<tr>
<td>Hydrochloric acid (HCl)</td>
<td>0.0005mM, 0.0005, 0.005, 0.05, 0.5</td>
<td>1, 30 and 60</td>
</tr>
</tbody>
</table>

A control PO and PS oligonucleotide (25 µM) was injected on to the HPLC in triplicate. The mean peak area was calculated to correspond to 100% intact PO and PS oligonucleotide.

Equal volumes of PO and PS oligonucleotides (50µM) was added to 0.01M NaOH (pH 11.41) in three separate micro tubes representing the three time points of analysis, time 1, 30 and 60 minutes, to give a final oligonucleotide concentration of 25 µM, and injected onto a 20 µl loop by manual injection. The effect of low pH was analysed in a similar manner using 0.0005 mM, 0.0005, 0.005, 0.05 and 0.5 M HCl. Each acidic/basic reaction mixture, at each time point, was analysed at ambient room temperature (25°C) in triplicate.

3.2.12 IP-RP LC analysis of the effect of pH in combination with elevated temperature on the stability of PO and PS oligonucleotides

The same procedure as described in section 3.2.11 was carried out for each acidic and basic concentration/pH over a period of 60 minutes at 40, 60 or 80°C and subsequently analysed by IP-RP LC.
3.2.13 Electrophoretic analysis of pH stability of PO and PS oligonucleotides

Incubation of the PO and PS oligonucleotide with HCl / NaOH was carried out as described in section 3.2.11. The composition of the aliquots withdrawn at appropriate intervals was subsequently determined in triplicate by PAGE as outlined in section 3.2.6.

3.2.14 Electrophoretic analysis of the effect of pH in combination with elevated temperature on the stability of PO and PS oligonucleotides

The experimental conditions as described in section 3.2.12 was carried out for each acidic and basic solution/pH over a period of 60 minutes at 40, 60 or 80°C and subsequently analysed by PAGE in triplicate followed by staining with silver staining and Stains-all.

3.2.15 IP-RP LC and electrophoretic analysis of the pH stability of PS oligonucleotides to sulphuric acid and nitric acid

Stock solutions of 1M sulphuric acid (H\textsubscript{2}SO\textsubscript{4}) and nitric acid (HNO\textsubscript{3}) were made up. Serial dilutions of both acidic solutions were made up and their respective pH values were recorded. The concentration employed in the analysis of the stability of PS oligonucleotides over a range of pH values are outlined in table 3.2.

| Table 3.2 Experimental conditions used in the analysis of the stability of PO and PS oligonucleotides to H\textsubscript{2}SO\textsubscript{4} and HNO\textsubscript{3} of varying concentrations. |
|---------------------------------------------|---------------------------------------------|
| Chemical reagent | Concentration (M/%) (Final reaction conc.) | Length of incubation (min) |
| Sulfuric acid (H\textsubscript{2}SO\textsubscript{4}) | 0.0005, 0.0025, 0.005, 0.05, 0.5M | 1, 30 and 60 |
| Nitric acid (HNO\textsubscript{3}) | 3.5, 5, 10, 15% | 1, 30 and 60 |

Similarly, equal volumes of PS oligonucleotides (50 µM) was added to sulphuric acid or nitric acid, at a dedicated concentration, in three separate micro tubes representing the three time points of analysis, time 1, 30 and 60 minutes and analysed in triplicate at ambient room temperature or 40°C via IP-RP LC and PAGE.

3.2.16 Analysis of nucleobase and DNTP mix (depurination products)

Nucleic acid bases thymine (T), guanine (G) and adenine (A) were made up to a concentration of 0.1 M in 1 M NaOH. Cytosine (C) was reconstituted in 0.5 M HCl to a stock solution of 0.1 M. Serial dilutions of each base, within the range of 10 to 250 µM,
were made up in water and analysed via HPLC in triplicate. A calibration curve was constructed for each nucleobase.

3.2.17 Statistical analysis

Data analysis, including calculation of mean, standard deviation and linear regression was conducted using Excel 2010 (Microsoft, USA).
3.3 Results and Discussion

3.3.1 Method development - Mobile phase composition

In order to establish a suitable gradient programme various mobile phase compositions were investigated. It is strongly recommended that the mobile phase B composition contains a “premix blend” of aqueous and organic solvents (e.g., Mobile Phase B = Acetonitrile/0.1 M TEAA, v/v) to minimize potential inadequate solvent mixing which can compromise component resolution (Waters Corporation, 2009). It was established that a mobile Phase consisting of Mobile phase A = 0.1 M TEAA and Mobile Phase B = Acetonitrile/0.1 M TEAA, 50/50, v/v) produced the clearest baseline with minimal background noise and also permitted the use of a more sensitive attenuation setting most likely due to the decrease in the percentage acetonitrile in mobile phase B.

3.3.2 Method Validation

3.3.2.1 Precision: Repeatability and reproducibility, specificity and identification

Precision is the degree of repeatability of an analytical methodology among individual test results obtained following multiple sampling of a homogenous sample under normal operational conditions (van Iterson, 2009; Kumar et al. 2010). The test analysis equivalent concentration (25 µM PS oligonucleotide) was injected onto the HPLC system six times. The precision was determined by the retention time the peak eluted off at and the peak area. The mean retention time was detected as 9.17 minutes (±0.07), (n=6). The mean peak area was 473234 and the percentage relative standard deviation was 0.8% significantly less than the acceptable range of 5% (Bartolomeo and Maisano, 2006; Harrison et al. 2003). From this, it was established that this method produced reproducible results.

The retention time precision is important, as not only is retention time the main method for peak identification, but also fluctuations can signify problems within the LC system (van Iterson, 2009). The specificity and identification was determined by comparing retention times of the test sample concentration (six samples). The retention time for the modified PS oligonucleotide was found to be 9.1 to 9.6 minutes and 6.0 to 6.3 minutes for PO oligonucleotides which allows confident highly specific, peak identification of both unmodified PO oligonucleotide and modified PS oligonucleotides.
3.3.2.2 Linearity

Using the optimized chromatographic conditions, a steady baseline was recorded and a linear relationship was evaluated across the range of the analytical procedure. Each concentration (0.25 – 200 µM equivalent to an absolute load of 5 pmol - 4 nmol) of PO or PS oligonucleotide was injected onto the HPLC column in triplicate, the mean peak area was calculated, and a calibration curve of concentration of PO or PS oligonucleotide versus peak area was constructed (figure 3.5 and 3.6). The peak area increased proportionately with increasing concentration of PO or PS oligonucleotides. Good straight line correlations between the concentration and the peak area were obtained. An $R^2$ value of 0.9885 and 0.999 was obtained for the standard curve of PO and PS oligonucleotides respectively which indicates good linear reliability. The results indicate that an excellent correlation exists between peak area and concentration of both PO and PS oligonucleotide within the specified concentration range.

![Figure 3.5 Calibration curve of peak area vs. concentration of PO oligonucleotides ranging from 5 pmol - 4 nmol.](image)

Standard PO oligonucleotide solutions (5 pmol - 4 nmol) were prepared in purified water. HPLC analysis of PO oligonucleotides was performed using liquid chromatography equipped with a UV detector ($\lambda = 260$ nm, a 20 µL loop injector and an X Bridge™ OST (Oligonucleotide Separation Technology) C$_{18}$ column, 2.5 µm particle size (4.6 x 50mm). Elution of the PO oligonucleotides was carried out using a gradient of 0.1M Triethylammonium acetate (TEAA), (pH 7.0), (A) and buffer solution (B) containing 0.1M TEAA and acetonitrile (50/50 V/V) at a flow rate of 1.0 ml/min. Each value represents mean ± SD (n=3).
Figure 3.6 Calibration curve of peak area vs. concentration of PS oligonucleotides ranging from 5 pmol – 4 nmol.

Standard PS oligonucleotide solutions (5 pmol - 4 nmol) were prepared in purified water. HPLC analysis of PS oligonucleotides was performed using liquid chromatography equipped with a UV detector (λ = 260nm), a 20 µL loop injector and an X Bridge™ OST (Oligonucleotide Separation Technology) C18 column, 2.5 µm particle size (4.6 x 50mm). Elution of the PS oligonucleotides was carried out using a gradient of 0.1M Triethylammonium acetate (TEAA), (pH 7.0), (A) and buffer solution (B) containing 0.1M TEAA and acetonitrile (50/50 V/V) at a flow rate of 1.0 ml/min. Each value represents mean ± SD (n=3).

3.3.2.3 Limit of detection and limit of quantitation

The Limit of Detection (LOD) and Limit of Quantification (LOQ) of the developed method were determined by injecting progressively lower concentrations of oligonucleotide using the developed IP-RP LC method. The detection/quantitation limit was established by the analysis of samples with known concentrations of analyte and by determining the minimum level at which the analyte can be reliably detected/quantified with acceptable accuracy and precision (ICH, 1996). LOD and LOQ were determined from residual standard deviation of the regression line and slope method according to ICH guideline (ICH, 1996). A specific calibration curve was examined using samples containing the analyte in the range of detection limit (DL) and quantitation limit (QL). The LOD and LOQ for PO oligonucleotides was found to be 0.1 and 0.3 µM (2.1 and 6.3 pmol). The LOD and LOQ for PS oligonucleotides was found to be 0.2 and 0.6 µM (3.7 and 11.1 pmol).
3.2.3.1 Comparison of PO and PS oligonucleotide by IP-RP LC

Figure 3.7 represents a chromatogram of the analysis of a co-injection of both PO and PS oligonucleotides at the outlined concentration and illustrates the differences observed between unmodified and modified oligonucleotides by IP-RP LC.

![Image](image.png)

**Figure 3.7 Chromatographic analysis of a co-injection of PO and PS oligonucleotides as analysed by IP-RP LC.**

Description of a typical chromatogram: Both 2 cm graduation and the time after starting analysis are printed on the time axis of the chromatogram. The integrator records the peak number, peak area, peak height and percentage concentration. The peak numbers are printed above each peak corresponding to the order in which the peak elutes and appears on the chromatogram. Peak detection markers / points and termination points are also indicated on the chromatogram.

The most significant difference between PO and PS oligonucleotides, observed by IP-RP LC, was the disparity in retention time (6.0-6.3 minutes versus 9.1-9.6 minutes, respectively). PS oligonucleotides are more hydrophobic in nature (Gilar et al. 1998b), and therefore require a greater percentage organic modifier (acetonitrile) to elute them from the stationary phase. No significant difference was observed in the peak area values over the 13 concentration levels analysed in the linearity of the system. The shape of the peak was somewhat broader for PS oligonucleotides than for PO oligonucleotides. This is characteristic of PS oligonucleotides and it is rational to presume that this broadness is due to the presence of the large number of diastereoisomers (Schweitzer and Engels, 1997; Murakami et al. 1994).

3.3.3 Electrophoretic detection and semi-quantification of oligonucleotides

Using the optimized electrophoretic conditions, 12 concentration levels ranging from 0.125 - 100 µM (equivalent to an absolute load of 1.25 - 1000 pmol) for PO oligonucleotides were loaded on the gels in triplicate and subsequently visualised by silver staining as presented in
Chapter Three

The same procedure was carried out to analyse modified PS oligonucleotides (figure 3.8 (b)). Differential levels of sensitivity of silver staining to both PO and PS were observed. It was observed that silver staining was significantly more sensitive to the analysis of PS oligonucleotides which had a limit of detection of 0.25 µM (2.5 pmol) in comparison to 12.5 µM (125 pmol) for PO oligonucleotides. It was also observed that the working range of PS oligonucleotides was between 1.25 and 25 µM (12.5 - 250 pmol) as at higher concentrations additional bands (due to impurities) were produced. The working range for PO oligonucleotides was between 37.5 and 100 µM (375 – 1000 pmol (and above)).

An alternative staining method was also investigated for the detection and semi-quantification of PO and PS oligonucleotides to establish the appropriate quantity of oligonucleotide required to produce a distinct, visible, sharp band and to allow for adequate visualisation. Figure 3.9 (a and b) present the results obtained following detection and semi-quantification of the oligonucleotides over the range of 0.125 – 100 µM (1.25 - 1000 pmol). Following staining with “Stains-all” it was observed that the PO oligonucleotides stained blue whilst the PS oligonucleotides stained purple. Similar results were observed Grajkowski et al. (2005) and Serge Beaucage, who stated that “Purple staining is truly characteristic of PS oligonucleotides” (Grajkowski et al. 2005; Serge Beaucage (FDA) personal communication, 2010). Little if any disparities were observed in the level of detection sensitivity between PO and PS oligonucleotides which had a limit of detection of 0.25 µM (2.5 pmol) and a working range between 1.25 and 25 µM (12.5 – 250 pmol) for both oligonucleotide chemistries. The PO oligonucleotide displays a slightly higher mobility in the gel matrix in contrast to the PS oligonucleotide and runs slightly further down the gel
as illustrated in figure 3.9 (c). Similar findings were reported by Gilar et al. (1997) who established that PS oligonucleotides exhibit a slower electrophoretic mobility in non-denaturing gels in contrast to their phosphodiester analogues.

**Figure 3.9 Electrophoretic analysis of PO and PS oligonucleotides by 20% denaturing PAGE followed by staining with Stains-all.**

PO oligonucleotides 1000–1.25 pmol, per lane (a), PS oligonucleotides 1000–1.25 pmol, per lane (b), and PO/PS oligonucleotides 250-12.5 pmol, per lane (c). The limit of detection was 2.5 pmol and the working range was between 12.5–250 pmol for both oligonucleotide chemistries.

### 3.3.4 Comparison of PO and PS oligonucleotide

Significant disparities were noted following the analysis of PO and PS oligonucleotides by PAGE following staining with silver staining (figure 3.8). Most noticeably, the method of silver staining is significantly more sensitive to the analysis of PS oligonucleotides in comparison to their unmodified counterparts. This may be due to the favoured metal interactions of the soft silver metal ions with the sulfur replacement. The limit of detection and quantification are significantly different for both oligonucleotides as described previously. Due to the variation in the sensitivity of this method of staining, conclusive determinations with regards to the disparities in thickness and migration of the bands
produced by both types of oligonucleotides were unable to be established. Staining of both oligonucleotide chemistries with Stains-all resulted in a characteristic purple band for PS oligonucleotides and a blue band which moved slightly further down the gel for the unmodified PO oligonucleotide (figure 3.9).

Both staining methods, silver staining and Stains-all, displayed similar detection sensitivities for the analysis of modified PS oligonucleotides with a LOD of 0.25 μM (2.5 pmol) following both staining methods. Sigma-Aldrich Co., (2008) reported that Stains-all is generally less sensitive than silver stains and coomassie blue most specifically to nonacidic proteins (Sigma-Aldrich Co., personal communication, 2008). However, little if any disparity in level of sensitivity was observed for the detection and quantification of PS oligonucleotides under the experimental conditions employed in this study. Additionally, staining with Stains-all was significantly more sensitive to the analysis of unmodified PO oligonucleotides (LOD 0.25 μM (2.5 pmol)), than staining with silver staining which had a LOD of 12.5 μM (125 pmol).

Based on these initial studies it was established that both analytical methods are suitable for the detection and quantification/semi-quantification of PS oligonucleotides and the degradation products produced following physicochemical treatment. Additionally, the application of both HPLC and PAGE allows the detection of desulfurization, (Chapter Four), by either a shift in retention time (HPLC) or a colour change (PAGE followed by staining with “Stains-all”) and degradation of the PS oligonucleotides. Strong anion exchange chromatography, \(^{31}\)PNMR, and HPLC-MS are the major analytical methods for determining the percentage P=O vs. P=S linkages (Capaldi and Scozzari, 2007). However, analysis via PAGE followed by staining with “Stains-all” offers an alternative, effective and simplified method to detect desulfurization by an associated colour change when specialised equipment may not be readily available.
3.3.5 IP-RP LC analysis of temperature stability of PO and PS oligonucleotides

The effect of temperature on the stability of PS oligonucleotides was analysed, over a wide temperature range, ranging from 25 to 100°C over a period of up to and including 8 hours. The PS oligonucleotides were made up in TE buffer pH 8.0, (10mM Tris pH 8, containing 1Mm EDTA), as this is the pH and buffering solution most commonly recommended for resuspension and storage of oligonucleotides as the covalent structure of DNA appears to have its maximum stability in aqueous solution at pH 8.0-8.5 (Lindahl and Nyberg, 1974). This is primarily due to the fact that the rate of deamination of cytosine residues in DNA is slowest at this pH range. In addition to this, the cleavage of purine-deoxyribose bonds also occurs at a minimal rate (Lindahl and Nyberg, 1974). Therefore, any significant change in peak area would be as a direct result of the influence of temperature. The effect of temperature on PS oligonucleotides was analysed by both IP-RP LC and PAGE as described in section 3.2.7 and 3.2.8.

![Figure 3.10](image)

**Figure 3.10** Thermal stability of PS oligonucleotide to temperatures ranging from 25 to 100°C over a period of 8 hours as assessed by IP-RP LC. Each value represents mean ± SD (n=3).

Figure 3.10 illustrates the trend in peak area over the analysis period of 8 hours at the respective elevated temperatures. No significant change in peak area was detected, indicating that the PS oligonucleotides are stable at pH 8 over a temperature range of 25 – 80°C over the period of up to and including 8 hours. Minimal degradation was observed for prolonged periods of 4 and 8 hours at the elevated temperature of 100°C. The graph in figure 3.10 illustrates a similar trend where the peak area value initially increased and subsequently decreased again after increased length of incubation. This trend was most pronounced at
elevated temperatures of 60, 80 and 100°C. The peak area of the PS oligonucleotide appears to increase with increasing length of incubation of 2 hours at 60°C. This may be due to the fact that initial breakdown of the PS oligonucleotide was beginning to occur, and that the degradation products formed were masked under the main peak and could not be fully resolved from the intact PS oligonucleotide. A similar trend was also observed after 0.5 hours incubation at 80°C where an increase in peak area was detected up to and including 2 hours of analysis. The peak of the intact PS oligonucleotide is wider at the base, (increasing in peak area), suggesting that slight degradation is beginning to occur and the presence of hidden degradation peaks which increase in number and magnitude with increasing length of incubation as highlighted by the peak detection and termination points. However, at time 4 hours a decrease in peak area is detected, indicating that these breakdown products are increasing in size and as a result are been resolved from the intact PS oligonucleotide. A similar trend and profile was observed at the elevated temperature of 100°C where additional degradation products were detected after heat treatment for prolonged periods of over 2 hours.

Figure 3.11 presents representative chromatographic results obtained at the highest temperature (100°C) of analysis after a period of 0.02, 4 and 8 hours. No significant change in response was detected after elevated temperature of 100°C after 1 hour. However, after increased length of incubation of 2 hours, degradation products were detected. It is evident from the chromatograms (figure 3.11 b and c) that the main peak starts to decrease in peak area as indicated by the blue arrow with the appearance of new, small degradation products (red arrows). An early eluting peak at 0.7 minutes increased in magnitude after 2 and 4 hours and to a greater extent after 8 hours as highlighted by the green arrow.
Figure 3.11 Chromatographic results of the effect of elevated temperature on the stability of PS oligonucleotides.

The analysis of thermal stability of PS oligonucleotides was performed in 10 mM Tris-HCl buffer pH 8, containing 1mM EDTA at 100°C for a). 0.02 h, b). 4 h, c). 8 h. The peak numbers are printed above each peak corresponding to the order in which the peak elutes and appears on the chromatogram. The retention time is denoted on the time axis of the chromatogram.

▲ = appearance of degradation products  ✔ = appearance of early eluting degradation products
▼ = decrease in peak area.
Unmodified PO oligonucleotides were also subjected to treatment with elevated temperature of 100°C, over a period of 8 hours, to evaluate if PO oligonucleotides are more susceptible to thermal degradation than modified PS oligonucleotides. It was established that PO oligonucleotides display a similar response to thermal stress. No significant degradation was observed over a period of up to and including 8 hours. Similar chromatographic results as outlined in figure 3.11 were observed under such experimental conditions. Figure 3.12 outlines the change, or lack or significant change, in peak area of the PO oligonucleotide following treatment with elevated temperature (100°C) over a period of 8 hours. What is evident is that the peak area increases with increasing length of incubation most significantly at time 1 hour where degradation of oligonucleotide is beginning to occur and the breakdown products are masked under the main peak. With increasing length of incubation the rate of degradation increased generating small breakdown products that are more easily resolved from the main peak thus decreasing the peak area of the main peak product.

**Figure 3.12 Thermal stability of PO oligonucleotide at 100°C over a period of 8 hours as assessed by IP-RP LC.** Each value represents mean ± SD (n=3).

### 3.3.6 Electrophoretic analysis of temperature stability of PO and PS oligonucleotides

Electrophoretic analysis was also carried out to assess the thermal stability of PO and PS oligonucleotides over a temperature range of 25, 40, 60, 80 or 100°C for periods of up to 8 hours. From electrophoretic analysis, followed by staining with Stains-all, it was established that both PO and PS oligonucleotides were stable over a temperature range of 25-80°C over the 8 hour period of analysis. However, at the elevated temperature of 100°C, after increased length of incubation, initial break down of the oligonucleotide was detected (figure 3.13), in
the form of smaller bands moving further down the gel, similar to results obtained by HPLC where initial degradation was observed after prolonged periods of 4 and 8 hours.

Figure 3.13 Electrophoretic analysis of the effect of elevated temperature of 100°C on PO oligonucleotides (a) and PS oligonucleotides (b).

Oligonucleotides were visualised upon staining the gel with Stains-all. C1: Oligonucleotide only; C2: TE buffer only (10mM Tris pH 8, containing 1mM EDTA).

3.3.7 IP-RP LC and electrophoretic analysis of the effect of steam sterilisation on PO and PS oligonucleotides.

Table 3.3 outlines the results obtained following treatment of PO and PS oligonucleotides with steam sterilisation (section 3.2.9 and 3.2.10). The experimental conditions employed, retention time and percentage degradation were recorded and the mean ± standard deviation calculated. Following steam sterilisation, significant degradation of both oligonucleotide chemistries was detected via HPLC analysis. Autoclaving conditions effectively degraded the PO oligonucleotide by 78.7% and the PS oligonucleotide by 77.8%. In addition to this, a slight shift in retention time was detected following steam sterilisation. This is most likely due to the decrease in peak area of the sample oligonucleotide.

Table 3.3 Analysis of the effect of steam sterilisation on PO and PS oligonucleotides by IP-RP LC

<table>
<thead>
<tr>
<th>Condition</th>
<th>PO Oligonucleotides</th>
<th>PS Oligonucleotides</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Retention Time (min)</td>
<td>Percentage degradation (%)</td>
</tr>
<tr>
<td>Control / Before autoclaving</td>
<td>6.3 ± 0.1</td>
<td>NA</td>
</tr>
<tr>
<td>After autoclaving</td>
<td>6.1 ± 0.2</td>
<td>78.7 ± 4.0</td>
</tr>
</tbody>
</table>

Figure 3.14 illustrates two chromatograms that represent a control, prior to steam sterilisation, and following steam sterilisation of the modified PS oligonucleotide. The red arrow highlights the decrease in peak area of the peak corresponding to that of the intact
oligonucleotide whilst the green arrow and black brace represents the breakdown products detected after thermal stress under autoclaving conditions. The main peak decreased significantly by 77.8% after steam sterilisation producing many subsequent breakdown products, eluting earlier than the main peak but with some additional breakdown products, more hydrophobic in nature, which eluted with a later retention time. Similar chromatographic results were obtained for the unmodified PO oligonucleotides.

![Figure 3.14 Chromatographic results on the effect of steam sterilisation of PS oligonucleotide.](image)

**Figure 3.14 Chromatographic results on the effect of steam sterilisation of PS oligonucleotide.**

- Decrease in peak area
- Appearance of early eluting peaks

The peak numbers are printed above each peak corresponding to the order in which the peak elutes and appears on the chromatogram. The retention time is denoted on the time axis of the chromatogram.
Figure 3.15 represents a PAGE profile of PO and PS oligonucleotide before and after steam sterilisation. After steam sterilisation, the band corresponding to that of the intact oligonucleotide has disappeared, for both oligonucleotide chemistries, with the appearance of smaller breakdown products further down the gel similar to results obtained via HPLC.

**Figure 3.15 Electrophoretic analysis of the effect of steam sterilisation on PO and PS oligonucleotide.** Oligonucleotides were visualised upon staining the gel with Stains-all.

- : before steam sterilisation, +: after steam sterilisation.

### 3.3.8 Discussion of results on the effect of temperature on PO and PS oligonucleotide stability

Similar results were obtained following analysis by both IP-RP LC and PAGE on the thermal stability of PO and PS oligonucleotides. No significant change in peak area or band intensity was detected, indicating that the oligonucleotides are stable at pH 8, over a temperature range of 25–80°C over the period of up to and including 8 hours. This is consistent with results obtained by Neto *et al.* (1992), who observed no significant cleavage upon heat treatment of DNA alone at 70°C. One abasic site was formed after 22 minutes of heating (Neto *et al.* 1992). Most effective degradation was observed at the elevated temperature of 100°C after increased length of incubation of 2, 4 and 8 hours, where initial breakdown of the oligonucleotide was detected by both analytical methodologies. Dr. Richard Hogreffe (TriLink BioTechnologies, personal communication) indicated that PS oligonucleotides are quite stable to high temperatures “unless you plan to exceed 100°C for any length of time”. It appears that for any significant degradation to occur, prolonged periods at elevated
temperatures of 100°C or higher is required. Peak et al. (1995) observed that DNA at 100°C would experience around 3,000 times more hydrolytic events than DNA at 37°C.

Many treatment processes heat waste streams to a temperature of around 60°C. At these temperatures, nucleic acids in microorganisms are not denatured and thus may not be inactivated (Cover et al. 2003). Similarly, Bergemann et al. (1995) established that although numerous microorganisms and cell cultures can be killed by heating at 80°C the DNA is however not destroyed under such conditions. In several biological laboratories in China, a common method for treating wastewater, containing recombinant DNA fragments, is by heating at 100°C (boiling water) for 5-10 minutes (Li et al. 2009). Similar to results obtained in this study, electrophoretic analysis suggested that the recombinant plasmid DNA was not degraded or destroyed effectively upon thermo treatment at 100°C (Li et al. 2009). However, it should be noted that the majority of studies were investigating heat treatment of significantly larger structures of nucleic acids in relation to the 21-mer oligonucleotide is this study.

The known methods documented by the Central Commission of Biological Safety (ZKBS) of the Federal Republic of Germany, for sterilization and cell inactivation which is steam sterilization for 20 minutes at 121°C (Cover et al. 2003; Bergemann et al. 1995) was also analysed for its effectiveness at degrading the PO and PS oligonucleotides. When the oligonucleotides were subjected to high temperatures of 121°C for 15 minutes (autoclaving conditions), a significant decrease in peak area of 78.7 and 77.8% for PO and PS oligonucleotides respectively was observed by IP-RP LC analysis. However, Doblhoff-Dier et al. (2000) established that traditional inactivation procedures (e.g. autoclaving) did not break DNA down to the monomer level. In this case the DNA was much larger than 21 base pairs.

Modern autoclaves consist of a cylindrical or rectangular chamber that range in size from small and simple desk-top systems to complex systems with large chambers for industrial scale processing, with capacities ranging from 400 to 800 litres (Sultana, 2007). This method of treatment would be suitable for the treatment of antisense waste products as autoclaves have the capacity to hold and sterilise large volumes. However, to achieve this level of degradation rather elaborate equipment is required and such treatment is very expensive especially at industrial scale. These processes are very cost intensive not only for apparatus but also due to the high energy consumption, increasing the carbon footprint, and
as a result may be impractical (Cover et al. 2003; Bergemann et al. 1995). Conversely, McDonnell, (2007), states that steam sterilisation is cost effective with low running costs. Moreover, care should be taken to safeguard that materials have effectively cooled down prior to handling. In some instances, the cooling down period is disadvantageous especially for the use of large vessels employed in manufacturing facilities (McDonnell, 2007). In contrast to this, it is reported that heat treatment is the most commonly employed method for sterilising solid or semi-solid waste and liquid effluent especially in large-scale operations (Doblhoff-Dier et al. 2000; McDonnell, 2007) involving destruction of enzymes and other essential cell constituents (Sultana, 2007). In addition to this, heat treatment has the advantage of being easy to validate and control, flexible for various applications, widely available, is easy to use, and does not give rise to the release of toxic chemicals into the environment (Doblhoff-Dier et al. 2000; McDonnell, 2007).

In addition to the significant breakdown obtained, following steam sterilisation and thermal stress after 4 and 8 hours at 100°C, a complex pattern of degradation products was formed (3.11 and 3.14). If neutral aqueous solutions of DNA are incubated at elevated temperatures, changes in the covalent structure occur and amass at a slow rate. This heat-induced internucleotide cleavage is not indiscriminate (Lindahl and Nyberg, 1974). Similar results were obtained by Daniel Capaldi, vice president of analytical and process development (ISIS Pharmaceuticals), (personal communication) by subjecting an antisense drug product to thermal stress to assess the long term stability of the drug substance. This was carried out over the prolonged period of 28 days at 80°C and 5 days at 70°C (pH 7.5). Similar chromatographic results were obtained when the PS oligonucleotide in this study was subjected to 100°C for 4 and 8 hours whereby no significant decrease of PS oligonucleotide peak area was observed but there was evidence of increasing early eluting peaks, small initial breakdown products and larger peaks close to the sample peak. Similar results were also obtained by Rentel et al. (2005), which attributed the complex pattern of degradation products, by mass spec, as a result of depurinated, deaminated, desulfurized, and length shortened oligonucleotides following analysis of thermal stress of oligonucleotides at 90°C for four weeks. Depurination was determined to be the main pathway of degradation under such conditions. Hydrolytic attack, at a small number of sensitive hotspots, especially at the N-glycosyl bonds (Lindahl and Nyberg, 1974), was supported by the increase in apurinic oligonucleotides detected in addition to the accumulation of the specific shorter oligonucleotides following cleavage at sites adjacent to the 2′-deoxyguanosine and 2′-
deoxyadenosine residues (Rentel et al. 2005). It seems likely that heat induced deamination of cytosine residues and the cleavage of glycosidic bonds are the two most frequent forms of hydrolytic degradation of the primary structure of DNA (Marguet and Forterre, 1994; Neto et al. 1992; Lindahl and Nyberg, 1974) and represent the majority of the break down products formed.

Hardee et al. (2001) also established that desulfurization results upon treatment of PS oligonucleotides at elevated temperature. From analysis in this study of the effect of thermal stress, no appearance of a peak at the earlier retention time of 6.0 to 6.3 (retention time corresponding to that of the intact PO oligonucleotide) or significant shift in retention time was detected with increased incubation time at each of the outlined temperatures. However, early eluting peaks were detected which may indicate partial desulfurization of the PS oligonucleotide following steam sterilisation and heat treatment of 100°C after 4 and 8 hours.

3.3.9 IP-RP LC and electrophoretic analysis of pH stability of PS oligonucleotides at ambient room temperature

The stability of PS oligonucleotides to acidic and basic conditions was investigated, at ambient room temperature, using NaOH and HCl solutions ranging in pH values from 0.32 to 11.41 (Final pH value of the reaction mixture) over a period of 60 minutes. The final pH values of the respective solutions are detailed in table 3.4. Analysis was carried out by IP-RP LC and PAGE as described in section 3.2.11 and 3.2.13. Percentage intact PS oligonucleotide was quantified and calculated based on the peak area response and ratio of band intensity corresponding to intact and degraded oligonucleotides. Results obtained are presented as percentage intact PS oligonucleotide over each pH value over the three time periods of analysis.

Table 3.4 pH values of HCl and NaOH solution(s).

<table>
<thead>
<tr>
<th>Chemical</th>
<th>Initial concentration (M)</th>
<th>pH value</th>
<th>Final treatment concentration (M)</th>
<th>Final pH value of the reaction mixture</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium hydroxide (NaOH)</td>
<td>0.01M</td>
<td>11.74</td>
<td>0.005</td>
<td>11.41</td>
</tr>
<tr>
<td>Hydrochloric acid (HCl)</td>
<td>1M</td>
<td>0.02</td>
<td>0.5</td>
<td>0.32</td>
</tr>
<tr>
<td></td>
<td>0.1M</td>
<td>0.98</td>
<td>0.05</td>
<td>1.23</td>
</tr>
<tr>
<td></td>
<td>0.01M</td>
<td>1.95</td>
<td>0.005</td>
<td>2.25</td>
</tr>
<tr>
<td></td>
<td>0.001M</td>
<td>3.02</td>
<td>0.0005</td>
<td>3.25</td>
</tr>
<tr>
<td></td>
<td>0.000001M</td>
<td>5.41</td>
<td>0.0000005</td>
<td>5.53</td>
</tr>
</tbody>
</table>
As can be seen from figure 3.16 the PS oligonucleotides are stable over a period of up to 60 minutes at pH 11.41, 5.53, 3.25 and 2.25, where no significant degradation was observed over the period of analysis. This is in agreement with results obtained by Martin Gilar, (Waters Corporation) (personal correspondence) who states that the stability of PS oligonucleotides to temperature and pH is very high. Deshmukh et al. (2000d) also established that ISIS 2302, a PS oligonucleotide, was stable at pHs ranging from 6.9 to 9.0 over a period of four months at both 25 and 40°C. A small reduction (less than 3%) in the fully thioated oligonucleotide was observed under such experimental conditions. The rate of length reduction in addition to desulfurization of the oligonucleotide increased to some extent at the lower pH of 6.0 and to a greater extent at pH 5.0 which was consistent with results obtained in this study. However, no desulfurization of the PS oligonucleotide was observed over the wide pH range analysed.

![Figure 3.16 The pH stability of PS oligonucleotide after treatment with acidic and basic solutions at ambient room temperature as assessed by IP-RP LC.](image)

The PS oligonucleotide was incubated with NaOH and HCl solutions ranging in pH values from 0.32 to 11.41 over a period of 60 minutes. pH values represent the final pH value of HCl/NaOH. Each value represents mean ± SD (n=3).

However, treatment with HCl at less than pH 2 (1.23 and 0.32) lead to initial degradation, up to 31.8%, after 30 minutes treatment with 0.05 M HCl. Consistent with results obtained by Lindahl and Nyberg, (1972), it was established that the rate of depurination and degradation of DNA increased with decreasing pH values, most notable at a concentration of 0.05 M HCl (figure 3.16). Similar to results obtained by Decosse and Aiello, (1966) and Bahr et al. (2009), the rate of hydrolysis was roughly proportional to the H⁺ concentration. However, treatment with 0.05 M HCl gave rise to increased degradation of the PS oligonucleotide as
compared to that obtained following treatment with 0.5 M HCl under similar experimental conditions. Interestingly, this observation also occurred following treatment of the PS oligonucleotides with 0.05 and 0.5 M H$_2$SO$_4$ (section 3.3.13). Such findings may be due to additional breakdown products that were masked and not fully resolved from the main sample peak which is consistent with results obtained following PAGE (figure 3.17). As expected the longer exposure to acidic conditions gave rise to slight variations between depurination and degradation levels.

Similar results were observed after analysis by PAGE. Figure 3.17 represents results obtained following incubation of the PS oligonucleotide in 0.005, 0.05 and 0.5 M HCl at the three respective times points of analysis, 1, 30 and 60 minutes at ambient room temperature. No significant degradation was observed after treatment with 0.005 M HCl however, following treatment with 0.05 M HCl the bands appear slightly fainter with the slight appearance of breakdown products. Following treatment at the higher concentration of 0.5 M HCl the bands appear more diffuse due to initial degradation of oligonucleotide beginning to occur. Most significant degradation of the oligonucleotide was observed following treatment with 0.05 M HCl similar to results obtained by HPLC.

![Figure 3.17 Electrophoretic analysis of the effect of low acid pH on PS oligonucleotides.](image)

*Figure 3.17 Electrophoretic analysis of the effect of low acid pH on PS oligonucleotides.*

Incubation of PS oligonucleotides with 0.005, 0.05 and 0.5 M HCl at ambient room temperature over a period of 1, 30 and 60 minutes. Oligonucleotides were visualised upon staining the gel with Stains-all.

**C1.** Control PS oligonucleotide only;

**C2.** 0.5 M HCl only.
Similar stability studies were also carried out by Meidan et al. (1997) to study the stability of radiolabelled oligonucleotides as a function of their chemistry (phosphodiester and phosphorothioate) and chain length in aqueous solutions at three different pH values (1, 2 and 7). It was observed that no degradation products were evident following densitometry of 20% PAGE autoradiographs for all oligonucleotides (at pH 1, 2 and 7) after 30 min, indicating that the radiolabeled 7-mer and 20-mer oligonucleotides of both PO and PS backbone chemistries remained stable in agreement with results from this study (figure 3.17). However, over extended time periods, oligonucleotide degradation was observed at acidic pH (2-3 h at pH 1 and after around 16 h at pH 2 for PO sequences) (Meidan et al. 1997).

3.3.10 IP-RP LC and electrophoretic analysis of the effect pH in combination with elevated temperature on PS oligonucleotides

The chemical stability of PS oligonucleotides over a broad range of pH (0.32, 1.23, 2.25, 3.25, 5.53 and 11.41) was also investigated at elevated temperatures of 40, 60 or 80°C as described in sections 3.2.12 and 3.2.14. Results obtained are calculated as percentage intact PS oligonucleotide and are summarised in figure 3.18 and 3.19.

![Figure 3.18 The pH stability of PS oligonucleotide in mild alkaline conditions (pH 11.41) over a temperature range of 25 to 80°C as assessed by IP-RP LC.](image)

Each value represents mean ± SD (n=3).

Figure 3.18 summarises that results obtained after incubation of PS oligonucleotides with 0.005 M NaOH (pH 11.41), simulating mild alkaline conditions, over a series of temperatures. As can be seen from the graph, very little degradation, (< 5%), was observed after 60 minutes treatment with 0.005 M NaOH over a temperature range of 25 to 80°C. This
coincides with findings by Adams et al. (1992); Parkins and Lashmar, (2000); Jelen et al. (1997) that DNA is not sensitive to mild alkaline hydrolysis. In addition to this, Capaldi et al. (unpublished results, personal communication) established that exposure of PS oligonucleotides to 20 mM NaOH for up to 7 days at room temperature did not give rise to detectable deamination. However, when subjected to 1 N NaOH at 25°C for 24 hours degradation via deamination was detected which increased with increasing length of incubation of up to 90 hours. The dominant mode of degradation for DNA in alkaline solution is cytosine deamination (Lindahl, and Nyberg, 1974). The rate of deamination is approximately 1000 times greater in 1 M NaOH than at pH 7.4 for single-stranded DNA. Other alkali catalysed changes in the covalent structure of DNA include, alkali-catalysed depurination and chain breakage and imidazole ring opening in adenine residues (Lindahl, and Nyberg, 1974).

The addition of elevated temperature of 40, 60 and 80°C did not appear to affect or accelerate the reaction. At temperatures of 60 and 80°C the peak area of the PS oligonucleotide in fact increased in peak area similar to the trend observed in the analysis of the stability of PS oligonucleotides to temperature in section 3.3.5. This increase in peak area is a direct consequence of elevated temperature and may indicate the initial breakdown of the PS oligonucleotides.

Analysis of the effect of acidic pH was also analysed in combination with elevated temperatures of 40, 60 and 80°C. Previous studies have also investigated the use of low acidic pH in combination with elevated temperature (60-80°C) to inactivate the biological activity of DNA, specifically recombinant DNA, with successful results (Bergemann et al. 1995; Popp et al. 1992). Following treatment with a given acidic solution, less than pH 4, revealed a consistent pattern throughout. It was observed that a combination of both low acidic pH and elevated temperature significantly decreased the stability of PS oligonucleotides thereby increasing the rate of degradation. At any given concentration of HCl when subjected to a combination of both low acidic pH and elevated temperatures of 40, 60 and 80°C the extent of degradation generally increased (figure 3.19).
The PS oligonucleotide was incubated with a) 0.0005 mM (pH 5.53), b) 0.0005 M (pH3.25), c) 0.005 M (pH 2.25), d) 0.05 M (pH 1.23) and e) 0.5 M (pH 0.32) HCl solutions over a period of 60 minutes. pH values represent the final pH value of HCl/NaOH. Each value represents mean ± SD (n=3).

The influence of temperature in combination with 0.0005 mM HCl, pH 5.53 (figure 3.19 a) does not appear to have any significant effect on the stability of the PS oligonucleotides. Similar to treatment with mild alkaline conditions, the peak area of the PS oligonucleotide increased in peak area at elevated temperatures of 60 and 80°C, which may indicate the initial breakdown products of degradation that cannot be fully resolved from the intact PS oligonucleotide.

Treatment with 0.0005 M HCl (figure 3.19 b), in combination with elevated temperature modestly accelerated the reaction. The more significant degradation upon heat treatment at
80°C can be interpreted as additional backbone degradation occurring at the weakened apurinic sites after initial depurination. Decosse and Aiello, (1966), established that loss of adenine and guanine is completed during early hydrolysis and that at optimum hydrolysis phosphate groups remain intact and cytosine and thymine are fully recoverable. Further hydrolysis subsequently results in progressive degradation of the residual polymeric DNA complex (Decosse and Aiello, 1966). A similar trend can be seen upon treatment of PS oligonucleotide with 0.005 and 0.05 M HCl in combination with elevated temperature (figure 3.19 (c and d)), which further accelerated the extent of degradation of the PS oligonucleotide by 41.5% following treatment with 0.05 M HCl at 80°C for 60 minutes.

In a study carried out by Dale, (2000) to investigate the stability of both modified and unmodified oligonucleotides it was observed that a 14-mer PS oligonucleotide was degraded by 35, 71, and 99% after 0.5, 1 and 2 hour after treatment with 0.1 N HCl (final pH of 1.5) at 39°C. Under similar conditions, at pH 1.23, the PS oligonucleotide was only degraded by 19.8 and 22.2% after 0.5 and 1 hour incubation. The disparity observed in results may be due to difference in experimental conditions, however, the overall trend is the same. The PS oligonucleotide in that study was shorter and incubated with a greater quantity of acid. In addition to this, after acid treatment, the PS oligonucleotide was further diluted 1:20 in an alkaline solution of NaOH and 0.3 M NaCl. From the literature it is commonly accepted that alkaline treatment converts apurinic sites into breaks and is continual until all apurinic sites have become breaks (Lafteur et al. 1981).

After treatment of the PS oligonucleotide with 0.5 M HCl an unexpected result was observed following treatment at elevated temperatures. As can be seen in figure 3.19 (e), most significant degradation of 84.9 and 87.9% was observed following treatment with 0.5 M HCl at 40°C after 30 and 60 minutes respectively. It appears that the PS oligonucleotides were more stable in 0.5 M HCl at higher temperatures of 60 and 80°C in comparison to 40°C.

Figure 3.20 represents chromatographic results of the untreated PS oligonucleotide and PS oligonucleotides subjected to acidic conditions of 0.5 M HCl at the elevated temperature of 40°C for 30 and 60 minutes where maximum degradation was detected.
Figure 3.20 Chromatographic results on the pH stability of PS oligonucleotides to 0.5 M HCl (pH 0.32) at 40°C over a period of 30 and 60m minutes.

The analysis of stability of PS oligonucleotides was performed in the a), absence and b), presence of 0.5 M HCl (pH 0.32) at the elevated temperature of 40°C, after 30 minutes and c), after 60 minutes. The peak numbers are printed above each peak corresponding to the order in which the peak elutes and appears on the chromatogram. The retention time is denoted on the time axis of the chromatogram.

= decrease in peak area of the PS oligonucleotide  = appearance of an early eluting peak
Over the 60 minute period of analysis, purine hydrolysis and subsequent hydrolysis of the backbone occurred at a considerably faster rate with the addition of elevated temperature as indicated by the increase in early eluting degradation products (green arrow) in addition to the decrease in peak area corresponding to that of the intact PS oligonucleotide (purple arrow). This is in agreement with results obtained by Neto et al. (1992), who established that heating DNA under acidic conditions induces the rate of depurination and thus the formation of abasic sites. It was observed that depurination is several fold higher than other forms of degradation such as depyrimidination or cytosine deamination under such conditions. The more significant degradation upon heat treatment can be interpreted as additional backbone degradation occurring at the weakened apurinic sites after initial depurination (Lindahl, 1996; Sheppard et al. 2000; Jonas et al. 2000).

Figure 3.21 illustrates the electrophoretic results obtained after analysis of the effect of 0.5 M HCl in combination with elevated temperature of 40, 60 or 80°C after a period of 1, 30 and 60 minutes. The results are consistent with results obtained following IP-RP LC where most significant degradation of the PS oligonucleotide was observed following treatment with 0.5 M HCl at 40°C.

Figure 3.21 Electrophoretic analysis of the effect of low acid pH (0.5 M HCl, pH 0.32) on PS oligonucleotides in combination with elevated temperature of 40, 60 and 80°C.

Oligonucleotides were visualised upon staining the gel with Stains-all.
C1). Control PS oligonucleotide only; C2). 0.5 M HCl only.

The influence of elevated temperature has the effect of decreasing the stability of the PS oligonucleotides thereby giving rise to breakdown of the PS oligonucleotides resulting in measurable shorting of the oligonucleotide strands. This is clearly evident by the ratio of
band intensity corresponding to intact and degraded oligonucleotides and the appearance of smaller degradation products which migrated further down the gel. Following treatment with 0.5 M HCl at 40°C, for 30 and 60 minutes, the band corresponding to the intact oligonucleotide is very faint in contrast to the control sample and treatment at the higher temperature of 60°C. Incubation of the oligonucleotide with 0.5 M HCl at the elevated temperatures of 60°C did however generate the appearance of smaller breakdown products and to a greater extent following incubation at 80°C.

3.3.11 IP-RP LC and electrophoretic analysis of the effect pH in combination with elevated temperature on PO oligonucleotides

Based on the results obtained from pH stability studies, the chemical stability of PO oligonucleotides to low acidic pH was investigated using 0.005, 0.05 and 0.5 M HCl at both room temperature and elevated temperature of 40, 60 and 80°C to assess if the unmodified oligonucleotides were more unstable to low acidic conditions. Treatment with low acidic pH did not give rise to significant degradation of the unmodified oligonucleotide following treatment with 0.005, 0.05 and 0.5 M HCl at ambient room temperature (figure 3.22).

![Figure 3.22 The pH stability of PO oligonucleotide at pH 2.25 (0.005 M HCl), pH 1.23 (0.05 M HCl) and pH 0.32 (0.5 M HCl) at ambient room temperature as assessed by IP-RP LC.](image)

Each value represents mean ± SD (n=3).

Maximum degradation of 26.0% was observed via HPLC analysis following treatment with 0.5 M HCl after a period of 60 minutes. Such results are comparable to results obtained following treatment of the modified PS oligonucleotide which is summarised in table 3.5.
However, maximum degradation (26.0%) of the PO oligonucleotides was obtained following treatment at the highest concentration of 0.5 M HCl in contrast to 0.05 M HCl for PS oligonucleotides (31.8%, 0.05 M HCl for 30 minutes).

Table 3.5 Analysis and comparison of the effect of HCl on PO and PS oligonucleotides by IP-RP LC
Each value represents mean ± SD (n=3).

<table>
<thead>
<tr>
<th>Final acid concentration (M)</th>
<th>Temperature (°C)</th>
<th>Length of incubation (min)</th>
<th>Phosphodiester oligonucleotide</th>
<th>Phosphorothioate oligonucleotide</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Intact (%)</td>
<td>Degradation (%)</td>
</tr>
<tr>
<td>0.005</td>
<td>R.T</td>
<td>1</td>
<td>96.7</td>
<td>3.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>30</td>
<td>95.8</td>
<td>4.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>60</td>
<td>95.4</td>
<td>4.6</td>
</tr>
<tr>
<td>0.05</td>
<td>R.T</td>
<td>1</td>
<td>89.8</td>
<td>10.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>30</td>
<td>93.5</td>
<td>6.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>60</td>
<td>92.9</td>
<td>7.1</td>
</tr>
<tr>
<td>0.5</td>
<td>R.T</td>
<td>1</td>
<td>79.2</td>
<td>20.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>30</td>
<td>75.2</td>
<td>24.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>60</td>
<td>74.0</td>
<td>26.0</td>
</tr>
</tbody>
</table>

The PO oligonucleotides were also subjected to treatment with 0.5 M HCl at the elevated temperatures of 40, 60 and 80°C. A consistent trend was observed under such experimental conditions as can be seen in figure 3.23.

![Figure 3.23](image)

**Figure 3.23 The pH stability of PO oligonucleotide at pH 0.32 over a temperature range of 25 to 80°C as assessed by IP-RP LC.**
Each value represents mean ± SD (n=3).

The influence of temperature in combination with low acidic pH of 0.32 had the effect of decreasing the stability and thereby increasing the rate of degradation of the unmodified oligonucleotide. Most significant degradation of 60.3 and 71.8% was observed following
incubation of the oligonucleotide in 0.5 M HCl at the elevated temperature of 80°C after 30 and 60 minutes respectively. This is in contrast to results obtained for the modified PS oligonucleotide where most significant degradation of 84.9 and 87.9% was observed following treatment with 0.5 M HCl at 40°C after 30 and 60 minutes, respectively.

Following confirmatory analysis via PAGE, similar results were obtained to HPLC analysis. Figure 3.24 (a) represents the results obtained following treatment of the unmodified oligonucleotide with 0.005, 0.05 and 0.5 M HCl at ambient room temperature and figure 3.24 (b) presents the results obtained following incubation of the oligonucleotide in 0.5 M HCl at 40, 60 and 80°C over a period of 1, 30 and 60 minutes as highlighted on both gel profiles.

![Figure 3.24](image)

**Figure 3.24 Electrophoretic analysis of the effect of low pH at ambient room temperature and in combination with elevated temperature on PO oligonucleotides.**

a). Incubation of PO oligonucleotides with 0.005, 0.05 and 0.5M HCl at ambient room temperature and b). Incubation of PO oligonucleotides with 0.5 M HCl at 40, 60 and 80°C. C1). Control PO oligonucleotide only; C2). 0.5 M HCl only. Oligonucleotides were visualised upon staining the gel with Stains-all.

Similar to results observed by HPLC, the PO oligonucleotides are stable over a period of 60 minutes at pH 2.25 (0.005 M HCl). However, treatment with 0.05 M HCl (pH 1.23) generates the initial appearance of degradation breakdown products and to a greater extent following treatment with 0.5 M HCl (pH 0.32) at ambient room temperature. The influence of temperature in combination with low acidic pH increased the rate of degradation as detected by the decrease in intensity of the main band and the appearance of smaller bands of degradation further down the gel. This was most evident at the highest temperature (80°C) where maximum degradation of 60.3 and 71.8% was quantified by HPLC following incubation for 30 and 60 minutes respectively.
3.3.12 Analysis of degradation products following pH stability studies

When the PS oligonucleotide was subjected to acidic conditions less than pH 5 in combination with elevated temperature, early eluting degradation products were produced. In addition to the decrease in the main peak corresponding to that of the intact PS oligonucleotide, an increase in the early eluting products was also detected which increased in magnitude upon increasing temperature and length of incubation at a specific experimental condition. This is clearly evident in figure 3.20 (as highlighted by the green arrow) which represents treatment of the PS oligonucleotide in 0.5 M HCl at 40°C after 30 and 60 minutes. As can be seen, the peaks detected at 0.5 and 0.7 minutes increased in peak area over the period of analysis. Similar chromatographic results were observed by ISIS Pharmaceuticals, Inc. (Daniel Capaldi, personal communication), when a solution of oligonucleotide (7 deoxypurines) was held at pH 3, 25°C for 2 hours. ISIS Pharmaceuticals, Inc. established that the main pathway of degradation was depurination and attributed the early eluting peaks as depurination products, which include pyrimidine mono- and oligodeoxynucleotides phosphorylated both at their 3’- and 5’- ends (Dallas et al. 2004), free bases and breakdown of deoxyribose (Adams et al. 1992). The oligonucleotide in our study comprises of the sequence of 5’-GCG TTT GCT CTT CTT CTT GCG-3’ which contains 5 purines (5 guanines), “hotspots” for acid catalysed hydrolysis.

To verify if such early eluting peaks were as a direct result of depurination, four nucleobases (Adenine, Guanine, Cytosine and Thymine) were subjected to HPLC analysis. A calibration curve representing peak area versus nucleobase concentration was constructed for each base as outlined in figure 3.25. Good straight line correlations between the peak area and nucleobase concentration were achieved for each individual nucleic acid base. The $R^2$ value for the calibration curve was 0.9997 for Adenine, 0.9998 for Guanine, 0.995 for Thymine and 1 for Cytosine. Each nucleobase had a slightly different retention time. As stated previously in section 3.1.5, oligonucleotides of equal length but with different base sequence have different retention times. Cytosine eluted at the earlier retention time of 0.646 minutes (±0.006), guanine at 0.794 minutes (±0.006), followed by Adenine at 0.954 minutes (±0.030) and Thymine at 1.034 minutes (±0.012). This is in agreement with results obtained by Gilar et al. (2002) who stated that the hydrophobicity contribution to the oligonucleotide retention increases in order of C<G<A<T (Gilar et al. 2002). The four peaks detected had a similar retention time to that of the early eluting breakdown products verifying that such peaks, which increase in peak area, are as a result of depurination.
Chapter Three

Figure 3.25 Calibration curve of peak area versus concentration of the four nucleobases, Adenine (a), Guanine (b), Cytosine (c) and Thymine (d) as assessed by IP-RP LC.

In addition to this, a deoxynucleotide solution mix (an equimolar solution of dATP, dCTP, dGTP and dTTP) was injected on to the HPLC column. Four individual peaks corresponding to the four nucleobases were identified with retention times of 0.5, 0.7, 0.8 and 0.9 minutes, similar retention times to the early eluting breakdown products (0.5 and 0.7 minutes) further verifying that such early eluting degradation peaks are as a result of depurination.

The application of PAGE could only detect degradation of the oligonucleotide by a disappearance of the main band corresponding to the intact oligonucleotide and the appearance of breakdown products of smaller size that ran further down the gel. This method could not detect depurination.

3.3.13 IP-RP LC and electrophoretic analysis of the effect of Sulfuric acid (H$_2$SO$_4$) on PS oligonucleotides.

A similar procedure as to that carried out in 3.2.11 was employed for the analysis of the influence of additional acids on the stability of PS oligonucleotides. Based on the extensive pH stability analysis and results obtained following treatment with HCl a second commonly employed industrial acid, sulfuric acid (H$_2$SO$_4$), was investigated for its efficacy at degrading PS oligonucleotides (Section 3.2.15). The final pH values of the respective solutions are detailed in table 3.6. Results obtained are illustrated in figure 3.26, 3.27 and 3.28.
Table 3.6 The initial and final pH values of H₂SO₄.

<table>
<thead>
<tr>
<th>Chemical</th>
<th>Initial concentration (M)</th>
<th>pH value</th>
<th>Final treatment concentration (M)</th>
<th>Final pH value of the reaction mixture</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sulfuric acid (H₂SO₄)</td>
<td>1</td>
<td>0.04</td>
<td>0.5</td>
<td>0.35</td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>0.95</td>
<td>0.05</td>
<td>1.17</td>
</tr>
<tr>
<td></td>
<td>0.01</td>
<td>1.81</td>
<td>0.005</td>
<td>2.04</td>
</tr>
<tr>
<td></td>
<td>0.005</td>
<td>2.06</td>
<td>0.0025</td>
<td>2.31</td>
</tr>
<tr>
<td></td>
<td>0.001</td>
<td>3.00</td>
<td>0.0005</td>
<td>3.29</td>
</tr>
</tbody>
</table>

Similar to results obtained following treatment with HCl most notable degradation of 27.7% was detected via HPLC after treatment with 0.05 M H₂SO₄ after 60 minutes incubation at ambient room temperature (figure 3.26). Based on the results obtained from HCl studies, the effect of 0.5 M H₂SO₄ at 40°C was also analysed. Under these conditions, degradation of 85.0 and 83.8% of the modified PS oligonucleotide was obtained following incubation for 30 and 60 minutes respectively.

Figure 3.26 The pH stability of PS oligonucleotide after treatment with H₂SO₄ as assessed by IP-RP LC.

Each value represents mean ± SD (n=3).

Results obtained are comparable to results detected, under similar experimental conditions, with 0.5 M HCl which gave rise to 84.9 and 87.9% degradation after 30 and 60 minutes as outlined in table 3.7. This therefore infers that both hydrochloric acid and sulfuric acid at a concentration of 0.5 M under the influence of low temperature of 40°C could be effective and could potentially be used to treat oligonucleotide containing waste streams.
### Table 3.7 Analysis and comparison of the effect of HCl and H\textsubscript{2}SO\textsubscript{4} on PS oligonucleotides by IP-RP LC

Each value represents mean ± SD (n=3).

<table>
<thead>
<tr>
<th>Final acid concentration (M)</th>
<th>Temperature (°C)</th>
<th>Length of incubation (min)</th>
<th>HCl Intact (%)</th>
<th>HCl Degradation (%)</th>
<th>H\textsubscript{2}SO\textsubscript{4} Intact (%)</th>
<th>H\textsubscript{2}SO\textsubscript{4} Degradation (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.005 R.T</td>
<td>1</td>
<td>94.2</td>
<td>5.8</td>
<td>96.2</td>
<td>3.8</td>
<td></td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>93.8</td>
<td>6.2</td>
<td>97.1</td>
<td>2.9</td>
<td></td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>94.5</td>
<td>5.5</td>
<td>94.5</td>
<td>5.5</td>
<td></td>
</tr>
<tr>
<td>0.05 R.T</td>
<td>1</td>
<td>74.5</td>
<td>25.5</td>
<td>83.3</td>
<td>16.7</td>
<td></td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>68.2</td>
<td>31.8</td>
<td>74.8</td>
<td>25.2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>69.8</td>
<td>30.2</td>
<td>72.3</td>
<td>27.7</td>
<td></td>
</tr>
<tr>
<td>0.5 R.T</td>
<td>1</td>
<td>91.4</td>
<td>8.6</td>
<td>92.1</td>
<td>7.9</td>
<td></td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>88.0</td>
<td>12.0</td>
<td>90.8</td>
<td>9.2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>87.8</td>
<td>12.2</td>
<td>90.1</td>
<td>9.9</td>
<td></td>
</tr>
<tr>
<td>0.5 40°C</td>
<td>1</td>
<td>85.4</td>
<td>14.6</td>
<td>81.9</td>
<td>18.1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>15.1</td>
<td><strong>84.9</strong></td>
<td>15.0</td>
<td><strong>85.0</strong></td>
<td></td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>12.1</td>
<td><strong>87.9</strong></td>
<td>16.2</td>
<td><strong>83.8</strong></td>
<td></td>
</tr>
</tbody>
</table>

Figure 3.27 outlines the chromatographic results observed under optimum treatment conditions. Treatment with 0.5 M H\textsubscript{2}SO\textsubscript{4} at 40°C had the effect of decreasing the peak area of the sample peak (purple arrow) with the appearance and increase of early eluting degradation products (green arrow) which increased in magnitude with decreasing pH and increasing length of incubation.
Figure 3.27 Chromatographic results on the pH stability of PS oligonucleotides to 0.5 M H$_2$SO$_4$ (pH 0.35) at 40°C over a period of 1, 30 and 60 minutes.

The analysis of stability of PS oligonucleotides was performed in the presence of 0.5 M H$_2$SO$_4$ (pH 0.35) at 40°C after a) 1 minute, b) 30 minutes and c) 60 minutes. The peak numbers are printed above each peak corresponding to the order in which the peak elutes and appears on the chromatogram. The retention time is denoted on the time axis of the chromatogram.

↓ = decrease in peak area of the PS oligonucleotide. ▶ = appearance of an early eluting peak.
Similar results were confirmed following analysis with PAGE. Figure 3.28 presents the results obtained following treatment of the PS oligonucleotides with 0.05, 0.5 M H$_2$SO$_4$ at ambient room temperature and 0.5 M H$_2$SO$_4$ at 40°C. It is clearly evident from the gel profile that the oligonucleotides display increased degradation at ambient room temperature following incubation with 0.05 M H$_2$SO$_4$ as detected by the slightly fainter bands in contrast to treatment with the higher concentration of 0.5 M H$_2$SO$_4$. Maximum degradation following incubation of the oligonucleotides in 0.5 M H$_2$SO$_4$ at 40°C after 30 and 60 minutes, as detected via HPLC, was non-quantitatively confirmed by PAGE analysis. Following treatment for 30 and 60 minutes the bands are very faint in contrast to the control PS oligonucleotide band (C1).

![Figure 3.28 Electrophoretic analysis of the effect of low acid pH at ambient room temperature and in combination with elevated temperature on PS oligonucleotides.](image)

3.3.14 IP-RP LC and electrophoretic analysis of the effect of Nitric acid (HNO$_3$) on PS oligonucleotides.

The third acid employed in this study was nitric acid to investigate if it could effectively degrade PS oligonucleotides within a period of 60 minutes. In addition to having a low pH nitric acid is also an oxidising agent, both properties which have been attributed to the degradation of PS oligonucleotides (Hardee et al. 2001). The PS oligonucleotide was treated with nitric acid solution at various percentages (3.5, 5, 10 and 15%) and samples were
removed at different time points and subsequently analysed by both IP-RP LC and PAGE as described in section 3.2.15.

Figure 3.29 represents the stability of the PS oligonucleotide upon treatment with various percentages (3.5, 5, 10 and 15%) of nitric acid at ambient room temperature as a function of concentration over the three time periods of analysis (1, 30 and 60 minutes). Degradation of the PS oligonucleotide proceeds significantly faster with increasing concentration of nitric acid and increasing length of incubation. The addition of 3.5% nitric acid to PS oligonucleotides had the effect of decreasing the peak area of the PS oligonucleotide by 26.8%. Following treatment with increased concentration of nitric acid, 5, 10 and 15% over the same period of analysis, the PS oligonucleotide was further degraded by 40.4, 72.4 and 81.9% respectively. The retention time at which the PS oligonucleotide eluted remained constant under all experimental conditions with the exception of the most extreme conditions of 15% nitric acid after 60 minutes. Increasing length of incubation increased the rate of breakdown of the PS oligonucleotide.

![Figure 3.29 The effect of nitric acid on PS oligonucleotides as assessed by IP-RP LC.](image)

The PS oligonucleotide was incubated with 3.5, 5, 10, and 15% nitric acid at ambient room temperature for 1, 30 and 60 minutes. Each value represents mean ± SD (n=3).

The identity of the degradation products was confirmed by comparison with retention times and chromatographic data of the control PS oligonucleotide. Representative chromatograms of the results observed upon treatment of the PS oligonucleotide with various concentrations of nitric acid after a period of 60 minutes at ambient room temperature are presented in figure 3.30. In contrast to the untreated PS oligonucleotide, a change in peak shape was
observed upon treatment with 3.5% nitric acid (figure 3.30 (a)). A shoulder at the end of the main peak was detected, (red arrow), indicating initial breakdown of the PS oligonucleotide after 60 minutes incubation.

Figure 3.30 (b and c) outline the chromatographic data obtained after treatment of the PS oligonucleotide with 10 and 15% nitric acid after a period of 60 minutes at ambient room temperature. As can be seen from the representative chromatograms, a significant change in peak profile was observed. The main peak, which decreased in peak area (blue arrow), split into two peaks after 60 minutes incubation with 10% nitric acid (figure 3.30b). Following treatment with increasing concentration of nitric acid (15%), the peak significantly decreased in magnitude (81.9%) after 60 minutes in contrast to the untreated PS oligonucleotide.

The most likely pathway of degradation of the PS oligonucleotides by nitric acid is acid catalysed hydrolysis, with minimal oxidative degradation, giving rise to significant degradation of 78.9 and 81.9% of the PS oligonucleotide following treatment with 15% nitric acid after 30 and 60 minutes. Due to the fact that nitric acid absorbs to some extent at 260 nm, an early eluting peak corresponding to that of nitric acid was detected with a retention time of less than 2 minutes. This early eluting peak made it difficult to detect and assess if any early eluting peaks, corresponding to depurination products (free purine bases characteristic of acid catalysed degradation), were produced.
Figure 3.30 Chromatographic results of the effect of nitric acid on PS oligonucleotides.

The reaction was performed in the presence of a) 3.5%, b) 10% and c) 15% nitric acid over a period of 60 minutes at ambient room temperature. The peak numbers are printed above each peak corresponding to the order in which the peak elutes and appears on the chromatogram. The retention time is denoted on the time axis of the chromatogram.

a: Formation of a shoulder on main peak.
= appearance of a new peak.  = Decrease in peak area of the PS oligonucleotide peak.
Figure 3.31 illustrates the electrophoretic results obtained following the analysis of the effects of nitric acid on the PS oligonucleotide. The semi-quantitative results obtained by PAGE confirmed the quantitative analyses recorded by IP-RP LC. No significant degradation of the intact PS oligonucleotide was observed after treatment with 5% nitric acid where minimum percentage decrease in peak area was detected by HPLC. Subsequent to treatment of the PS oligonucleotide with 10% nitric acid, where significant decomposition of 13.6, 54.6 and 72.4% of the PS oligonucleotide was detected by IP-RP LC after 1 30 and 60 minutes respectively, the bands appear fainter in intensity with the appearance of small breakdown products. Similar to results obtained by HPLC noticeable degradation was observed by PAGE following treatment with 15% after 30 and 60 minutes. The bands decreased in intensity following incubation with 15% nitric acid after 30 minutes and to a greater extent following 60 minutes incubation.

![Figure 3.31 Electrophoretic analysis of the effect of nitric acid on PS oligonucleotide after a period of 1, 30 and 60 minutes at ambient room temperature.](image)

Treatment with 5, 10, and 15% nitric acid.
C1: PS oligonucleotide only; C2: Nitric acid only (made up in water). Oligonucleotides were visualised upon staining the gel with Stains-all.

3.3.15 The potential benefits and limitations of chemical treatment over physical heat treatment at industrial scale.

Treatment via mild acid hydrolysis offers a distinct advantage over physical heat treatments as it is very quick, effective, efficient, and highly sensitive, less than 60 minutes application. Additionally, all three acids (hydrochloric acid, sulphuric acid and nitric acid) are commonly used and widely available laboratory reagents having a wide range of industrial applications.
Moreover, they present as a more cost effective treatment of oligonucleotide containing waste streams. The primary disadvantage associated with the application of acids is the corrosive nature of the acid and its potential environmental implications. However, in this study relatively low concentrations of 0.5 M HCl and H₂SO₄ were used to give rise to greater than 80% degradation of the modified PS oligonucleotides. Additional advantages and limitations are outlined in Chapter Six.

3.4 Concluding remarks

This study entailed the development and optimisation of two analytical methodologies, polyacrylamide gel electrophoresis (PAGE) and ion-pair reversed phase liquid chromatography (IP-RP LC), to detect and quantify PS oligonucleotides and their degradation products. An appropriate analytical gradient IP-RP LC methodology was adapted and optimised to quantitatively detect both PO and PS oligonucleotides and their subsequent degradation products following physicochemical treatment. Method specification was demonstrated by investigating the parameters outlined in section 3.2.5. Given the excellent reproducibility of retention times and peak area responses the method proved highly specific for the proposed application.

The influence of temperature (25-100°C) on oligonucleotide stability was assessed for periods of up to 8 hours and no significant degradation resulted, suggesting that the oligonucleotides are heat stable over this temperature range. However, when the oligonucleotides were subjected to high temperatures of 121°C for 15 minutes (steam sterilization), a significant decrease in peak area of 78.7 and 77.8% was observed by HPLC analysis for PO and PS oligonucleotides respectively. The stability of PS oligonucleotides in acidic and basic conditions was investigated, at ambient room temperature, using NaOH and HCl solutions ranging in pH values from 0.32 to 11.41. The PO and PS oligonucleotides are stable over a period of up to 60 minutes at pH 11.41, 8.82, 5.53, 3.25 and 2.25. However, treatment with HCl less than pH 2 (1.23 and 0.32) lead to initial degradation of 26.0% of the PO oligonucleotides following treatment with 0.5 M HCl for 60 minutes and 31.8% degradation of the PS oligonucleotides following incubation with 0.05 M HCl for 30 minutes at ambient room temperature.

The stability of PO and PS oligonucleotides over a broad range of pH was also investigated at elevated temperatures of 40, 60 or 80°C. It was observed that a combination of both low pH and elevated temperature significantly decreased the stability of the oligonucleotides
thereby increasing the extent of degradation. Maximum degradation of 61.2 and 70.9% was quantified by HPLC following incubation of the PO oligonucleotides for 30 and 60 minutes with 0.5 M HCl 80°C whilst maximum degradation 84.9 and 87.9% of the modified PS oligonucleotides was detected following treatment with 0.5 M HCl at 40°C. It can be concluded that under acidic conditions oligonucleotide degradation occurs and its extent depends on temperature and exposure time.

Two additional acids (sulphuric acid and nitric acid) were chosen and systemically analysed to assess their ability to effectively degrade PS oligonucleotides. Similar results were obtained following analysis of the effect of H$_2$SO$_4$ on the stability of PS oligonucleotides. Maximum degradation of 85.0% was quantified following incubation of the oligonucleotides in 0.5 M H$_2$SO$_4$ at 40°C for 30 minutes. The third acid, nitric acid, which is also an oxidising agent, was investigated at concentrations ranging from 3.5 to 17.5%. Most significant degradation of 78.9 and 81.9% was observed following treatment with 15% nitric acid for 30 and 60 minutes respectively.

In addition to the significant degradation (>80%), quantified via HPLC, it was also established that one of the main pathways of degradation observed, under the aforementioned methodologies, was depurination, which can be defined as the loss of the purine bases from nucleosidyl units through cleavage of the glycosidic bond between the base and the sugar of DNA (Simon et al. 1999). Loss of purine bases from an antisense oligonucleotide may be adequate treatment of the oligonucleotide containing waste streams as the removal of nucleobases will inactivate the biological activity of the oligonucleotide. It is through the mechanism of complementarity of base pairs that antisense bind and hybridise to their target mRNA sequence to bring about their therapeutic property.

This study reveals that pH and temperature are important oligonucleotide degrading factors and confirm the results of studies on DNA degradation previously documented in the scientific literature. Treatment via mild acid hydrolysis offers a distinct advantage over physical heat treatments as it is very quick, effective, efficient, and highly sensitive, less than 60 minutes application. Additionally, all three acids (hydrochloric acid, sulphuric acid and nitric acid) are commonly used and widely available laboratory reagents having a wide range of industrial applications. Based upon the concentration of reagents required, treatment duration, economic considerations and potential environmental impacts, it is likely that treatment with low acidic pH (0.5 M HCl and H$_2$SO$_4$) in combination with elevated
temperature (40°C) present the most effective methods of degrading PS oligonucleotides and could potentially be effective on an industrial scale when used to pre-treat a waste treatment influent stream.
Chapter Four: Detection and Quantification of Phosphorothioate Oligonucleotide Degradation by selected Chemical Means
Chapter Four: Detection and Quantification of Phosphorothioate Oligonucleotide Degradation by Selected Chemical Means.

4.1 Introduction

The experimental work presented in this chapter aims at highlighting a variety of chemical processes, in a systematic degradation study of PS oligonucleotides. Approaches include the application of soft metal ions, chemical oxidation technologies and disinfectants whereby knowledge of the mechanism of action assists in designing optimal degradation outcomes. Key governing features of these interactions include the physicochemical characteristics of the chemical agent and the physiological status of the oligonucleotide. This work highlights the rationale for choosing such chemical processes and the efficacy of the treatment method as analysed via both analytical methodologies IP-RP LC and PAGE to treat PS oligonucleotide waste product streams.

4.1.1 The effects and interactions of metal ions with nucleic acids

Metals can react with nucleic acids in a variety of ways which can ultimately lead to many modifications of the DNA and RNA molecules. DNA is polymorphic and such metal ions can either promote or destroy the multi-stranded helical structure of DNA and synthetic polynucleotides (phosphate binding of metals in general stabilizes, and base binding destabilizes the ordered helical conformations) (Butzow et al. 1990). Therefore, in addition to structural roles, most polyvalent metal ions (M ≥2+) can, under certain conditions, have deleterious effects and can induce cleavage (i.e. breakage, fragmentation, scission, rupture or depolymerisation) of nucleic acids. In addition to this, metal ions can also promote cleavage via modification of nucleic acid sugar residues and or nucleobase, resulting in destabilisation of the N-glycosidic bond between these moieties (Dallas et al. 2004; Sreedhara and Cowan, 2001). These reactions can be either non-specific or dependent on the chemical nature of nucleotide residues, secondary and or/tertiary structure or nucleic acid sequence.

The specificity of these reactions is dependent on both the nucleic acid conformation and metal binding modes in addition to the properties of the metal ions themselves (Dallas et al. 2004). The cleavage of nucleic acids can be catalysed by metal ions through a variety of different mechanisms such as direct interaction of the metal ion catalysts with either the atoms flanking the cleavable bonds or the nucleic acid functional groups participating in the cleavage chemistry (Dallas et al. 2004).
The nature of the reaction of metal and nucleic acid depends heavily upon the site to which the metal ion is bound. The electron donor atoms to which metals can be liganded are the nitrogens (and sometimes oxygens or sulfurs) on the bases, the phosphate oxygens, the sulphur, and rarely the 2'-oxygen on the ribose of RNA which is not available in DNA (Butzow et al. 1990). Metals differ in their relative affinity for these binding sites for example “hard” alkali and alkaline earth metals prefer phosphate binding, whereas “soft” metals such as platinum or mercury favour the nucleobases. “The interaction of metallic elements with living systems is dominated by the properties of metal ions as Lewis acids” (Duffus, 2002). The classification of metals should include assessment of the behaviour of metal ions as electron acceptors as this ultimately determines the possibilities of complex formation. Metal ions can be described as Class A, Class B or borderline as illustrated in figure 4.1. This classification of metals by their Lewis acidity signifies the form of bonding in their complexes (Duffus, 2002).

![Figure 4.1 The periodic table representing classification of metals](image)

as: **Class A**: hard metals (darkest grey); **Class B**: soft metals (lightest grey); and **borderline** / intermediate metals (intermediate grey) (Duffus, 2002).

Class A, are hard metal ions, or nonpolarizable, which preferentially form complexes with similar nonpolarizable ligands, mainly oxygen donors. The bonding in these complexes is primarily ionic. Class B or soft metal ions preferentially bind to polarizable, soft ligands to produce slightly more covalent bonding. As a general rule, it is evident that hard–hard or soft–soft combinations are preferred wherever possible (Duffus, 2002). This principle...
therefore forms the basis of the experimental analysis to investigate the effect of soft metal ions on PS oligonucleotides. One of the most noticeable differences between modified PS oligonucleotides and unmodified PO oligonucleotides is the different metal ion interactions and the favoured association and affinity of the modified PS oligonucleotide for soft metal ions (Eckstein, 2002; Strobel and Shetty, 1997). As stated, sulfur is a “soft” atom that coordinates preferentially with “soft” metal ions, whereas oxygen is a “hard” atom that coordinates to “hard” metal ions (Verma and Eckstein, 1998). Numerous studies have established the susceptibility of PS linkages (of the bridging and non-bridging positions) to silver nitrate and mercuric chloride mediated chemical cleavage (Kuimelis and McLaughlin 1995; Mag et al. 1991; Verma and Eckstein, 1998; Vyle et al. 1992; Xu and Kool, 1998; Connelly and Rider, 1985). Exceptionally soft metals such as silver and mercury salts are known to cause rapid cleavage of bridging 3’- and 5’-phosphorothioates in DNA (Kuimelis and McLaughlin 1995). To the best of our knowledge, no systematic study has been carried out to investigate the effects of soft metals ions such as silver (nitrate) on full length 21-mer PS oligonucleotides. Only phosphoromonothioate analogues, dinucleotides and single modified linkage incorporation in both the bridged and non-bridging positions have been investigated (Vyle et al. 1992).

### 4.1.2 Oxidising agents – Their effects on nucleic acids and applications in industry

Oxidation is defined as the process of electron removal. Oxidising agents (or oxidants) are substances that remove electrons (oxidation) from a substrate, thereby gaining electrons themselves (McDonnell, 2007; Sloop, 2010). In addition to a variety of chemical applications, oxidising agents have potent antimicrobial activities (McDonnell, 2007; Lazur, 2009; U.S. Peroxide, LLC, 2011; EPA, 1999; DuPont Company®, 2011; Rutala, 1996; Antec International Limited, 1994; Bagchi et al. 2002; Fournier and Meyer, 1975; Health Protection Agency, 2007; Yoa et al. 2006; Fatemi and Frank 1999). Biocides that possess oxidising agent ability are extensively used and include the halogens (chlorine, iodine and bromine), peroxygens (hydrogen peroxide and peracetic acid) and other forms of oxygen (chlorine dioxide and ozone) (McDonnell, 2007). Chemical disinfectants are widely used in industry, laboratory settings, clinical and medical settings and also in the household.

Oxidising agents have numerous effects on nucleic acids, proteins, carbohydrates and lipids that lead to the loss of their functions and structures. Specific effects include changes in
structure, breakdown of these macromolecules into smaller constituents, and transformation of structural and functional groups, in addition to certain effects leading to cross linking within and between the molecules (McDonnell, 2007). Oxidation represents a further pathway of degradation of nucleic acid–derived pharmaceuticals. A significant portion of the present knowledge on nucleotide oxidation comes from the biochemical literature where the oxidative modification of DNA has been recognised as a noteworthy hallmark accompanying conditions of aging or oxidative stress in vivo (Pogcki and Schöneich, 2000). Oxidants have dramatic effects on DNA and RNA structures. They readily attack both the sugar phosphate backbone and the nucleotide bases (section 1.74, figure 1.14). These effects result in strand breakage and may also cause reactions between sugars or converted bases and other molecules associated with the nucleic acid, including the formation of adducts (McDonnell, 2007). Oxidation of DNA can cause damage to all four bases and to the deoxyribose. Disruption of the 3', 4' bond of deoxyribose can lead to a strand interruption (Lindahl, 1996). DNA pyrimidines are specifically sensitive to ring saturation, fragmentation, and condensation brought about by active oxygen. Guanine is the most susceptible among the DNA bases and is the preferential target of one-electron oxidising agents. The main oxidative product is 8-oxoGua which is the main mutagenic oxidative lesion in the genome (Aust and Eveleigh, 1999; Cadet et al. 2003; Pogcki and Schöneich, 2000; Lindahl, 1996). These effects can disrupt the functions of polynucleotides, including DNA, RNA, and other, related nucleotides monomeric structures (McDonnell, 2007).

4.1.3 The effect of oxidising agents on PS oligonucleotides

The main premise for the use of oxidising agents in this study is that instability of PS oligonucleotides has been largely attributed to two degradation mechanisms, desulfurization and acid-catalysed hydrolysis (Chapter Three) (Bennett et al. 2004; Hardee et al. 2001). Conversion of organophosphates containing P=S or P=Se bonds into their oxo-derivatives (P=O) has interested several researchers involved in stereochemical correlations aiming at the elucidation of organic and enzymatic reaction mechanisms, studies on the metabolism of organophosphorus pesticides, or detoxification of organophosphorus warfare agents (Wozniak et al. 1998). Various methodologies and chemical agents have been reviewed throughout the literature which can effectively desulfurize PS oligonucleotides, a method for replacing the sulphur atoms of PS oligonucleotides to attain phosphodiesters, as illustrated in figure 4.2 (Wozniak et al. 1998; Schweitzer and Engels, 1997; Capaldi et al. 2003; Schuette et al. 1995; Wyrzykiewicz, 1997; Gish and Eckstein, 1988; Schuette et al. 1995). Such
studies were primarily carried out to convert the PS oligonucleotide back to the unmodified PO counterpart for subsequent sequencing of the oligonucleotide by chemical or enzymatic means.

![Figure 4.2 Diagram illustrating the inter conversion of natural phosphodiester oligonucleotide to phosphorothioate.](image)

Numerous methods available for desulfurization of phosphorothioates have been carried out employing reagents such as 2-iodoethanol, sodium metaperiodate, iodine-bicarbonate, cyanogens bromide (BrCN), bromine, and N-bromosuccinimide (Wyrzykiewicz, 1997). However, such procedures were directed towards oligonucleotides containing only one or two phosphorothioates and such procedures may not be practical as pharmaceutical formulations generally contain more nucleotide units (Wyrzykiewicz, 1997). A variety of additional chemical reagents have been identified as effective oxidants of phosphothioates or phosphoselenoates, including potassium peroxymonosulfate, nitric acid (HNO₃), dinitrogen tetroxide/nitrogen peroxide (N₂O₄), hydrogen peroxide (H₂O₂), potassium permanganate (KMnO₄), chloral or organic peracids, of which only sodium periodate or iodine/water can withstand the requirements of non-destructive oxidation of PS oligonucleotides (Wozniak et al. 1998).

A careful examination of the literature discloses the basis of oxidative action and offers a fertile area for scientific exploration with the potential to establish principals by which these technologies could be employed in this study. Seven commonly available oxidising agents/disinfectants were chosen on their ability to effectively desulfurize and subsequently degrade PS oligonucleotides and this was analysed by quantitative IP-RP LC and semi-quantitative PAGE.
4.2 Materials and Methods

4.2.1 Source of chemical reagents
As described in section 3.2.1
Oligonucleotides were obtained from Eurofins MWG Operon (London, UK), and reconstituted and stored as described in section 3.2.2. IP-RP LC and electrophoretic equipment employed in this study were previously outlined in section 3.2.4 and 3.2.6.

4.2.2 IP-RP LC analysis of the effect of soft metal ions - Silver nitrate (AgNO₃) on PS oligonucleotides.
Each standard PS oligonucleotide (50 µM) was made up in its corresponding diluent and mixed with silver nitrate (AgNO₃) (50/50 v/v), in three separate micro tubes representing the three time points of analysis, time 1, 30 and 60 minutes. Each micro tube was vortexed briefly and injected onto the HPLC column. Each concentration, at each time point, was analysed in triplicate. Table 4.1 outlines the experimental conditions, time points and concentrations of silver nitrate analysed. Results obtained upon treatment with each concentration of silver nitrate were then compared quantitatively against the control ‘untreated’ standard 50 µM PS oligonucleotide (made up in purified water (MiliQ) to a final test sample concentration of 25 µM) representing 100% intact PS oligonucleotide prior to treatment.

Table 4.1 Experimental conditions employed in the analysis of the effect of silver nitrate on PS oligonucleotides.
Reactions were undertaken at ambient room temperature and subsequently analysed by IP-RP LC and PAGE.

<table>
<thead>
<tr>
<th>Concentration (mM) (Final reaction conc.)</th>
<th>Time points of analysis (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.25, 0.5, 2.5, 12.5, 25, 50, 250, 500</td>
<td>1, 30 and 60</td>
</tr>
</tbody>
</table>

4.2.3 Electrophoretic analysis of the effect of soft metal ions - Silver nitrate (AgNO₃) of PS oligonucleotides.
Following incubation of the PS oligonucleotide with silver nitrate as described in section 4.2.2, the reaction mixture was analysed by PAGE and subsequently stained by silver staining, as described in section 3.2.6.
4.2.4 IP-RP LC and electrophoretic analysis of the effect of oxidising agents on PS oligonucleotides.

PS oligonucleotide degradation reactions (final reaction volume = 60 µl) were performed with a 21-mer PS oligonucleotide (final concentration 25 µM) at ambient room temperature with seven commonly employed oxidising agents, iodine, potassium permanganate, acidic potassium dichromate, sodium hypochlorite, peracetic acid, hydrogen peroxide, and Virkon®. Degradation reactions were initiated by the addition of equal volumes (30 µl) of freshly prepared solution of an oxidising agent of varying concentrations as outlined in table 4.2. The reactions were performed over a period of 1, 30 and 60 minutes and subsequently analysed by both IP-RP LC and PAGE in triplicate.

Table 4.2 Experimental conditions used in the analysis of the effects of seven oxidising agents on PS oligonucleotides.

Reactions were undertaken at ambient room temperature and subsequently analysed by IP-RP LC and PAGE.

<table>
<thead>
<tr>
<th>Chemical reagent</th>
<th>Concentration (mM/M/%) (Final reaction conc.)</th>
<th>Length of incubation (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Iodine (I₂)</td>
<td>0.25, 2.5, 5, 12.5 and 25 mM</td>
<td>1, 30 and 60</td>
</tr>
<tr>
<td>Potassium permanganate (KMnO₄)</td>
<td>1.25, 2.5, 5, 10, 25 and 50 mM</td>
<td>1, 30 and 60</td>
</tr>
<tr>
<td>Acidic potassium dichromate (K₂Cr₂O₇ / H₂SO₄)</td>
<td>0.0125, 0.025, 0.0625, and 0.125 M</td>
<td>1, 30 and 60</td>
</tr>
<tr>
<td>Sodium hypochlorite (NaOCl) (% available chlorine)</td>
<td>0.125, 0.25, 0.5, 1.25, 2.5 and 5 %</td>
<td>1, 30 and 60</td>
</tr>
<tr>
<td>Peracetic acid (CH₃COOOH) (% w/v)</td>
<td>0.0025, 0.025, 0.25, 1.25, 2.5, 5 and 12.5 %</td>
<td>1, 30 and 60</td>
</tr>
<tr>
<td>Hydrogen peroxide (H₂O₂) (% w/v)</td>
<td>0.5, 2.5, 5, 7.5, 10 and 15 %</td>
<td>1, 30 and 60</td>
</tr>
<tr>
<td>Virkon® (% w/v)</td>
<td>0.0025, 0.025, 0.05, 0.25 0.5 and 2.5%</td>
<td>1, 30 and 60</td>
</tr>
</tbody>
</table>

4.2.5 Statistical analysis

Data analysis, including calculation of mean, standard deviation and linear regression was conducted using Excel 2010 (Microsoft, USA).
Chapter Four

4.3 Results and discussion

4.3.1 IP-RP LC analysis of the effect of soft metal ions - Silver nitrate (AgNO₃) on PS oligonucleotides.

A control PS oligonucleotide of a concentration of 25 µM was injected on to the HPLC column (figure 4.3). The retention time (9.31 ± 0.02 minutes) and peak area (442458 ± 5.11 RDS) remained unchanged thus indicating that PS oligonucleotides were stable over the period of analysis. The mean average peak area value, of the three analyses, was calculated to represent 100% intact oligonucleotide. This figure was then used to calculate the overall percentage degradation of the PS oligonucleotide treated with various concentrations of silver nitrate over the various time points of analysis as illustrated in figure 4.5. Representative chromatograms of the results observed upon treatment of the PS oligonucleotide with 0.5, 25 and 250 mM silver nitrate after a period of 60 minutes are presented in figure 4.4.

Figure 4.3 Chromatograph representing the untreated PS oligonucleotide (25 µM).

Figure 4.4 (a) represents the chromatographic results obtained following treatment of the PS oligonucleotide with 0.5 mM silver nitrate after a period of 60 minutes at ambient room temperature. The chromatograms were compared with those of the relevant untreated samples (figure 4.3). The control PS oligonucleotide produced good sharp intense peaks, as is expected for fragments having a homogeneous molecular mass, charge, and/or structure (figure 4.3). However, upon treatment with 0.5 mM silver nitrate the peak profile was somewhat broader and shorter than that of the untreated PS oligonucleotide.
Figure 4.4 Chromatographic results of the effect of silver nitrate on PS oligonucleotides.

The reaction was performed in the presence of a) 0.5 mM, b) 25 mM and c) 250 mM silver nitrate over a period of 60 minutes at ambient room temperature. The peak numbers are printed above each peak corresponding to the order in which the peak elutes and appears on the chromatogram. The retention time is denoted on the time axis of the chromatogram.

↑ = Emergence of new peak that increases in peak area over the various time points.
↓ = Decrease in starting intact PS oligonucleotide material.
The peak profile changed dramatically with increasing concentration of 25 mM silver nitrate over a period of 60 minutes, revealing a peak corresponding to the PS oligonucleotide with a broad, longer retained migrating shoulder (figure 4.5 (b)) indicative of either a range of molecular masses, charges, and/or structures for the affected fragments. A similar trend was observed by Hartzell and McCord, (2005) in the analysis of divalent metal ions on DNA by Capillary Electrophoresis (CE). The peak area of the newly formed complex, with a retention time of 12.0 minutes, increased with increasing concentration of silver nitrate, as highlighted by the red arrow, whereas the intensities of the starting material response decreased by 43.6%, 49.1% and 52.3% after 1, 30 and 60 minutes respectively. Increasing concentration of 250 mM silver nitrate gave rise to a significant decrease in peak area of 77.1%, 81.8% and 81.3% after 1, 30 and 60 minutes incubation (figure 4.4 (c)). This indicates that while the concentration of the PS oligonucleotide appeared to be decreasing over time, a new product with a later retention time was increasing in peak area with increasing treatment concentration of silver nitrate.

Figure 4.5 represents a graph illustrating the results obtained by IP-RP LC of percentage decrease in peak area of the PS oligonucleotide as a function of silver nitrate concentration over the three time points of analysis (1, 30 and 60 minutes). No significant decrease in peak area was observed after treating the PS oligonucleotide with 0.5 mM silver nitrate at room temperature over a period of 60 minutes.

![Figure 4.5](image)

**Figure 4.5 Analysis of the decrease in peak area of the PS oligonucleotide as a function of concentration of silver nitrate as assessed by IP-RP LC.**

The PS oligonucleotide was incubated with silver nitrate ranging in concentration from 0.25, 0.5, 2.5, 12.5, 25, 50, 250 and 500 mM at ambient room temperature for 1, 30 and 60 minutes. Percentage degradation of PS oligonucleotides = % intact PS oligonucleotide after treatment for the indicated time relative to the untreated PS oligonucleotide. Each value represents mean ± SD (n=3).
The trend in figure 4.5 shows a consistent increase in the level of degradation, at each time point, upon treatment with up to and including 50 mM silver nitrate. With increasing concentration of silver nitrate the peak corresponding to that of the intact PS oligonucleotide decreased by 15.3% upon treatment for 1 minute with 2.5 mM silver nitrate up to 71.1% following treatment with 50 mM silver nitrate after a period of 30 minutes. At high metal ion concentrations of 250 and 500 mM, the cleavage rate appears to reach a plateau and level off to a constant value, presumably due to saturation of the starting material with the metal ion, consistent with results obtained by Ora et al. (1998). Under the experimental conditions, maximum degradation of 89.5% was observed following treatment of the oligonucleotide with 500 mM silver nitrate after 30 minutes. The effect of silver nitrate is immediate and does not appear to statistically change with increasing length of incubation. A decrease of 88.2 ± 4.1%, 89.5 ± 1.2% and 88.8 ± 1.4% in peak area was detected after 1, 30 and 60 minutes treatment with 500 mM silver nitrate. However, this does not necessarily equate to degradation of the PS oligonucleotide, as a newly formed peak with a later retention time of 12.0 minutes was detected, which increased with increasing concentration of silver nitrate and length of incubation.

Figure 4.6 summarises the relationship of the decrease in peak area of the PS oligonucleotide material and the appearance of a new peak product which eluted with a later retention time of 12.0 minutes with increasing concentration of silver nitrate. The graph presents results obtained following 60 minutes incubation.

![Graph showing the relationship between peak area and concentration of silver nitrate](image)

**Figure 4.6** Analysis of the change in peak area of the intact PS oligonucleotide versus the newly formed complex upon treatment with silver nitrate for 60 minutes. Each value represents mean ± SD (n=3).
With increasing concentration of silver nitrate, a concurrent decrease in peak area of the PS oligonucleotide starting material occurs, with an increase in peak area of the newly formed product, to a point where almost (90%) of the starting material has disappeared. This suggests that a new product was formed, a unique metal ion complex, of the PS oligonucleotide bound to the soft metal ion silver. Under certain conditions, divalent metal ions, such as Zn\(^{2+}\), Ni\(^{2+}\) and Co\(^{2+}\) are capable of being incorporated into DNA to form complexes termed M-DNA (Hartzell and McCord, 2005). The shift in retention time may be as a result of both structural change in the DNA upon metal intercalation and as a consequence of the altered charge and mass of the molecules. The extent of both peak-broadening and retention time shifts were found to be dependent on the metal ion concentration, and to a lesser extent the length of incubation prior to injection.

4.3.2 Electrophoretic analysis of the effect of soft metal ions - Silver nitrate (AgNO\(_3\)) on PS oligonucleotides.

HPLC results are consistent with and further supported by PAGE analysis. PAGE not only provided an insight into the quality of but also the extent and concentration at which this complex was formed. Figure 4.7 displays the electrophoretic results of the chemical stability of PS oligonucleotide as evaluated at the following relevant conditions of 0.25, 0.5, 2.5, 12.5, 25, 50 and 250 mM silver nitrate, at ambient room temperature over a period of 60 minutes.

![Figure 4.7 Electrophoretic analysis of the effect of silver nitrate (0.25, 0.5, 2.5, 12.5, 25, 50 and 250 mM) on PS oligonucleotide after a period of 60 minutes at ambient room temperature.](image)

Oligonucleotides were visualised upon staining the gel with silver staining. 
**C1**: PS oligonucleotide only; and **C2**: 250 mM silver Nitrate only.
Following incubation of the PS oligonucleotide with increasing concentration of silver nitrate the electromobility of the samples significantly decreased as indicated by the green arrow. Following treatment with increasing concentration of silver nitrate the bands are located higher up the gel, with a lower electromobility, than the free unbound PS oligonucleotide (C1). Direct comparison of the control and treated bands reveal that they have different mobilities, and therefore are two distinct complexes. This is consistent with the principle that a complex would have a lower mobility in a gel than the uncomplexed free PS oligonucleotide. Similar results were observed by Hartzell and McCord, (2005), using capillary electrophoresis, that some of the metal-complexed DNA molecules had different electrophoretic mobilities than their normal DNA counterparts which they attributed to the divalent cations causing structural changes in the single-stranded DNA (Hartzell and McCord, 2005).

The bands displaying the lowest mobility were less defined and broader which indicates that this newly formed complex may have been difficult to resolve. It is evident from the gel profile in figure 4.7 (last lane) that the intensity of the band significantly decreased and appeared as smeared bands at higher concentrations of treatment in contrast to the control PS oligonucleotide (C1). This observation was noted by Vyle et al. (1992) where cleavage with 20 mM aqueous silver nitrate under such conditions were incompatible with the analysis of large molecular weight DNA by agarose gel electrophoresis and resulted in weak, smeared bands that were consistent with silver complexation by the DNA (Arya and Yang, 1975). At the highest concentration of 250 mM silver nitrate it is difficult to conclusively state if the PS oligonucleotide was in fact degraded or if the small pore size would allow this complex to be qualitatively analysed by PAGE. Alternatively, the lack of intensity of the band (250 mM silver nitrate) may indicate the diminished ability of the silver ions to remain electrostatically associated with the PS oligonucleotide as it migrates through the gel and this may explain why no band is evident in the last lane.
4.3.3 IP-RP LC and electrophoretic analysis of the effect of Iodine (I$_2$) on PS oligonucleotides.

The effect of iodine was systematically analysed for its ability to effectively desulfurize and subsequently degrade the PS oligonucleotide by quantitative IP-RP LC and PAGE analysis as described in section 4.2.4. Figure 4.8 presents the chromatographic data following incubation of the PS oligonucleotides with a). 2.5 mM, b). 12.5 mM and c). 25 mM iodine. In contrast to the untreated control PS oligonucleotide (figure 4.3) there is a significant change in peak morphology and decrease in peak area with increasing treatment concentration of iodine solution. In addition to this, a sharp shift in retention time at which the treated oligonucleotide eluted was detected.

With an iodine concentration greater than 5 mM, an increase in background noise was detected, which interfered with some of the analysis of its effect on PS oligonucleotides and gave rise to complex chromatographic profiles (figure 4.8 (b and c)). Control analysis of iodine only (1:2 dilution to simulate experimental conditions) indicated that iodine was detected at 260nm. This is consistent with observations by Cosstick and Vyle, (1990) who found that iodine caused interference with HPLC analysis following treatment of PS oligonucleotides. Due to this, the extent of degradation may in fact be greater than that recorded.
Figure 4.8 Chromatographic results of the effect of iodine on PS oligonucleotides.

The reaction was performed in the presence of a). 2.5 mM, b). 12.5 mM and c). 25 mM iodine solution over a period of 60 minutes at ambient room temperature. The peak numbers are printed above each peak corresponding to the order in which the peak elutes and appears on the chromatogram. The retention time is denoted on the time axis of the chromatogram.

= Shift in retention time of the sample PS oligonucleotide.
Percentage degradation of PS oligonucleotide following treatment with increasing concentration of 0.25, 2.5, 5, 12.5 and 25 mM iodine solution was analysed over the three time points of analysis, time 1, 30 and 60 minutes and the results obtained are represented in figure 4.9.

**Figure 4.9 The effect of iodine on PS oligonucleotides as assessed by IP-RP LC.**

The PS oligonucleotide was incubated with iodine solution ranging in concentration from 0.25, 2.5, 5, 12.5 and 25 mM at ambient room temperature for 1, 30 and 60 minutes. Percentage degradation of PS oligonucleotides \(=\%\) intact PS oligonucleotide after treatment for the indicated time relative to the untreated PS oligonucleotide. Each value represents mean ± SD (n=3).

The addition of iodine solution of increasing concentration had the effect of decreasing the peak area of intact PS oligonucleotide. Initial degradation proceeds slowly where a steady increase in the extent of degradation was observed ranging from 13.0, 25.1 and 29.8\% following treatment with 0.25, 2.5 and 5 mM iodine respectively. At low iodine concentration, the amount of reagent is presumably not sufficient enough to react with all the available phosphorothioate groups. Upon treatment with an increased concentration of 12.5 mM iodine, a significant decrease of intact PS oligonucleotide was detected, degrading the PS oligonucleotide by up to 84.4\% after 30 minutes. The extent of decomposition started to slow down following a 2 fold increase in treatment concentration (25 mM) where maximum degradation of 93.0\% was observed following incubation for 30 minutes. The reaction was fast and effective with no statistical difference with respect to treatment over the various time points of analysis at a given concentration. Iodine (25 mM) degrades the PS oligonucleotide by 91.6 ± 4.0, 93.0 ± 4.0 and 90.7 ± 2.9\% after 1, 30 and 60 minutes treatment respectively.
In addition to the structural degradation of the PS oligonucleotide, detected by HPLC, an additional pathway of degradation was observed. PS oligonucleotide has a characteristic retention time of between 9.1 and 9.6 (section 3.3.2.1). A notable shift in retention time was detected following treatment with increasing concentration of iodine (figure 4.10). This was derived to indicate initial/partial desulfurization of the PS oligonucleotide by the oxidant back to its natural PO counterpart as previous analysis established that the retention time of the unmodified PO oligonucleotide, of the same length and sequence, was between 6.0 and 6.3 minutes. A concurrent shift in retention time was detected with increasing iodine treatment concentration and length of incubation. The retention time of the sample peak following incubation for 60 minutes with 0.25, 2.5, 5 and 25 mM iodine solution was 8.3 ± 0.1 minutes, 7.5 ± 0.0 minutes, 7.7 ± 0.1 minutes and 7.6 ± 0.1 minutes respectively.

Figure 4.10 Graph representing the relationship of the change in retention time of PS oligonucleotide peak after treatment with iodine solution as assessed by IP-RP LC. Each value represents mean ± SD (n=3).

Figure 4.11 (a) and (b) display the electrophoretic results following treatment with 0.25, 2.5 and 5 mM iodine (a) and 12.5 and 25 mM iodine (b) followed by staining with Stains-all. Treatment with 0.25 mM iodine had no effect on the PS oligonucleotides. However, with increasing concentration of 2.5 mM iodine and greater the bands appear blue in colour, larger and with a slightly higher mobility than that of the control PS oligonucleotide (C1). Such bands are characteristic of unmodified PO oligonucleotides. Similar results were obtained by Wyrzykiewicz, (1997), who observed that migration of the desulfurized oligonucleotides on PAGE gels was faster, in comparison to the phosphorothioates. At this concentration (2.5 mM) a shift in retention time was detected by HPLC analysis. This method therefore
reinforces and corroborates the findings via HPLC analysis. Following incubation of the oligonucleotides with the higher concentrations of iodine (12.5 and 25 mM) (figure 4.11 (b)), the bands still remain blue with the appearance of smaller breakdown products that run lower down the gel. Additionally, under optimum treatment conditions, where maximum degradation of >90% was noted by HPLC analysis the bands are much fainter in contrast to the control PS oligonucleotide band. The shift in colour from purple to blue indicates that iodine desulfurizes PS oligonucleotides back to their unmodified counterparts at concentrations greater than or equal to 2.5 mM iodine solution. At the higher treatment concentrations of 12.5 and 25 mM iodine, structural degradation of the oligonucleotide appears to occur.

![Electrophoretic analysis](image)

**Figure 4.11 Electrophoretic analysis of the effect of iodine solution on PS oligonucleotides after a period of 1, 30 and 60 minutes at ambient room temperature.**

Oligonucleotides were visualised upon staining the gel with Stains-all.

Gel a), 0.25, 2.5 and 5 mM iodine solution; Gel b), 12.5 and 25 mM iodine solution.

C1: PS oligonucleotide only; and C2: 5 mM iodine (gel a) and 25 mM iodine (gel b).
4.3.4 IP-RP LC and electrophoretic analysis of the effect of potassium permanganate (KMnO₄) on PS oligonucleotides.

The extensively used oxidant potassium permanganate was used in this study to investigate its ability over a range of concentrations to desulfurize and subsequently degrade the PS oligonucleotide. The effect of potassium permanganate (1.25 mM - 50 mM) on PS oligonucleotide stability was analysed by IP-RP LC and PAGE as described in section 4.2.4. Percentage PS oligonucleotide degradation was calculated based on the peak area response after treatment of the PS oligonucleotide relative to the untreated intact PS oligonucleotide.

Figure 4.12 represents the chromatographic results obtained after treatment of the PS oligonucleotide with 1.25, 5 and 50 mM potassium permanganate for 60 minutes. Figure 4.12 (a) represents the lowest concentration of potassium permanganate (1.25 mM) employed in this study. In contrast to the peak of the untreated PS oligonucleotide which was a tall, sharp peak, eluting at a retention time of 9.6 minutes, the peak in chromatogram figure 4.12 (a) was much broader in shape with a noticeable decrease in peak height and a slight decrease in peak area of 19.1%. What is most evident is the significant shift in retention time, indicative of initial desulfurization of the PS oligonucleotide, from 9.6 minutes to 8.0 minutes after treatment for 60 minutes, as indicated on the time axis of the corresponding chromatogram.

Figure 4.12 (b) illustrates the results observed after incubation of the PS oligonucleotide for 60 minutes with 5 mM potassium permanganate in chromatographic form. The initial early peak can be assigned to the peak corresponding to potassium permanganate, which increased with increasing concentration. A control with only potassium permanganate was also carried out which verified that this peak was in fact due to potassium permanganate. Similar to figure 4.12 (a), the oligonucleotide peak changed in shape and size where an increase in percentage degradation of 40.0% was detected. Additionally, the retention time shifted from 9.5, to 7.3 and 6.9 minutes following treatment for 1, 30 and 60 minutes respectively.

Figure 4.12 (c) represents the chromatographic results obtained following treatment with the highest concentration of 50 mM potassium permanganate after a period of 60 minutes incubation at ambient room temperature. The peak corresponding to that of the intact PS oligonucleotide decreased in peak height, peak area and also eluted at the earlier retention time of 7.0 minutes. Such conditions gave rise to the most significant degradation of the PS oligonucleotide (79.6%) after 60 minutes.
Figure 4.12 Chromatographic results of the effect of potassium permanganate on PS oligonucleotides.

The reaction was performed in the presence of a) 1.25 mM, b) 5 mM and c) 50 mM potassium permanganate over a period of 60 minutes at ambient room temperature. The peak numbers are printed above each peak corresponding to the order in which the peak elutes and appears on the chromatogram. The retention time is denoted on the time axis of the chromatogram.

= Shift in retention time of the sample PS oligonucleotide.
Percentage degradation of the PS oligonucleotide was analysed as a function of potassium permanganate concentration over the three time periods of analysis as illustrated in figure 4.13. The extent of degradation of PS oligonucleotide increased with increasing concentration of potassium permanganate. Similar to the effect of the previous oxidant iodine, the extent of PS oligonucleotide degradation was minimal following treatment with concentrations of potassium permanganate ≤ 10 mM. Following treatment with concentrations greater than 10 mM potassium permanganate the extent of degradation increased significantly where maximum degradation of 80.1, 77.6 and 79.6% of the PS oligonucleotides was obtained upon treatment with 50 mM potassium permanganate after 1, 30 and 60 minutes respectively. Similar to the action of silver nitrate and iodine, the action of potassium permanganate was very fast, with little or no significant statistical change in percentage degradation observed over the three time points of analysis.

![Figure 4.13 The effect of potassium permanganate on PS oligonucleotides as assessed by IP-RP LC.](image)

The PS oligonucleotide was incubated with potassium permanganate ranging in concentration from 1.25, 2.5, 5, 10, 25 and 50 mM at ambient room temperature for 1, 30 and 60 minutes. Percentage degradation of PS oligonucleotides = % intact PS oligonucleotide after treatment for the indicated time relative to the untreated PS oligonucleotide. Each value represents mean ± SD (n=3).

The graph in figure 4.14 displays the relationship between the shift in retention time at which the PS oligonucleotide elutes as a function of treatment concentration of potassium permanganate. The rate at which the retention time changed over the three time points, and thus the extent of desulfurization increased with increasing concentration of potassium permanganate up to and including a concentration of 10 mM potassium permanganate. However, at concentrations greater than this (25 and 50 mM potassium permanganate)
although the initial retention time at 1 minute is lower than previous retention times detected, beyond this time point, there is no further significant change in retention time over the next two time points of analysis. This can be attributed to the fact that the first and initial pathway of degradation by potassium permanganate is desulfurization followed by cleavage and structural degradation of the PS oligonucleotide. Treatment with a concentration of potassium permanganate less than 10 mM lead to degradation of less than 40%. However, an increase in concentration of 25 and 50 mM gave rise to 70 and 80% degradation of the PS oligonucleotide respectively. Therefore, at lower concentrations of potassium permanganate it appeared that the PS oligonucleotide was initially desulfurized. However, upon treatment with an increased concentration of potassium permanganate, the increased rate of desulfurization levelled off and increased degradation of the PS oligonucleotide backbone occurred.

![Graph representing the relationship of the change in retention time of PS oligonucleotide peak after treatment with potassium permanganate as assessed by IP-RP LC.](image)

Each value represents mean ± SD (n=3).

Figure 4.15 presents the electrophoretic results observed following the analysis of the effect of (a): 0.5, 2.5, 5 and (b): 10, 25 and 50 mM potassium permanganate on PS oligonucleotides at ambient room temperature over a period of 1, 30 and 60 minutes. The application of PAGE provided consistent results to HPLC. Treatment with the lowest concentration (0.5 mM) resulted in no significant change in band colour or shape to that of the control lane (C1). However with increasing treatment concentration of 2.5 mM potassium permanganate the band after 1 minute treatment remains purple and progressively changed to a blue/purple band to a blue band after 30 and 60 minutes respectively. This is in
agreement with results obtained via HPLC analysis (figure 4.14) where the oxidising ability of potassium permanganate at these concentrations increased with increasing length of incubation as detected by the shift in retention time. A similar trend appears subsequent to incubation of the PS oligonucleotides with 5 mM potassium permanganate where the band at 1 minute remains purple whilst there is a significant conversion in colour and morphology of the band after 30 and 60 minutes respectively.

![Figure 4.15 Electrophoretic analysis of the effect of potassium permanganate on PS oligonucleotides after a period of 1, 30 and 60 minutes at ambient room temperature.](image)

Oligonucleotides were visualised upon staining the gel with Stains-all.

Treatment with a) 0.5, 2.5 and 5 mM; b) 25, 50 and 100 mM.

C1: PS oligonucleotide only; C2: 5 mM potassium permanganate (gel a), 100 mM potassium permanganate (gel b).

With increasing concentration of potassium permanganate (25, 50 and 100 mM) the results became difficult to interpret. The bands remain predominately blue but appear as a smear down the gel. Despite the fact that potassium permanganate does not stain when run alone in the gel (C2) it does appear to interfere with electrophoretic analysis at higher concentrations. Unlike the HPLC system which could elute the permanganate component from the test sample at its relative retention time the application of electrophoresis was unable to do this. What is evident from the gel profiles is that the bands are much fainter in intensity which indicates significant loss of oligonucleotide material from the test sample comparable with results obtained via HPLC analysis where degradation of up to 80% was determined.
4.3.5 IP-RP LC and electrophoretic analysis of the effect of acidic potassium dichromate (K$_2$Cr$_2$O$_7$ / H$_2$SO$_4$) on PS oligonucleotides.

Potassium dichromate is an orange, crystalline solid, soluble in water, which is a strong oxidizing agent in the presence of dilute sulphuric acid (Hammer and Hammer, 2008). A solution of 0.25 M potassium dichromate was acidified with dilute 0.05 M sulphuric acid. Dilutions of the acidic solution were made up (1:2, 1:5 and 1:10) and incubated with equal volumes of 50 μM PS oligonucleotide at ambient room temperature and subjected to analysis by both IP-RP LC and PAGE as described in section 4.2.4. The stability of PS oligonucleotides to acidic potassium dichromate was investigated at final treatment concentration of 0.125, 0.0625, 0.025 and 0.0125 M. All treated samples were then compared quantitatively and qualitatively against the untreated standard PS oligonucleotide made up in water.

Figure 4.16 (a and b) represents the optimum treatment chromatographic results obtained after the analysis of the effect of 0.125 M acidic potassium dichromate on PS oligonucleotides upon treatment for 30 and 60 minutes respectively. A peak corresponding to potassium dichromate eluted with an early retention time as highlighted by the red arrow. In contrast to the untreated sample (figure 4.3) treatment with 0.125 M potassium dichromate after 30 minutes generated a significant decrease in peak area as highlighted by the blue arrow. This equated to substantial degradation of the PS oligonucleotide by 81.3% and to a greater extent (94.7%) following incubation under similar conditions for 60 minutes (figure 4.16 (b)).
Figure 4.16 Chromatographic results of the effect of acidic potassium dichromate on PS oligonucleotides.

The reaction was performed in the presence of 0.125 M, acidic potassium dichromate over a period of 30 minutes a), and 60 minutes b), at ambient room temperature. The peak numbers are printed above each peak corresponding to the order in which the peak elutes and appears on the chromatogram. The retention time is denoted on the time axis of the chromatogram.

= Decrease in starting intact PS oligonucleotide material.
Percentage degradation of the PS oligonucleotide was analysed as a function of acidic potassium dichromate concentration at the three respective time points of analysis 1, 30 and 60 minutes as displayed in figure 4.17. Increasing concentration of acidic potassium dichromate resulted in accelerated decomposition of the PS oligonucleotide from 22.7% subsequent to treatment with the most dilute concentration of 0.0125 M potassium dichromate to 32.7 and 34.4% for 0.025 and 0.0625 M acidic potassium dichromate respectively after 60 minutes incubation. Most significant degradation of 81.3 and 94.7% was observed following treatment with 0.125 M acidic potassium dichromate after 30 and 60 minutes respectively. The length of incubation also had a dramatic effect on the extent of degradation most notably between 1 and 30 minutes, for example, 19.6, 81.3 and 94.7% degradation was observed after incubation with 0.125 M acidic potassium dichromate at time 1, 30 and 60 minutes respectively. This is comparable to results obtained using nitric acid (section 3.3.14). No change in retention time was detected under the experimental conditions, indicating that, despite the fact that chromic acid is a strong oxidising agent no desulfurization was observed.

![Figure 4.17](image)

**Figure 4.17 The effect of acidic potassium dichromate on PS oligonucleotides as assessed by IP-RP LC.**

The PS oligonucleotide was incubated with 0.0125, 0.025, 0.0625 and 0.125 M acidic potassium dichromate at ambient room temperature for 1, 30 and 60 minutes. Percentage degradation of PS oligonucleotides = % intact PS oligonucleotide after treatment for the indicated time relative to the untreated PS oligonucleotide. Each value represents mean ± SD (n=3).

Acidic potassium dichromate is made up of two components sulphuric acid and potassium dichromate. To determine if the degradation observed was primarily due to one of the components, a solution of 0.25 M potassium dichromate was made up in water to investigate
its effect alone, at varying concentrations, on the stability of PS oligonucleotides. Figure 4.18 displays the results obtained following a study to investigate the effect of 0.0125, 0.25, 0.0625 and 0.125 M potassium dichromate (made up in water, with no acid) on PS oligonucleotides over a period of 1, 30 and 60 minutes. Under the experimental conditions, no significant degradation (>2.0%) of the oligonucleotide was observed. Similarly, treatment with sulphuric acid, at the higher concentration of 0.05 M gave rise to less than 30% degradation, at ambient room temperature (section 3.3.13). It can therefore, be deduced that the increased percentage degradation was a result of the synergistic effect of potassium dichromate made up in an acidic medium.

Figure 4.18 The effect of potassium dichromate (water) on PS oligonucleotides as assessed by IP-RP LC.

The PS oligonucleotide was incubated with 0.0125, 0.025, 0.0625 and 0.125 M potassium dichromate (water) at ambient room temperature for 1, 30 and 60 minutes. Percentage degradation of PS oligonucleotides = % intact PS oligonucleotide after treatment for the indicated time relative to the untreated PS oligonucleotide. Each value represents mean ± SD (n=3).

Comparable results were obtained following analysis via PAGE followed by staining with Stains-all. Figure 4.19 (a and b) depict the results attained following treatment of the PS oligonucleotide with 0.025, 0.0625 and 0.125 M potassium dichromate made up in an acidic medium (4.19a) or in water (4.19b) after a period of 1, 30 and 60 minutes.

No significant degradation was observed after incubation of the PS oligonucleotide with 0.025 M acidic potassium dichromate after a period of up to 30 minutes. However, minimal degradation was observed after 60 minutes as evidenced by a decrease in band intensity where 32.7% degradation was determined by HPLC analysis. Upon treatment with increased
concentration of 0.0625 M acidic potassium dichromate a notable change in band intensity was observed. After 1 minute of incubation the band appeared to have faded slightly in intensity. After an increased length of incubation, the band at 30 minutes appeared very faint and was only slightly visible after 60 minutes. Maximum degradation was observed following treatment with 0.125 M acidic potassium dichromate, where no band was evident upon incubation for 30 and 60 minutes respectively, consistent with results detected by HPLC analysis, where maximum degradation of 81.3 and 94.7% of the intact PS oligonucleotide was detected after 30 and 60 minutes. Under the experimental conditions analysed in this study, no change in colour of the bands was detected confirming that the oligonucleotides were not desulfurized to the oxygenated form.

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**Figure 4.19** Electrophoretic analysis of the effect of acidic potassium dichromate (a) and potassium dichromate (water) (b) on PS oligonucleotides after a period of 1, 30 and 60 minutes at ambient room temperature.

- Treatment with 0.025, 0.0625 and 0.125 M acidic potassium dichromate (Gel a)
- Treatment with 0.025, 0.0625 and 0.125 M potassium dichromate (water) (Gel b)
- C1: PS oligonucleotide only; C2: 0.125 M acidic potassium dichromate only (Gel a); C2: 0.125 M acidic potassium dichromate only (Gel b). Oligonucleotides were visualised upon staining the gel with Stains-all.

Figure 4.19b also confirms analysis by HPLC whereby no degradation was observed following incubation of the oligonucleotide with potassium dichromate (made up in water) at concentrations ranging from 0.0125 to 0.125 M. Treatment of the PS oligonucleotide with potassium dichromate (made up in water) led to no change in band shape, colour or size.
4.3.6 IP-RP LC and electrophoretic analysis of the effect of Sodium hypochlorite (NaOCl) on PS oligonucleotides.

The PS oligonucleotide was treated with sodium hypochlorite, a potent viral agent and disinfectant, at various percentages (0.125, 0.25, 0.5, 1.25, 2.5 and 5% (% available chlorine)) and samples were removed at different time points (1, 30 and 60 minutes) and subsequently analysed by both IP-RP LC and PAGE as described in section 4.2.4. The identity of the degradation products was confirmed by comparison with retention times and chromatographic data of control PS oligonucleotide analysis.

Figure 4.20 represents the chromatographic results obtained after treatment of the PS oligonucleotide with 0.125, 0.5 and 1.25% sodium hypochlorite following treatment for 60 minutes at ambient room temperature. In figure 4.20 (a) clear, sharp peaks were produced which eluted at a retention time characteristic of the intact PS oligonucleotide. What is evident from the chromatogram is the increased width at the base of the peak which may be attributed to initial products of desulfurization/degradation as they were detected as individual peaks with earlier retention times than the main sample peak.

After analysis of the effect of 0.5% sodium hypochlorite on the PS oligonucleotide upon treatment for 60 minutes, (figure 4.20 (b)), a significant pattern emerged whereby the main peak corresponding to that of the intact PS oligonucleotide was detected at two main retention times. With increasing length of incubation the peak shifts in retention time and eluted with a lower percentage of organic modifier characteristic of PO oligonucleotides. This shift in retention time is indicative of the effect of the oxidising agent at desulfurizing the PS oligonucleotide.

Upon treatment with the increasing concentration of 1.25% sodium hypochlorite (figure 4.20 c) after 60 minutes, a single peak with an early retention time of 6.13 minutes was detected, similar to the characteristic retention time of PO oligonucleotides established from earlier analysis to be between 6.0 and 6.3 minutes.
Figure 4.20 Chromatographic results of the effect of sodium hypochlorite on PS oligonucleotides.

The reaction was performed in the presence of a) 0.125%, b) 0.5% and c) 1.25% (available chlorine) sodium hypochlorite over a period of 60 minutes at ambient room temperature. The peak numbers are printed above each peak corresponding to the order in which the peak elutes and appears on the chromatogram. The retention time is denoted on the time axis of the chromatogram.

↓ = appearance of new peak with earlier retention time. ← = Shift in retention time of the sample PS oligonucleotide.
Figure 4.21 shows the chromatographic data observed on the effect of 2.5% sodium hypochlorite on the PS oligonucleotide. One main peak eluted at an earlier retention time which significantly decreased in both peak height and peak area (38.5%). The peak appeared broad and round in shape which may indicate additional degradation products masked/hidden under the main peak which may signify increased degradation of the PS oligonucleotide than that calculated and documented.

**Figure 4.21 Chromatographic results of the effect of 2.5 % sodium hypochlorite on PS oligonucleotides.**

The reaction was performed in the presence of 2.5% (available chlorine) sodium hypochlorite over a period of 60 minutes at ambient room temperature. The peak numbers are printed above each peak corresponding to the order in which the peak elutes and appears on the chromatogram. The retention time is denoted on the time axis of the chromatogram.

\[ \text{Shift in retention time of the sample PS oligonucleotide.} \]

Figure 4.22 illustrates the results obtained following treatment with 2.5% sodium hypochlorite which gave rise to 41.4, 39.2 and 38.5% degradation after 1, 30 and 60 minutes respectively. Similar to the effects of the other oxidising agents, the reaction was fast, leading to maximum degradation of 41.4% following 1 minute incubation with the oxidant.

No significant degradation of the PS oligonucleotide was observed after treatment with 0.125, 0.25, 0.5 and 1.25% sodium hypochlorite (<10%) over the period of analysis. However, despite the fact that a similar and consistent trend was observed under each experimental condition, in triplicate, results obtained with lower concentrations of sodium
hypochlorite gave rise to the generation of two peaks as shown in figure 4.20. For this reason only the results obtained following treatment with 2.5% sodium hypochlorite, which gave rise to maximum degradation of the PS oligonucleotide under experimental conditions, are represented in figure 4.22.

Figure 4.22 The effect of 2.5% sodium hypochlorite on PS oligonucleotides as assessed by IP-RP-LC.

The PS oligonucleotide was incubated with 2.5% (available chlorine) sodium hypochlorite at ambient room temperature for 1, 30 and 60 minutes. Percentage degradation of PS oligonucleotides = % intact PS oligonucleotide after treatment for the indicated time relative to the untreated PS oligonucleotide. Each value represents mean ± SD (n=3).

Figure 4.23 represents a graph to further investigate the rate and extent of desulfurization as detected by a change in retention time at which the PS oligonucleotide eluted as a function of sodium hypochlorite concentration. With increasing concentration of 0.125, 0.25 and 0.5% sodium hypochlorite a slight but noticeable shift in retention time was detected which occurred to a greater extent with increasing length of analysis. The most significant extent of change in retention time and therefore desulfurization was noted at a concentration of 1.25%. The increased rate of desulfurization observed at 1.25% in contrast to 2.5% may be due to the fact that the main pathway of degradation, at lower concentrations of sodium hypochlorite, was desulfurization followed by no significant degradation of the oligonucleotide. However, at the higher concentration of 2.5% sodium hypochlorite, the initial pathway of degradation appears to be desulfurization in combination with the additional breakdown of the desulfurized material.
Figure 4.23 Graph representing the relationship of the change in retention time of PS oligonucleotide peak after treatment with sodium hypochlorite as assessed by IP-RP LC.

Each value represents mean ± SD (n=3).

Figure 4.24 (a and b) presents the electrophoretic results obtained following analysis of the effect of 0.25, 0.5 and 1.25% sodium hypochlorite (figure 4.24a) and 2.5, and 5% sodium hypochlorite (figure 4.24b) on PS oligonucleotides. There was no distinct change in colour of the band from purple, for the control PS oligonucleotide (C1), following treatment with 0.25 and 0.5% sodium hypochlorite. Upon treatment with 1.25% sodium hypochlorite, the bands at all three time points of analysis appear purple with a bluish tinge located slightly lower down the gel. This is indicative of the initial stage of desulfurization where maximum desulfurization was observed via HPLC analysis (figure 4.23). It would be expected that these bands would be bluer in colour. An increase in treatment concentration of 2.5 and 5% sodium hypochlorite increased the rate of desulfurization as indicated by the appearance of blue bands in gel b. It is, however, difficult from the gel analysis, to conclusively verify maximum degradation of 41%, as evidenced by HPLC analysis, following treatment with 2.5% sodium hypochlorite. However, the bands appear slightly fainter in intensity with the appearance of smaller breakdown products which ran further down the gel.
Figure 4.24 Electrophoretic analysis of the effect of sodium hypochlorite on PS oligonucleotides after a period of 1, 30 and 60 minutes at ambient room temperature. Oligonucleotides were visualised upon staining the gel with Stains-all.

Treatment with a) 0.25, 0.5 and 1.25% sodium hypochlorite; b) 2.5 and 5% sodium hypochlorite.

C1: PS oligonucleotide only; C2: 1.25% sodium hypochlorite (gel a), 5% sodium hypochlorite (gel b).

4.3.7 IP-RP LC and electrophoretic analysis of the effect of Peracetic acid (CH₃COOOH) on PS oligonucleotides.

The application of peracetic acid (PAA) was investigated on the stability of phosphorothioate oligonucleotides at final treatment concentrations of 0.0025, 0.025, 0.25, 1.25, 2.5, 5 and 12.5% (w/v) over a period of 60 minutes as described in section 4.2.4

Figure 4.25 (a and b) represent treatment of the PS oligonucleotide with 0.025 and 0.25% PAA for 60 minutes respectively. Figure 4.25(a) represents the chromatographic results where maximum desulfurization of the PS oligonucleotide was detected by the notable shift in retention time to 6.8 ± 0.1 minutes as indicated on the time axis. Maximum degradation of 38.5% was obtained following incubation of the PS oligonucleotide with 0.25% PAA (figure 4.25 (b)). The main sample peak decreased in peak height and peak area in addition to eluting at an earlier retention time.
Figure 4.25 Chromatographic results of the effect of Peracetic acid on PS oligonucleotides.
The reaction was performed in the presence of a). 0.025% (w/v) and b). 0.25% (w/v) peracetic acid after 60 minutes at ambient room temperature. The peak numbers are printed above each peak corresponding to the order in which the peak elutes and appears on the chromatogram. The retention time is denoted on the time axis of the chromatogram.

= Shift in retention time of the sample PS oligonucleotide.
Under the experimental conditions analysed most significant degradation of 38.5% was observed following treatment with 0.25% PAA after 60 minutes (figure 4.26). Treatment at higher concentrations resulted in a lower level of degradation which may be due to the pH of the solution increasing upon subsequent dilutions. At the higher concentration of PAA (2.5 and 12.5%) maximum degradation of 20.2% was observed.

In addition to the decrease in peak area observed following analysis via HPLC, incubation with increasing concentrations of PAA had the effect of decreasing the retention time at which the PS oligonucleotide eluted from the column as presented in figure 4.27 similar to results obtained following treatment with the other oxidising agents. This concurrent decrease in retention time is indicative of the oxidising ability of PAA at desulfurizing the modified PS oligonucleotide back to the unmodified PO oligonucleotides. This was most evident following incubation the oligonucleotide with 0.025% PAA after 60 minutes which resulted in a shift in retention time from the control untreated PS oligonucleotide at 10.3 minutes to 6.8 minutes. Upon increasing treatment concentration (>0.025%) the rate of desulfurization increased only at time 1 minute. For increased lengths of incubation (30 and 60 minutes) the rate of desulfurization did not statistically change following treatment with concentrations of 0.025% PAA and greater.
Chapter Four

Figure 4.27 Graph representing the relationship of the change in retention time of PS oligonucleotide peak after treatment with peracetic acid as assessed by IP-RP LC.

Each value represents mean ± SD (n=3).

Similar results were obtained following analysis by PAGE as presented in figure 4.28. Desulfurization as observed by a change in colour from the characteristic purple band of the control PS oligonucleotide (C1) to a blue band was detected at concentration equal to 0.025% peracetic acid and greater comparable to findings obtained by HPLC outlined in figure 4.27.

Figure 4.28 Electrophoretic analysis of the effect of peracetic acid on PS oligonucleotides after a period of 1, 30 and 60 minutes at ambient room temperature.

Oligonucleotides were visualised upon staining the gel with Stains-all. Treatment with a) 0.0025, 0.025 and 0.25% (w/v) peracetic acid; b) 2.5, 5 and 12.5% (w/v) peracetic acid. C1: PS oligonucleotide only; C2: 0.25% (w/v) peracetic acid (gel a), 12.5% (w/v) peracetic acid (gel b).

The band was also characteristically slightly lower down the gel than the control sample. Treatment with 0.25% peracetic acid resulted in the formation of blue bands fainter in intensity indicating loss of oligonucleotide material. Under these conditions maximum
degradation of 38.5% was detected by HPLC analysis. Analysis of the effect of higher concentrations of peracetic acid resulted in a complex gel profile. Despite the fact that PAA alone (C2) does not stain, and thus does not interfere with subsequent analysis at higher concentration, the bands ran in an irregular manner. This may be due to the low pH of the sample matrix running through the gel.

4.3.8 IP RP LC and electrophoretic analysis of the effect of hydrogen peroxide (H$_2$O$_2$) on PS oligonucleotides.

The widely recognised and employed oxidant hydrogen peroxide was used in this study to investigate its ability over a range of concentrations to desulfurize and subsequently degrade PS oligonucleotides. Analysis of the effect of 0.5, 2.5, 5, 7.5, 10 and 15% (w/v) hydrogen peroxide on PS oligonucleotides was carried out by IP-RP LC and PAGE as described in section 4.2.4. Percentage PS oligonucleotide degradation was calculated based on the peak area response after treatment of the PS oligonucleotide relative to the untreated intact PS oligonucleotide.

Figure 4.29 (a) displays the chromatographic results of the effect of 0.5% hydrogen peroxide on PS oligonucleotides upon treatment for 60 minutes at ambient room temperature. At this low concentration no significant change in retention time was detected. However, a change in peak shape was observed, as the peak height decreased and appeared somewhat broader in size in contrast to the untreated PS oligonucleotide. Figure 4.29 (b) represents the chromatographic results obtained after treatment of the PS oligonucleotide with 7.5% hydrogen peroxide. In contrast to figure 4.29 (a), a notable shift in retention time was recorded after 60 minutes (7.1 minutes). Similar chromatographic profiles were obtained upon treatment with 15% hydrogen peroxide for 60 minutes (figure 4.29 (c)) where by the retention time shifted from 9.2 to 7.3 and 7.0 minutes after 1, 30 and 60 minutes respectively.
Figure 4.29 Chromatographic results of the effect of hydrogen peroxide on PS oligonucleotides.

The reaction was performed in the presence of a) 0.5% (w/v), b) 7.5% (w/v) and c) 15% (w/v) hydrogen peroxide over a period of 60 minutes at ambient room temperature. The peak numbers are printed above each peak corresponding to the order in which the peak elutes and appears on the chromatogram. The retention time is denoted on the time axis of the chromatogram.

= Shift in retention time of the sample PS oligonucleotide.
Figure 4.30 presents a graph representing the percentage degradation of the PS oligonucleotide following treatment with 0.5, 2.5, 5, 7.5, 10 and 15% hydrogen peroxide over a period of 1, 30 and 60 minutes. With increasing treatment concentration there was an increase in the rate of degradation. Similar to the effect of iodine and potassium permanganate there was little disparity in the extent of decomposition over the three time points of analysis. Minimal degradation was observed under the experimental conditions where a maximum percentage of 19.2% was detected after treatment of the PS oligonucleotides with 15% hydrogen peroxide after 60 minutes.

![Graph showing percentage degradation of PS oligonucleotide with increasing hydrogen peroxide concentration.]

**Figure 4.30 The effect of hydrogen peroxide on PS oligonucleotides as assessed by IP-RP LC.**

The PS oligonucleotide was incubated with 0.5, 2.5, 5, 7.5, 10 and 15% (w/v) hydrogen peroxide at ambient room temperature for 1, 30 and 60 minutes. Percentage degradation of PS oligonucleotides = % intact PS oligonucleotide after treatment for the indicated time relative to the untreated PS oligonucleotide. Each value represents mean ± SD (n=3).

Figure 4.31 graphically summarises the relationship of the shift in retention time at which the treated oligonucleotide eluted as a function of increasing concentration of hydrogen peroxide. No significant difference in retention time was observed over all concentrations of hydrogen peroxide analysed after 1 minute. However, after subsequent treatment for increased incubation periods of 30 and 60 minutes a trend began to emerge. With increasing length of incubation and increasing hydrogen peroxide concentration, the extent of change in retention time at which the PS oligonucleotides eluted off and thus desulfurization increased. This shift in retention time is due to the oxidising ability of hydrogen peroxide at desulfurizing the PS oligonucleotide which increased proportionally with increasing concentration. The extent of desulfurization levelled off at the highest concentration of 15% hydrogen peroxide.
Figure 4.31 Graph representing the relationship of the change in retention time of PS oligonucleotide peak after treatment with hydrogen peroxide as assessed by IP-RP LC.
Each value represents mean ± SD (n=3).

To further verify if the shift in retention time was due to the oxidative ability of hydrogen peroxide on PS oligonucleotides, unmodified PO oligonucleotides were treated with hydrogen peroxide under similar conditions. A concentration of 7.5% was chosen as it represents an intermediate concentration of hydrogen peroxide employed which also gave rise to a noticeable shift in retention time at which the PS oligonucleotide eluted after increasing length of incubation.

Table 4.3 presents a table summarising the results obtained following analysis of the effect of 7.5% hydrogen peroxide on the unmodified PO oligonucleotide. The retention time at which the control PO oligonucleotide control eluted off was 6.0 minutes. After treatment of the PO oligonucleotide with 7.5% hydrogen peroxide no change in retention time was detected over the three time points of analysis. Three independent experiments reproducibly demonstrated consistent results. Thus, the only logical interpretation of this data is that the specific partial desulfurization of the PS linkage in the PS oligonucleotide occurs due to the oxidising ability of hydrogen peroxide. This therefore, reinforces the relationship between the change in retention time and the effect of the oxidising agent at effectively and efficiently desulfurizing the PS oligonucleotide back to the unmodified PO oligonucleotide form.
Table 4.3 Table of results summarising the effect of hydrogen peroxide (H$_2$O$_2$) on PO oligonucleotides as assessed by IP-RP LC.

Each value represents mean ± SD (n=3).

<table>
<thead>
<tr>
<th>Final reaction</th>
<th>Conc. H$_2$O$_2$ (% w/v)</th>
<th>Length of analysis (min)</th>
<th>Retention Time ± SD (n=3)</th>
<th>Peak Area ± %RSD</th>
<th>Description of peak</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>NA</td>
<td>6.0 ± 0.0</td>
<td>508490 ± 1.0</td>
<td>One single peak</td>
<td></td>
</tr>
<tr>
<td>7.5%</td>
<td>1</td>
<td>6.0 ± 0.0</td>
<td>515939 ± 1.8</td>
<td>One single peak</td>
<td></td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>5.9 ± 0.1</td>
<td>518136 ± 1.8</td>
<td>One single peak</td>
<td></td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>6.0 ± 0.1</td>
<td>510857 ± 1.1</td>
<td>One single peak</td>
<td></td>
</tr>
</tbody>
</table>

Figure 4.32 represents the electrophoretic results obtained after analysing the effects of hydrogen peroxide on PS oligonucleotides over a period of 60 minutes at ambient room temperature. From electrophoretic analysis the effect of the oxidant and the extent of desulfurization is evident following treatment with 0.5% hydrogen peroxide where all bands on the gel profile appear blue. This is not in agreement with HPLC analysis where minimal desulfurization was observed (figure 4.31). The bands following treatment with increased concentration of 2.5% hydrogen peroxide appear blue but smaller in intensity than that of the control PS oligonucleotide indicative of partial degradation of the oligonucleotide material. Following treatment with higher concentrations of 5, 10 and 15% hydrogen peroxide (figure 4.32 (b)) the bands appear much fainter in intensity with the appearance of what may be smaller break down products that move lower down the gel. This does not concur with HPLC analysis, where maximum degradation of <20% was detected. The electrophoretic analysis would indicate that greater than 20% degradation occurred.

![Figure 4.32 Electrophoretic analysis of the effect of hydrogen peroxide on PS oligonucleotides after a period of 1, 30 and 60 minutes at ambient room temperature.](image)

Oligonucleotides were visualised upon staining the gel with Stains-all.

Gel a). 0.05, 0.5 and 2.5% (w/v) hydrogen peroxide; Gel b). 5, 10 and 15% (w/v) hydrogen peroxide.

C1: PS oligonucleotide only; and C2: 2.5% (w/v) hydrogen peroxide (gel a) and 15% (w/v) hydrogen peroxide (gel b).
4.3.9 IP-RP LC and electrophoretic analysis of the effect of Virkon® on PS oligonucleotides.

Virkon®, a disinfectant commonly used in laboratories at a concentration of 1% was employed in this study to investigate its effect on PS oligonucleotide stability over a range of concentrations ranging from 0.0025 - 2.5% (w/v) Virkon®. The active ingredient in Virkon® is potassium peroxymonosulfate, an oxidiser which was used in a study carried out by Wozniak et al. (1998) to desulfurize PS oligonucleotide to enable sequencing via enzymatic methods.

Figure 4.33 represents the chromatographic data obtained following incubation of the oligonucleotide with 0.0025 (a), 0.025 (b) and 0.5% (c) Virkon® after 60 minutes. Incubation of the oligonucleotide with the lowest concentration of Virkon (0.0025%) after 60 minutes (figure 4.33 (a)) had no significant effect on the retention time at which the sample product eluted at. The peak appeared tall and sharp and slightly broader than that of the control PS oligonucleotide. The increased concentration of the oxidant at 0.025 and 0.5% Virkon® had the effect of increasing the extent of desulfurization resulted in a shift in retention time. The peak profile (figure 4.33 b and c) demonstrates that the PS oligonucleotide is almost completely converted back to its unmodified counterpart and eluted at an earlier retention time as indicated by the time axis on the representative chromatograms. This relationship is further highlighted in the graph in figure 4.35.
Figure 4.33 Chromatographic results of the effect of Virkon® on PS oligonucleotides.

The reaction was performed in the presence of a), 0.0025% (w/v), b), 0.025% (w/v) and c), 0.5% (w/v) Virkon® over a period of 60 minutes at ambient room temperature. The peak numbers are printed above each peak corresponding to the order in which the peak elutes and appears on the chromatogram. The retention time is denoted on the time axis of the chromatogram.

→ = Shift in retention time of the sample PS oligonucleotide.
Unlike treatment with the aforementioned oxidants, which gave rise to extensive degradation of the modified oligonucleotide, incubation with the disinfectant Virkon® after a period of up to 60 minutes had no significant effect on the peak area of the sample product in contrast to the control. Under the experimental conditions analysed in this study, the peak area remained almost constant as highlighted in the graph in figure 4.34. The only apparent and significant observation was the shift in retention time (figure 4.35).

![Graph showing effect of Virkon® on PS oligonucleotides as assessed by IP-RP LC.](Figure 4.34)

The PS oligonucleotide was incubated with 0.0025, 0.025, 0.25, 0.5% (w/v) peracetic acid at ambient room temperature for 60 minutes. Each value represents mean ± SD (n=3).

Similar to the effects of iodine, potassium permanganate, sodium hypochlorite, peracetic acid and hydrogen peroxide treatment with increasing concentration of Virkon® gave rise to a notable shift in retention time as is evident in the graph representation in figure 4.35. This disinfectant was very effective at desulfurizing the PS oligonucleotide almost completely back to its unmodified form. However, despite the fact that a similar and consistent trend was observed under each experimental condition, in triplicate, results obtained after 30 minutes were not as consistent. For this reason only time point (time 60 minute) is presented. The retention time of the control PS oligonucleotide (made up in water) was 9.4 ± 0.1 minutes which remained constant (9.4 ± 0.0) following treatment with 0.0025% Virkon®. A notable shift in retention time from 9.4 minutes (control) to 7.6 ±0.1 (0.025%), 7.1 ± 0.1 (0.05%), 6.5 ± 0.1 (0.25%) and 6.3 ± 0.2 (0.5%) following treatment with increasing concentration of the oxidant was detected.
This chemical transition and reaction was also observed and confirmed with analysis via PAGE followed by staining with Stains-All. Results obtained are outlined in figure 4.36.

Treatment with increasing concentration of the disinfectant gave rise to a sharp change in colour of the intact band from purple to blue. Similar to results obtained by HPLC (figure 4.35) where initial desulfurization was observed by a slight shift in retention time following incubation with 0.025% Virkon®, the band at 60 minutes appeared blue in colour. Similarly, an increase in treatment concentration led to appearance of bands more blue in intensity.
indicative of further desulfurization. The bands following treatment with all concentrations of Virkon® (0.0025 - 2.5%) remained the same size and intensity as the control PS oligonucleotide. This further corroborates results obtained via HPLC that Virkon® does not give rise to any significant degradation under the experimental conditions analysed in this study.
4.3.10 Discussion of results of the effects of oxidising agents on PS oligonucleotides

4.3.10.1 Effectiveness and mode of action of the chemical oxidative treatment process

Several oxidative procedures using for example potassium peroxymonosulfate (oxone) and iodine have been reviewed throughout the literature primarily to convert the PS oligonucleotide back to its unmodified PO counterpart for subsequent sequencing by chemical or enzymatic means (Reese and Yan, 2003; Wozniak et al. 1998). Based on this research, seven commonly employed oxidising agents/disinfectants (iodine, potassium permanganate, potassium dichromate, sodium hypochlorite, peracetic acid, hydrogen peroxide, and Virkon®) were chosen to investigate their ability to effectively desulfurize and subsequently degrade PS oligonucleotides, at varying concentrations, over a period of 60 minutes at ambient room temperature. The methods were systematically analysed by quantitative IP-RP LC and semi-quantitative PAGE. To the best of our knowledge, no research has been carried out to investigate the effects of the aforementioned oxidants for the treatment of PS oligonucleotides and prevention of the release of such active products into the environment. There are relatively few reports on the use of mild chemical reagents for the specific cleavage of oligodeoxyribonucleotides or DNA containing modified phosphodiester linkages or sugars (Vyle et al. 1992). The parameters investigated in this study, that influence efficacy of the oxidising agents, were oxidant concentration and contact time (time 1, 30 and 60 minutes). The results expressed as percentage degradation of the PS oligonucleotides following treatment with oxidizing agents under empirically established optimal treatment conditions are outlined in table 4.4 in order of most effective in addition to the likely mode of action and occurrence of oxidative degradation (desulfurization). For most oxidants investigated, the efficacy at degrading the PS oligonucleotides increased proportionally with increasing treatment concentration with only marginal disparities observed over the three time points of analysis.
Table 4.4 Percentage degradation of PS oligonucleotides following treatment with oxidizing agents under empirically established optimal treatment conditions.

<table>
<thead>
<tr>
<th>Optimum treatment conditions</th>
<th>Treatment duration (min)</th>
<th>% degradation*</th>
<th>Likely mode of degradation</th>
<th>Desulfurization (yes/no)</th>
</tr>
</thead>
<tbody>
<tr>
<td>K2Cr2O7 (0.125 M K2Cr2O7 in 0.025 M H2SO4)</td>
<td>60</td>
<td>94.7 ± 3.1</td>
<td>Acid catalysed hydrolysis</td>
<td>No</td>
</tr>
<tr>
<td>I2 (25 mM)</td>
<td>1</td>
<td>91.6 ± 4.0</td>
<td>Desulfurization followed by chain cleavage</td>
<td>Yes</td>
</tr>
<tr>
<td>KMnO4 (50 mM)</td>
<td>1</td>
<td>80.1 ± 11.7</td>
<td>Desulfurization followed by chain cleavage</td>
<td>Yes</td>
</tr>
<tr>
<td>NaOCl (2.5 %)</td>
<td>1</td>
<td>41.4 ± 1.7</td>
<td>Desulfurization followed by minimal chain cleavage</td>
<td>Yes</td>
</tr>
<tr>
<td>Peracetic acid (PAA) (CH3COOOH) (0.25%)</td>
<td>60</td>
<td>38.5 ± 6.5</td>
<td>Desulfurization followed by minimal chain cleavage</td>
<td>Yes</td>
</tr>
<tr>
<td>H2O2 (15 %)</td>
<td>60</td>
<td>19.3 ± 1.9</td>
<td>Desulfurization followed by minimal chain cleavage</td>
<td>Yes</td>
</tr>
<tr>
<td>Virkon® (0.5%)</td>
<td>60</td>
<td>&lt; 10%</td>
<td>Desulfurization</td>
<td>Yes</td>
</tr>
</tbody>
</table>

Simple iodine solutions are prepared by dissolving iodine, potassium iodide, or sodium iodide in alcohol or water (Mc Donnell, 2007). For the purpose of this study iodine, made up in water, was purchased from Sigma-Aldrich Co., Dublin, Ireland and subsequent dilutions were prepared freshly accordingly. This solution was chosen for two main reasons; to investigate the effect of iodine alone and to prevent the occurrence and prevalence of additional chemicals (additional pollutants) in the waste stream. The halogen, iodine (I2), which chemically has a high electronegativity is also highly reactive as an oxidising agent (Mc Donnell, 2007). Active iodine species, as reactive oxidising agents, react with nucleic acids (McDonnell, 2007; Rutala, 1996). More specifically, phosphorothioate linkages are susceptible to preferential cleavage with iodine (Huang et al. 1992). The reaction of iodine with sulfur is known to be a highly selective process (Bollmark and Stawinski, 1997), and it is widely accepted that iodine in various forms (Iodine, idoethanol, iodine in pyridine, iodine in methanol or ethanol, THF/water/12-methylimidazole/iodine) can effectively desulfurize (figure 4.37b) and selectively cleave PS oligonucleotides in both the bridging and non-bridging positions (figure 4.37a) (Heidenreich et al. 1993; Strobel, 1999; Polo et al. 1997; Serebrov and Pyle, 2004; Cummins and Potter, 1985; Conrad et al.1995; Strobel and Shetty, 1997; Bollmark and Stawinski 1997; Boudvillain and Pyle, 1998; Wyrzykiewicz and Cole, 1994). It is based on the susceptibility and reactivity of the internucleotide phosphorothioate
linkages with iodine which reacts rapidly and results in chain breakages (Schatz et al. 1991). The mechanism of iodine promoted desulfurization is undetermined but it almost certainly involves the initial formation of the corresponding oxaphosphoranesulfenyl iodine (Figure 4.37b), which undergoes a subsequent nucleophilic substitution at the phosphorous centre (Bollmark and Stawinski, 1997).

![Diagram](image.png)

**Figure 4.37** Iodine cleavage of phosphorothioate internucleotide group (a); reaction resulting in cleavage of the phosphorus-sulfur bond (i); reaction resulting in cleavage of the internucleotidic linkage (i). Iodine promoted desulfurization (b). (adapted from Heidenreich et al. 1993; Bollmark and Stawinski, 1997).

Iodine effectively degraded the PS oligonucleotide giving rise to most significant degradation of 91.6, 93.0 and 90.7% upon treatment with 25 mM iodine after 1, 30 and 60 minutes respectively. The reaction was fast and the main pathway of degradation appears to have been desulfurization followed by subsequent cleavage and breakdown of the PS oligonucleotide as evidenced by HPLC and PAGE. The alkylation of phosphorothioate-containing DNA with 2-idoethanol leads mainly to desulfurization but is accompanied by a small extent of chain cleavage (Gish and Eckstein, 1988).

Potassium permanganate is a powerful and potent oxidizing agent (Rubin and Schmid, 1980; Sloop, 2010) which can oxidize a wide variety of inorganic and organic substances (EPA, 1999). In contrast to iodine, this chemical has not been used to investigate its ability to
oxidise and thus desulphurise the PS oligonucleotide to the best of our knowledge. Desulphurization accompanied by cleavage of the PS oligonucleotide was observed by both IP-RP LC and PAGE. Most significant degradation was obtained upon treatment with 50 mM potassium permanganate which degraded the PS oligonucleotide by 80.1, 77.6 and 79.6% after 1, 30 and 60 minutes incubation respectively at ambient room temperature. Similar to the effects of iodine, the reaction was fast, consistent with findings by EPA, (1999), where reaction rates for the oxidation of constituents found in natural waters by potassium permanganate were relatively fast (EPA, 1999). In addition to the strong oxidizing ability of potassium permanganate, which lead to partial desulphurization of the PS oligonucleotides, studies have also demonstrated that it is used to selectively react with thymine residues in DNA (Rubin and Schmid, 1980; Terashima et al. 1999; Gogos et al. 1990; Tabone et al. 2006; Spicuglia et al. 2004), with a marked preference for nucleotides that are located within single-stranded DNA (Spicuglia et al. 2004). The proposed reaction mechanism is a straightforward glycolization of the carbon 5, 6 double bonds in pyrimidines followed by oxidation to carboxylic acid and/or aldehyde products and thus opening the pyrimidine ring structure (Rubin and Schmid, 1980; Tabone et al. 2006). The sequence of the 21-mer all PS oligonucleotide used in this study is 5’-GCG TTT GCT CTT CTT CTT GCG -3’, comprising of 10 thymines. Therefore, this could account for the additional degradation observed upon treatment with increasing concentration of potassium permanganate.

Potassium dichromate is a soluble hexavalent chromium salt with a strong oxidising ability (Levis et al. 1978; Hammer and Hammer, 2008). Potassium dichromate was made up in an acidic medium using 0.05 M sulphuric acid. Most significant degradation of 81.3 and 94.7% was observed following treatment with 0.125 M potassium dichromate in 0.025 M sulphuric acid after 30 and 60 minutes respectively. Treatment with 0.125 M potassium dichromate (made up in water) gave rise to no significant degradation, (<5%). Similarly, treatment with sulphuric acid, at the higher concentration of 0.05 M gave rise to less than 30% degradation. Therefore, the increased percentage degradation is a result of the synergistic effect of potassium dichromate made up in an acidic medium. A large number of studies established that chromium (VI) induces oxidative stress, DNA damage, altered gene expression and apoptotic cell death upon exposure of cells to chromium (VI) (Bagchi et al. 2002; de Flora, 2000). However, this mechanism is most likely to work in whole cells only when OH radicals generation occurs in close contact with DNA. Numerous types of DNA damage
occur in chromium (VI)-exposed cells, including single-strand breaks, oxidative nucleotide changes, DNA-DNA interstrand crosslinks, DNA-protein crosslinks, chromium-DNA adducts, and chromosomal aberrations (de Flora, 2000).

In contrast to treatment with iodine solution or potassium permanganate the reaction with acidic potassium dichromate is not as immediate. Degradation of PS oligonucleotides increased with increasing concentration of acidic potassium dichromate in addition to increasing length of incubation. Despite the fact that acidic potassium dichromate is a widely accepted oxidising agent (Levis et al. 1978; Hammer and Hammer, 2008) no significant desulfurization was observed under the experimental conditions outlined.

The second halogen used in this study was chlorine. Chlorine and chlorine-releasing agents are extremely active and potent oxidising agents (McDonnell, 2007; New Mexico Environment department, 2011; McDonnell and Russell, 1999). The principal sources of chlorine are hypochlorites, chloramines and chlorine gas (McDonnell, 2007). Sodium hypochlorite, an active ingredient in many household bleach such as Milton and Domestos, was used in this study (Antec International Limited, 1994). The specificity of the reaction of sodium hypochlorite is comparable to that observed by iodine solution and potassium permanganate. The main pathway of degradation was desulfurization followed by minimal degradation less than 42% as evidenced by IP-RP LC analysis. Maximum degradation of 41.4, 39.2 and 38.5% was detected following incubation of the oligonucleotide with 2.5% sodium hypochlorite after a period of 1, 30 and 60 minutes respectively. Interestingly, household bleach solutions generally contain ~ 5% sodium hypochlorite (McDonnell, 2007).

The mode of action of chlorine has been extensively investigated, and it undoubtedly has numerous modes of action by oxidation of proteins, lipids and carbohydrates (McDonnell, 2007). HOCI can produce hydrolysis, oxidation and deamination reactions with a variety of chemical substrates, and produces physiological lesions that may affect multiple cellular processes. Nucleotides are extremely vulnerable to oxidative degradation by HOCI (WHO, 2004). Deleterious effects of chlorine-releasing agents that involve the formation of chlorinated derivatives of nucleotide bases have been described on bacterial DNA (McDonnell and Russell, 1999; Dennis et al. 1979; Dukan and Touati, 1996; McDonnell, 2007).
Two peroxygens namely peracetic acid and hydrogen peroxide were employed in this study. Hydrogen peroxide and peracetic acid (the peroxide of acetic acid) are strong oxidising agents widely used for medical, industrial, and household applications (Yoa et al. 2006; Fraise, 1999; McDonnell, 2007). Similar to the effects of sodium hypochlorite, the peroxygens were not as effective as iodine, potassium permanganate and potassium dichromate at degrading the PS oligonucleotide. The primary pathway of degradation was desulfurization followed by minimal degradation (< 40%) upon treatment with both oxidants. Most significant degradation of 38.5% was observed following treatment with 0.25% PAA for 60 minutes. Under the experimental conditions of this study hydrogen peroxide was less effective giving rise to maximum degradation of 19.2% after incubation with 15% hydrogen peroxide for 60 minutes. Hydrogen peroxide acts as an oxidant by the production of destructive hydroxyl free radicals which can attack essential cell components, including DNA, proteins and lipids and cause oxidative damage (Willerslev et al. 2004; Fraise, 1999; Rutala, 1996; McDonnell and Russell, 1999). Significant research has been carried out on the effect of hydrogen peroxide on isolated DNA and it was established that hydrogen peroxide alters DNA to liberate all four bases resulting in sugar phosphate backbone breakage (Anathaswamy and Eisenstark, 1977). However, the majority of these studies were carried out on isolated DNA using relatively high concentrations of hydrogen peroxide (0.05 to 0.1M) after a long period of incubation in the presence of FeCl₃ (Anathaswamy and Eisenstark, 1977).

Peracetic acid is deemed to be a more potent biocide than hydrogen peroxide (McDonnell and Russell, 1999). Similar results were obtained in this study whereby peracetic acid was more effective at desulfurizing the PS oligonucleotide at lower concentrations than hydrogen peroxide. Additionally, it led to greater degradation at lower treatment concentrations. The mode of action of PAA is similar to that of hydrogen peroxide and deleterious effects of nucleic acids, including both DNA and RNA strand breakage, have been reported (McDonnell, 2007).

Scioli et al. (1997) states that Virkon NF (non foam), an oxidising agent, has a synergic effect on nucleic acids. Unlike most chemical poisons Virkon® is a mixture of six different biocides all designed to work together in unison against different physical and chemical structures within the microorganism. Such a synergic association reacts as a broad biocidal action mechanism, oxidative and denaturing towards nucleic acids, polypeptides, glycoproteins and membrane proteins of numerous structures which form microorganisms
Chapter Four

(Scioli et al. 1997; Antec International Limited, 1994). Virkon® was employed in this study as it is a disinfectant that is readily available and used in most laboratories and in the industry. Its active ingredient is potassium peroxymonosulfate, an oxidising agent which was used in a study carried out by Wozniak et al. (1998) to investigate its application to desulfurize PS oligonucleotides for subsequent sequencing via enzymatic methods with successful results. The concentration of Virkon® (1%-final treatment concentration 0.5%) that gave rise to maximum desulfurization is in compliance with standard recommendations currently used in industry.

It was established, from previous analysis, that the retention time of the PS oligonucleotide is later than that of their isosequential unmodified PO counterparts (section 3.3.2.1). When co-injected, the PO oligonucleotide and PS oligonucleotide eluted at 6.0–6.3 and 9.0–9.6 minutes respectively. Incubation of the PS oligonucleotide with iodine, potassium permanganate, sodium hypochlorite, peracetic acid, hydrogen peroxide and Virkon® demonstrated that they were partially desulfurized into oligonucleotides bearing phosphodiester linkage(s) as they eluted at different (earlier) retention times between the retention time of initial all PS oligonucleotide and the corresponding PO oligonucleotide on the HPLC chromatograms. The progressive replacement of sulfur by oxygen induced a concomitant reduction of the retention time observed following treatment with the above mentioned oxidants. This shift in retention time is indicative of the effect and oxidising ability of the oxidant at desulfurizing the PS oligonucleotide back to its natural PO counterpart. The application of PAGE followed by staining with “Stains-all” provided consistent results. Treatment with increasing concentrations of the oxidants resulted in a progressive shift in colour change from purple (PS oligonucleotides) to blue (PO oligonucleotides) (section 3.3.3).

Despite the fact that significant degradation was not observed upon treatment with either hydrogen peroxide, sodium hypochlorite, peracetic acid or Virkon® it was observed that such oxidants desulfurized the PS oligonucleotides to their natural PO counterpart thus making them less stable and more susceptible to nuclease degradation by naturally secreted nucleases in activated sludge and environment.

PS oligonucleotides conversions into natural oligonucleotides under conditions that do not affect nucleobases or the sugar-phosphate backbone appear to be attractive for sequencing
Chapter Four

(Wozniak et al. 1998). Much research has centred on the use of oxidising agents as a pre-treatment method for subsequent sequencing of the increased nuclease resistant PS oligonucleotides (Schuette et al. 1995; Wozniak et al. 1998). Additionally, Wozniak et al. (1998), reports that analysis of natural oligonucleotide (e.g., cloning or sequencing) is considerably more reliable than that of PS oligonucleotides. Several oxidative procedures involving, for example, potassium peroxyomonosulfate (oxone), and iodine have previously been used to convert unprotected PS oligonucleotides into the corresponding unmodified oligonucleotides (Schuette et al. 1995; Wozniak et al. 1998). Subsequent to oxidation, exonucleases which successively cleave individual bases from the 3’ or 5’ terminus of the oligonucleotide or base-specific cleavage chemicals are employed to help identify the sequence of the oligonucleotide. PAA (0.025%) and Virkon® (0.25%) could potentially be used to facilitate sequencing of PS oligonucleotides, as at low concentrations, it was observed that both oxidants efficiently desulfurized the modified oligonucleotide almost completely back to its unmodified structure, under conditions that do not affect the sugar-phosphate backbone or nucleobases, as evidenced by IP-RP LC and PAGE. We do not necessarily recommend these two new reactions as substitutions for the standard desulfurization reactions to enable ease of sequencing, but as potential alternatives. In addition to this they are cheap, readily available reagents which display relatively low toxicity. These properties make them attractive alternative reagents for chemical DNA sequencing followed by subsequent enzymatic or chemical treatment.

4.3.10.2 Advantages and limitations of each proposed oxidative chemical process

As discussed, all chemical treatments were effective either at generating > 70% degradation (iodine, potassium permanganate and potassium dichromate) or at partially or almost fully desulfurizing the PS oligonucleotides back to the more unstable unmodified form (sodium hypochlorite, peracetic acid, hydrogen peroxide and Virkon®). One common property that all the chemical oxidants display is that they are widely available and commonly employed chemical reagents in industry. Additionally, oxidative processes are commonly employed in waste water treatment facilities (WHO, 2004). Although it is evident that all chemical technologies will have limitations, understanding the limitations that may occur is critical for appropriate application of potential treatment processes. When choosing a suitable chemical treatment there are several additional factors such as environmental safety, user safety, by product formation and cost which need to be taken into account in assessing an appropriate treatment method for the successful removal of active antisense products. Table 4.5 lists the
advantages and limitations of the application of each of the seven oxidants employed in this study.

Table 4.5 The potential advantages and limitations of the application of the seven oxidising agents to treat PS oligonucleotide containing waste streams.


<table>
<thead>
<tr>
<th>Chemical oxidant</th>
<th>Advantages</th>
<th>Limitations</th>
</tr>
</thead>
<tbody>
<tr>
<td>Iodine</td>
<td>Widely available.</td>
<td>Specificity for PS modification.</td>
</tr>
<tr>
<td></td>
<td>Easy to prepare.</td>
<td>Generates brown stains on surfaces.</td>
</tr>
<tr>
<td></td>
<td>Fast (&lt;10 minutes), effective decomposition (&gt;90%) of the PS oligonucleotides at relatively low concentrations.</td>
<td>Gives water a slight straw colour at high levels.</td>
</tr>
<tr>
<td></td>
<td>Desulfurization.</td>
<td>At high concentrations (&gt;5%) iodine solutions are poisonous and may cause irritation to broken skin and mucous membranes, especially in combination with alcohol.</td>
</tr>
<tr>
<td></td>
<td>May have some short-lived persistent activity, remaining after application to provide residual degradation activity.</td>
<td>Generally unstable and surface incompatibility with some metals (corrosion) and plastic surfaces.</td>
</tr>
<tr>
<td></td>
<td>Does not require energy.</td>
<td></td>
</tr>
<tr>
<td>Potassium permanganate</td>
<td>Commercially available disinfectant</td>
<td>Strong oxidizer and should therefore be handled with care.</td>
</tr>
<tr>
<td></td>
<td>Easy to prepare, apply, transport and store as it is commercially available in powder or crystal form.</td>
<td>It is toxic and irritating to mucous membranes and skin.</td>
</tr>
<tr>
<td></td>
<td>Reactive, fast-acting oxidiser which gave rise to significant degradation (~80%) of the PS oligonucleotides</td>
<td>Will stain easily producing brown discolouration of clothing and skin</td>
</tr>
<tr>
<td></td>
<td>Desulfurization.</td>
<td>It is not desirable to maintain a residual of potassium permanganate as it tends to give water a pink shade.</td>
</tr>
<tr>
<td></td>
<td>Unlike iodine KMnO₄ is not PS chemistry specific</td>
<td>May also be a source of manganese which ought to be treated and/or controlled to minimise finishing water manganese levels</td>
</tr>
<tr>
<td></td>
<td>Does not interfere with other treatment processes or plant conditions at the water treatment facility.</td>
<td></td>
</tr>
<tr>
<td>Potassium dichromate</td>
<td>Widely available.</td>
<td>Chromium is on the top priority list of toxic pollutants identified by the US EPA.</td>
</tr>
<tr>
<td></td>
<td>Fast (60 minutes), effective decomposition (&gt;90%) of the PS oligonucleotides at relatively low concentrations.</td>
<td>Generates stoichiometric amounts of heavy-metal waste and Cr (VI), a proven toxin, carcinogen and mutagen.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Requirement of additional step for the removal and conversion of Cr (VII) to Cr(III) followed by removal or recycle of Cr(III) prior to discharge to avoid these deleterious effects.</td>
</tr>
<tr>
<td>Sodium hypochlorite</td>
<td>Widely used disinfectant.</td>
<td>Lead to moderate degradation (41%) of the PS oligonucleotides.</td>
</tr>
<tr>
<td></td>
<td>Commercially available / industrial disinfectants e.g. Milton, Domestos</td>
<td>Can cause corrosion of metal surfaces, predominantly at high concentrations.</td>
</tr>
<tr>
<td></td>
<td>Readily available, easily applied and cheaper than other oxidizing agents such as potassium permanganate, ozone or chlorine dioxide</td>
<td>Light, Heat and moisture increase the rate of loss of free chlorine.</td>
</tr>
<tr>
<td></td>
<td>Colourless and easy to handle</td>
<td>At higher concentrations, chlorine is irritating and may lead to hypersensitivity and concentrated solutions may be toxic to humans.</td>
</tr>
<tr>
<td></td>
<td>Lead to desulfurization converting the modified PS oligonucleotide back to its</td>
<td></td>
</tr>
</tbody>
</table>
### Chapter Four

<table>
<thead>
<tr>
<th>Peracetic acid (PAA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>▪ Widely used in industry with multiple applications.</td>
</tr>
<tr>
<td>▪ Lead to significant desulfurization.</td>
</tr>
<tr>
<td>▪ It is environmentally friendly at low concentrations.</td>
</tr>
<tr>
<td>▪ Decomposes into safe, non-toxic by-products of a low concentration of acetic acid water and oxygen.</td>
</tr>
<tr>
<td>▪ It is reported to have no harmful health or environmental effects.</td>
</tr>
<tr>
<td>▪ Compatible with stainless steel and other surfaces but is dependent on the formulation and application.</td>
</tr>
<tr>
<td>▪ Didn’t give rise to significant degradation (39%) of the PS oligonucleotide.</td>
</tr>
<tr>
<td>▪ Considerably less stable than hydrogen peroxide solutions particularly when diluted.</td>
</tr>
<tr>
<td>▪ PAA has a strong, pungent (vinegar-like) odour at concentration of 5 to 37% which can be irritating to the respiratory system, mucous membranes and eyes.</td>
</tr>
</tbody>
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<table>
<thead>
<tr>
<th>Hydrogen peroxide</th>
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</thead>
<tbody>
<tr>
<td>▪ Widely used in industry with multiple applications.</td>
</tr>
<tr>
<td>▪ Lead to partial desulfurization.</td>
</tr>
<tr>
<td>▪ Hydrogen peroxide is environmentally friendly and nontoxic, as it can rapidly degrade into the innocuous by-products water and oxygen resulting in no toxic residues and harmful emissions.</td>
</tr>
<tr>
<td>▪ Concentrations from 3 to 6% peroxide solutions are considered safe for use directly on the skin and other surfaces.</td>
</tr>
<tr>
<td>▪ Is reasonably stable, but most dilutions contain stabilizers to prevent decomposition.</td>
</tr>
<tr>
<td>▪ No significant degradation observed (&lt;20%).</td>
</tr>
<tr>
<td>▪ Hydrogen peroxide can cause bleaching of surfaces.</td>
</tr>
<tr>
<td>▪ Contact with various surfaces such as organic materials, copper, brass and iron, and cellulosic materials can lead to the rapid degradation of peroxide</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Virkon®</th>
</tr>
</thead>
<tbody>
<tr>
<td>▪ Commercially available high level disinfectant.</td>
</tr>
<tr>
<td>▪ Virkon® was rapidly effective almost completely desulfurizing the PS oligonucleotides upon treatment with 0.5% treatment concentration Virkon®.</td>
</tr>
<tr>
<td>▪ Powder form - Virkon® is more compact than costly liquid disinfectants, easy to store, transport and prepare.</td>
</tr>
<tr>
<td>▪ Ease of use.</td>
</tr>
<tr>
<td>▪ Environmentally friendly and low toxicity.</td>
</tr>
<tr>
<td>▪ Poses no harmful effect to waste treatment plants.</td>
</tr>
<tr>
<td>▪ Virkon® is compatible with all surfaces and materials including metals, plastics, laminates and textiles.</td>
</tr>
<tr>
<td>▪ No significant degradation observed under the experimental conditions.</td>
</tr>
</tbody>
</table>
4.4 Concluding remarks

This study was carried out to investigate and provide alternative, appropriate and suitable methodologies for treating PS oligonucleotides thus rendering the waste water stream free of active drug product. The fate of PS oligonucleotides in the presence of soft metal ions (silver nitrate) and various oxidants (iodine, potassium permanganate, acidic potassium dichromate, sodium hypochlorite, peracetic acid, hydrogen peroxide and Virkon®) was established through quantitative and semi-qualitative analysis of the intact 21-mer all PS oligonucleotide by IP-RP LC and PAGE.

The theory that PS oligonucleotides, due to their soft metal sulfur replacement, can interact and coordinate preferentially with the soft metal ions such as silver and to a greater extent at higher concentrations was conclusively established and verified by IP-RP LC and PAGE. The application of both analytical techniques provided consistent and valuable information. Both techniques highlight the strong affinity of the soft metal ions for PS oligonucleotides as indicated by an intense band with lower mobility than that of the uncomplexed PS oligonucleotide in analysis by PAGE and the appearance of the newly formed peak at 12.0 minutes as detected by IP-RP LC. It is proposed that the apparent disparities in affinity may be due to changes in the steric or electrostatic environment engendered by the PS modification (Christian et al. 2002). The application of immobilized metal ion adsorption chromatography (IMAC) using silver ions presents a method by which the PS oligonucleotides could be “trapped” isolated from other waste material and subsequently treated in a suitable manner prior to release to the waste water stream. A similar study was carried out by Eriksson and Johansson, (2004, 2008) to isolate fully thioated single stranded oligonucleotides from a biological solution and/or to separate the fully thioated oligonucleotides from incorrectly thioated antisense oligonucleotides in the solution. In this study the metals of choice were Fe$^{3+}$ and Zr$^{2+}$.

Oxidation appears to be the best remediation technology whereas soft metal ion treatment appears to be less effective. Thus, this study verified that instability of PS oligonucleotides is mainly attributed to one of the two degradation mechanisms, desulfurization as observed by Bennett et al. 2004; Hardee et al. 2001; the other being acid catalysed hydrolysis (Chapter Three).

Iodine, potassium permanganate and acidic potassium dichromate present the most effective methods, resulting in almost complete degradation of the PS oligonucleotides, suitable levels
for the subsequent disposal of these wastes which can therefore be passed with less danger into the wastewater treatment plant. Potassium dichromate was the most effective at degrading the PS oligonucleotides by 94.7% followed by iodine (91.6%) and potassium permanganate (80.1%) under the experimental conditions analysed. Based upon the concentration of reagents required, treatment duration, economic considerations and potential environmental impacts, it is likely that iodine would be the most environmentally manageable and suitable treatment method which could potentially be effective on an industrial scale. From the four reagents (sodium hypochlorite, peracetic acid, hydrogen peroxide and Virkon®) that gave rise to minimal degradation of the PS oligonucleotide (≤42%) peracetic acid and Virkon® present as the most effective and environmentally friendly at effectively desulfurizing the PS oligonucleotides back to the more unstable unmodified form making them less stable and more susceptible to nuclease degradation by naturally secreted nucleases in the activated sludge and environment. Additionally, PAA (0.025%) and Virkon® (0.25%) could potentially be used to facilitate sequencing of PS oligonucleotides, as at low concentrations, it was observed that both oxidants efficiently desulfurized the modified oligonucleotide almost completely back to its unmodified structure, under conditions that do not affect the sugar-phosphate backbone or nucleobases. The potential advantage of such proposed methods of treatment is that the chemicals employed in this study are inexpensive, readily available reagents and allow ease of use. Such methods could potentially be effective on an industrial scale when used to pre-treat a waste treatment influent stream thus rendering it free of active drug product.
Chapter Five: The Use of Commercially Available Nucleases for the Degradation of Antisense Oligonucleotides.
Chapter Five: The Use of Commercially Available Nucleases for the Degradation of Antisense Oligonucleotides.

5.1 Introduction

This chapter outlines the methodologies employed in a study of the detection, quantification and analysis of the enzymatic stability of unmodified PO oligonucleotides and modified PS oligonucleotides to commercially available nucleases. In this chapter the applications of enzymes in industry and most specifically nucleases are discussed to investigate their application to hydrolyse and degrade PS oligonucleotides in waste product streams thus preventing the possibility of further environmental implications. Treatment via enzymatic methods using nucleases offers an alternative to physical/chemical based antisense degradation (Chapter Three and Four). It may present as a more environmentally friendly and energy saving alternative than the addition of more hazardous chemical reagents.

5.1.1 Enzyme technologies and applications in industry

‘White biotechnology’ describes the biotechnological production of chemical compounds using both enzymes and microorganisms (Jaeger, 2004). Enzymes, naturally-occurring proteins, are biocatalysts that facilitate chemical reactions in biological systems (Leisola et al. 2002; Jayaraman 2010; Mishra, 2002). For thousands of years enzymes have been used in many biotechnological processes, which include the production of foods and beverages such as wine, beer, cheese and vinegar and commodity products such as leather and linen. All these processes were carried out with microorganisms or crude cell extracts without having any previous knowledge about the existence of enzymes (Jaeger, 2004). Following their discovery about 200 years ago researchers have started to purify and characterize a large variety of different enzymes and in many instances cloned their respective genes during the past few decades. By this time it became evident that many enzymes were superior catalysts capable of accepting a variety of different substrates, displaying exquisite substrate specificity, exhibiting high regioselectivity and enantioselectivity (Jaeger, 2004).

In 1914 isolated enzymes were first used in detergents, their protein nature was proven in 1926 and their large-scale microbial production was initiated in the 1960’s. Today, several chemical companies are using white biotechnology to manufacture a wide range of products for the chemical market including bulk organic chemicals, pharmaceuticals and agrochemicals, as well as food ingredients and bioplastics (Jaeger, 2004). Over 500
commercial products are manufactured using enzymes (Johannes and Zhao, 2006). Some applications of industrial enzymes are summarised in Table 5.1. In these applications, enzymes possess numerous benefits including improvement of quality and health characteristics, reduction of manufacturing costs, and enhancement of sustainability profile of manufacturing processes (Jayaraman, 2010).

Table 5.1 Selected examples of enzymes which have found industrial application (Leisola et al. 2002).

<table>
<thead>
<tr>
<th>Industry</th>
<th>Enzyme</th>
<th>Application</th>
</tr>
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<tbody>
<tr>
<td>Animal feed additive</td>
<td>Xylanase</td>
<td>Fibre solubility</td>
</tr>
<tr>
<td></td>
<td>Phytase</td>
<td>Release of phosphate</td>
</tr>
<tr>
<td>Baking</td>
<td>Xylanase</td>
<td>Dough conditioning</td>
</tr>
<tr>
<td></td>
<td>Alpha-amylase</td>
<td>Loaf volume; shelf-life</td>
</tr>
<tr>
<td></td>
<td>Glucose oxidase</td>
<td>Dough quality</td>
</tr>
<tr>
<td>Brewing</td>
<td>Glucanase</td>
<td>Filter aid</td>
</tr>
<tr>
<td></td>
<td>Papain</td>
<td>Haze control</td>
</tr>
<tr>
<td>Dairy</td>
<td>Rennin</td>
<td>Protein coagulation</td>
</tr>
<tr>
<td></td>
<td>Lactase</td>
<td>Lactose hydrolysis</td>
</tr>
<tr>
<td>Detergent</td>
<td>Proteinase</td>
<td>Protein degradation</td>
</tr>
<tr>
<td></td>
<td>Lipase</td>
<td>Fat removal</td>
</tr>
<tr>
<td></td>
<td>Cellulase</td>
<td>Colour brightening</td>
</tr>
<tr>
<td>Fruit juice</td>
<td>Pectinase</td>
<td>Juice clarification</td>
</tr>
<tr>
<td></td>
<td>Cellulase,</td>
<td>Juice extraction</td>
</tr>
<tr>
<td></td>
<td>Xylanase</td>
<td></td>
</tr>
<tr>
<td>Pulp and paper</td>
<td>Xylanase</td>
<td>Biobleaching</td>
</tr>
<tr>
<td>Starch</td>
<td>Amylases</td>
<td>Glucose formation</td>
</tr>
<tr>
<td></td>
<td>Glucose isomerase</td>
<td>Fructose formation</td>
</tr>
<tr>
<td>Textile</td>
<td>Cellulase</td>
<td>Microfibril removal</td>
</tr>
<tr>
<td></td>
<td>Laccase</td>
<td>Colour brightening</td>
</tr>
</tbody>
</table>

The development of recombinant DNA technology had a significant effect on production levels of enzymes, as it was possible to clone genes encoding enzymes from microbes and express them at levels hundreds of times higher than those produced naturally and move enzyme production from strains unsuitable for industry into industrial strains (Galante and Formantici, 2003). Over half of the enzyme market is provided by recombinant enzymes (Sanchez and Demain, 2011). Due to improved production technologies, engineered enzyme properties and novel application fields the industrial enzyme business is now steadily growing as an interesting green alternative to replace traditional chemical processes (Leisola et al. 2002; Sanchez and Demain, 2011).
5.1.2 Nucleases and nucleic acids

The International Union of Biochemistry (IUB), have divided enzymes into six classes: oxidoreductases, transferases, hydrolases, lyases, isomerases, and ligases (Sanchez and Demain, 2011). Nucleases, are enzymes of the class hydrolase, and are defined as a group of enzymes capable of cleaving the phosphodiester bonds between the nucleotide subunits of nucleic acids (figure 1.14) (Desai and Shankar, 2003; Mishra, 2002). Nucleases are ubiquitous in living organisms (Weir, 1993) and display very diverse functions (Friedhoff et al. 1999). They are present in prokaryotes, eukaryotes, several fungal species and viruses. It has been reported that the majority of organisms produce multiple extracellular and intracellular nucleases with different endonuclease and exonuclease activities (Mouratou et al. 2002). Numerous varieties of nucleases provide functions and are involved in numerous aspects of DNA metabolism, including recombination, replication and repair (Desai and Shankar, 2003; Mouratou et al. 2002; Rangarajan and Shankar, 1999). Extracellular nucleases have been reported to play a role in nutrition, have been involved in “scavenging” of nucleotides and phosphates for the growth and metabolism and in the recycling of DNA building blocks from the substrate mycelium (Brnáková et al. 2007). As a result, all living organisms have nucleases that are capable of interacting with nucleic acids and subsequently hydrolysing the phosphodiester linkages. Some of the properties of nucleases and their possible biological roles have been described previously (Nicholson, 1999; Zuo and Deutscher, 2001; Hsia et al. 2005; Shevelev and Hübscher, 2002; Ceska and Sayers, 1998; Nishino and Morikawa, 2002; Marti and Fleck, 2004). Some nucleases also possess other enzymatic functions in addition to their main property of catalysing the hydrolysis of phosphodiester linkages such as ligase, DNA polymerase, kinase or helicase and other functions such as repressor (Mishra, 2002).

5.1.3 Nucleases and nuclease classification

The enzymatic degradation of nucleic acids was initially observed and demonstrated in the early twentieth century and the term ‘nucleases’ was coined for enzymes involved in this (Mishra, 2002). It was not until 1940 that Kunitz reported two groups of nucleases based on sugar specificity designated as deoxyribonuclease (DNase) or ribonuclease (RNase) capable of hydrolysing deoxyribonucleic acids and ribonucleic acids, respectively (Kunitz 1940; Kunitz 1950). With the discovery of multifunctional nucleases such as micrococcal nuclease and snake venom phosphodiesterases, that attacked both DNA and RNA, the classification of nucleases into DNase and RNase, appeared to be somewhat inappropriate (Laskowski,
As a result, a new class of sugar-nonspecific nucleases was established and added to the list of nucleases. This method of classification gave rise to a trend whereby the terms DNase and RNase were used for the sake of convenience (Mishra, 2002). Consequently, with the increased discovery of newer nucleases and multifunctional enzymes, variations of schemes of classification were suggested. As a result Bernard (1969) and Laskowski (1982) suggested that specificity of a particular nuclease be classified on the basis of:

1. The nature of the substrate being hydrolysed, either DNA or RNA
2. The kind of nucleolytic attack, exonuclease and/or endonuclease
3. The nature of hydrolytic products produced i.e. mono or oligonucleotides terminating in a 3’- or a 5’-phosphate
4. The type of bond hydrolysed.

However, within each of these categories, in the classification schemes, there are exceptions which are due to the complex and varied nature of the catalytic activities of unique nucleases (Desai and Shankar, 2003). This scheme however did not take into consideration additional criteria such as structure-selectivity, site-specificity, the nature of the substrate DNA and functional ability to restructure DNA molecules (i.e., to facilitate genetic recombination). These were added to this list at a later stage (Mishra, 2002). Incorporating most of these aspects, Linn (1982) devised the most acceptable system of classification (Brnakova and Godany, 2003). A schematic diagram of the classification of nucleases is illustrated in figure 5.1.
Figure 5.1 A schematic diagram of the classification of nucleases (Rangarajan and Shankar, 2001).

5.1.2.2 Site of cleavage and mode of action

Nucleases and phosphodiesterases cleave the bond between phosphorus and oxygen in the internucleotide linkage of the nucleic acid as illustrated in figure 5.2 (Mishra, 2002). The site of cleavage can be on either side of the phosphate bond generating a 5’ or a 3’ monoesterified product (Weir, 1993). No enzymes, to date, have been discovered that can split the internucleotide bond on both sides (Weir, 1993). Enzymes that hydrolyse nucleic acids are often characterized based on whether they act on the 5’ or 3’ position.
Nucleases are categorised as exonucleases and/or endonucleases. Exonucleases cleave residues beginning at one end of the polynucleotide chain, from either the 3’ or the 5’ end. Endonucleases hydrolyse the phosphodiester linkages at sites internally in the chain. Cleavage may occur at either the 5’ (A) or the 3’ (B) side of the phosphodiester bond.

A further characterisation of these enzymes is based on whether the nucleic acid chain is cleaved internally (endonuclease) or whether a terminal residue is cleaved (exonuclease), or both (endo-exonuclease) (figure 5.2). Exonuclease refers to a nuclease enzyme that degrades a nucleic acid chain sequentially starting from one or both ends of the chain (Lorenz, 2008). As stated previously, cleavage may be either in the 3’ to 5’ direction releasing 5’ phosphomononucleotides or in the 5’ to 3’ direction yielding 3’ phosphomononucleotides (Weir, 1993). The products of hydrolysis are predominantly mononucleotides and mode of attack is processive (Desai and Shankar, 2003). Exonucleases generally require a free terminus for their action (Brnakova and Godany, 2003). An example of a widely used exonuclease is exonuclease III from Escherichia coli (Weir, 1993) or snake venom phosphodiesterase. Conversely, the phosphodiester bonds of nucleic acids are cleaved internally by endonucleases with or without termini. They display a distributive mode of action and the main products of hydrolysis are mononucleotides and/or oligonucleotides (Brnakova and Godany, 2003; Lorenz, 2008). Pancreatic DNase is an example of an endo-
DNase (Weir, 1993). An endonuclease will not digest DNA molecules to completion - to nucleotide monomers. It is only when exonuclease activity is present will DNA be digested to completion (Weir, 1993). Endo-exonucleases hydrolyse both DNA and/or RNA, either endonucleolytically or exonucleolytically but they demonstrate different modes of action on these substrates. Most of these enzymes degrade nucleic acids to oligonucleotides whilst some degrade them further to mononucleotides (Brnakova and Godany, 2003). Nuclease P1 is an example of an endo-exonuclease.

As with the majority of enzyme families, nucleases demonstrate selectivity/specificity for the substance on which they act. Structure specificity and strand preference are most relevant to this study. Nucleases appear to have a “preference” for cleavage of single-stranded DNA or double-stranded DNA substrates (Weir, 1993). As the application of the nuclease required in this study is on single stranded oligonucleotides it would be expected that a single stand specific nuclease would be most effective. To date, a large number of single strand-specific and sugar non-specific nucleases have been recognised and isolated from a wide variety of sources including animals, plants and micro-organisms. Their widespread use has paved the way for the isolation of greater than 30 single strand specific nucleases from a variety of sources. However, to date, only a small number of enzymes such as S1 nuclease from Aspergillus oryzae, P1 nuclease from Penicillium citrinum, BAL 31 nuclease from Alteromonas espejiana, Neurospora crassa, Ustilago maydis and mung bean nucleases have been adequately characterized (Rangarajan and Shankar, 1999; Naik and Raghavan, 2008). Bovine pancreatic DNase I, on the other hand, has a strong preference for double stranded DNA. Some nucleases are however, able to degrade both single and double stranded nucleic acids, such as the extracellular endonuclease from Serratia marcescens, extracellular endonuclease from Anabaeba sp, mitochondrial endoexonuclease from Neurospora crassa, or the mitochondrial endonuclease from yeast (Freidhoff et al., 1994).

5.1.2.3 Applications of nucleases

The fact that nucleases can be obtained in a purified form, in large quantities, has led to their application in science, biotechnology and medicine (Mishra, 2002). Their application depends on the mode of action and specificity of a particular enzyme (Reddy and Shankar, 1993). Some of their applications are outlined in table 5.2. All these novel methods of genetics, in which nucleases play critical roles, hold great potential for the future of mankind through the development of science, technology, and commerce (Mishra, 2002).
Table 5.2 Selected examples of nucleases which have found industrial application


Applications of Nucleases

- Single stranded nucleases are used as analytical tools for the determination of nucleic acid structure.
- They are used as probes for the structural determination of nucleic acids, investigating the interactions of DNA with a variety of intercalating agents and mapping mutations.
- RNases are used in molecular biological study, food and pharmaceutical industry.
- Many RNases are highly cytotoxic and display several biological roles, such as antiviral and antitumor activity, and may be considered as alternative chemotherapeutic drugs in the future.
- Used in forensic science in DNA fingerprinting/DNA profiling.
- Industrial use of nucleases involves the production of certain sweetener and flavouring materials.
- The discovery of a new class of nucleases called restriction endonucleases (1970) resulted in the development of recombinant DNA technology, DNA sequencing methodology and new methodologies for genetic mapping and the extensive mapping of human chromosomes.
- Nucleases pose great potential to be used in medicine and agriculture via the application of recombinant DNA technology and in overcoming many environmental problems as an indicator of environmental pollutants or infectants.
- They are employed for the diagnostic purposes in identifying the genes controlling human and other diseases.

5.1.2.4 Nucleases and their ability to degrade phosphorothioate oligonucleotides

One of the most notable functional attributes of PS oligonucleotides is that they are generally resistant to nucleases (Brautigam and Steitz, 1998; Eckstein, 1985; Spitzer and Eckstein, 1988) but are not nuclease proof (Stein, 1996; Toulmé et al. 1997). Oligodeoxynucleotides, containing a PS backbone, display increased nuclease resistance but are ultimately degraded (Tang et al. 1993). As resistance towards destructive nucleases is an essential requirement for antisense therapeutics, numerous studies have been carried out to investigate the nuclease resistance and stability of various oligonucleotide analogues (McKay et al. 1996; Stein et al. 1988; Spritzer and Eckstein, 1988; Morita et al. 2002; Ito et al. 2003; Murata et al. 2003; Gilar et al. 1998b; Agrawal et al. 1997; Iyer et al. 1996).

Current automated synthetic methods used to prepare PS oligonucleotides are non-stereospecific and consists of a mixture of 2^n diastereomers where n is a number of phosphorothioate internucleotide linkages (Wilk and Stec, 1995; Stec et al. 1995). The diastereotopicity of these oxygens gives rise to a pair of diastereomeric phosphorothioates which are designated as having either the Rp or Sp diastereomer orientation (section 1.5.1) (Almer et al. 1996). It has been demonstrated that nucleases are extremely diastereoselective (Koziolkiewicz and Stec, 1992), that interact and exert action on PS oligonucleotides in a diastereomeric selective manner (Yu et al. 2000). “Stereospecificity of nucleases for one or the other diastereoisomers of the phosphorothioate linkages varies” (Wyrzykiewicz, 1997).
Of the two possible configurations of a phosphorothioate diastereomer, generally only one configuration will be cleaved by a particular enzyme at a reasonable rate. However, it is generally not possible to envisage which one this may be. Therefore, amongst the non-specific DNA endonucleases, there are enzymes which can cleave Rp linkages and those which can cleave Sp linkages (Potter and Eckstein, 1984; Wyrzykiewicz, 1997). The diastereoselectivity of many nucleolytic enzymes towards PS-oligonucleotides is well documented. SVPD and *Serratia marcescens* nuclease recognise and hydrolyse phosphorothioates of Rp configurations (Koziolkiewicz *et al.* 1999; Koziolkiewicz *et al.* 2001; Stec *et al.* 1995; Tamura *et al.* 1998; Wilk and Stec, 1995). Conversely, nuclease P1 and nuclease S1 preferentially hydrolyse internucleotide Sp phosphorothioates (Stec *et al.* 1995; Potter *et al.* 1983a; Stein *et al.* 1988; Wyrzykiewicz, 1997; Romier *et al.* 1998; Murakami *et al.* 1994; Almer *et al.* 1996).

The focus of this study is on the enzymatic degradation of the nuclease resistant PS oligonucleotide to find a nuclease(s) that will hydrolyse PS oligonucleotides at a reasonable rate, achieving significant/total degradation within 60 minutes, and thereby investigating their application to degrade oligonucleotides in waste product streams ultimately. Treatment via enzymatic methods, using nucleases, offers an additional alternative to the degradation and subsequent removal of antisense oligonucleotides from waste streams. Finding a suitable enzyme for a specific application necessitates an understanding of specific process variables such as pH, temperature and time, the chemistry of components and presence of inhibitors. Various reoccurring nucleases have been identified in the literature that are capable of cleaving PS oligonucleotides. The primary enzymes are nuclease S1, nuclease P1, *Serratia marcescens* nuclease and Snake venom phosphodiesterase. Five specific nucleases (Benzonase®, DNase I, nuclease S1, nuclease P1 and Snake Venom Phosphodiesterase (SVPD)) were chosen for comparative degradation studies. A comprehensive list of the physical properties of the five selected nucleases employed in this study is outlined in table 5.3. To establish the most effective nuclease, that would be most suitable for treating antisense oligonucleotide waste streams, both unmodified and modified (PS) oligonucleotides of identical sequence and length were subjected to treatment with the selected nuclease. The susceptibility of the oligonucleotides to nucleolytic digestion was determined by polyacrylamide gel electrophoresis on slab gels. A time point of 60 minutes was chosen as a feasible time scale from an industrial point of view to evaluate the application and effectiveness of each nuclease at degrading PS oligonucleotides and to determine the optimal operation parameters of the most effective nuclease.
Table 5.3 Substrate specificity, mode of action and properties of the five selected nucleases used for the degradation of PS oligonucleotides.


<table>
<thead>
<tr>
<th>Name of enzyme</th>
<th>Relevant information on the nuclease</th>
</tr>
</thead>
</table>
| **Deoxyribonuclease I** (DNase I) | - DNase I is a glycoprotein with a molecular mass of 31x10^3 dalton.  
- Non-specifically cleaves DNA to release 5'-phosphorylated di-, tri-, and oligonucleotide products.  
- The endonuclease DNase I requires divalent metal ions for DNA hydrolysis with maximum activity in the presence of Ca^{2+} and either Mg^{2+} or Mn^{2+}.  
- The mode of action and specificity of the enzyme are dependent on the type of divalent cations used. |
| Bovine pancreas |  |
| **Snake venom phosphodiesterase** (SVPD) | - Snake venom phosphodiesterase (SVPD) was obtained from the exotic species of *Crotalus adamanteus* (a northern American rattlesnake).  
- Phosphodiesterases have been described in snake venoms since the 1930's and after 5'-nucleotidases, ADPases, ATPases, exonucleases, endonucleases, and acid and alkaline phosphatases have also been described.  
- SVPD catalyses the exonucleolytic cleavage in the 3'-5' internucleotide phosphate bonds from the 3' terminus, yielding nucleotide 5'-monophosphates. |
| *Crotalus adamanteus* |  |
| **Benzonase®** *Serratia marcescens* | - *Serratia marcescens* nuclease, commercially available as Benzonase®, is an extracellular sugar non-specific endonuclease genetically engineered from the pathogenic Gram-negative bacteria *Serratia marcescens*.  
- It is produced and purified from *E. coli* strain W3110, a mutant of strain K12, containing the proprietary pNUC1 production plasmid.  
- Benzonase® degrades all forms of DNA and RNA (single stranded, double stranded, linear and circular), producing 5'-monophosphate-terminated di-, tri-, tetra-, and pentanucleotides, together with a very small fraction (less than 2%) of mononucleotides.  
- The enzyme prefers GC-rich regions in DNA while avoiding d(A)/d(T) tracts.  
- It displays a broad pH range from 6 to 10 (optimal at 8-8.5), has a wide temperature optimum between 37°C and 44°C and like many nucleases requires Mg^{2+} as a cofactor, with an optimum concentration between 5 and 10 mM. |
| **Nuclease S1** *Aspergillus oryzae* | - Nuclease S1 is a heat stable, multifunctional, extracellular enzyme secreted from fungus *Aspergillus oryzae*.  
- It is a monomeric protein with a molecular weight of 30 x 10^3 dalton.  
- It is a single-strand specific endonuclease that degrades single-stranded DNA or RNA to yield 5'-phosphate mono-oligonucleotides.  
- Under optimal conditions, the rates of nuclease S1 catalysed hydrolysis of single- and double-stranded nucleic acids differ by a factor of 75000.  
- The nuclease displays a sharp acid pH optimum at pH 4.2 and is most active at pH 4.0-4.3, with half-maximal rates at pH 3.3 and 4.9.  
- The enzyme requires Zn^{2+} for optimal activity. |
| **Nuclease P1** *Penicillium citrinum* | - Nuclease P1 is an extracellular glycoprotein purified from the fungi *Penicillium citrinum*.  
- It is a zinc-dependent glyco-enzyme with a molecular mass of 36 kDa.  
- It cleaves both single-stranded DNA and RNA, in both endo- and exonucleolytic modes by leaving the diester bond between the phosphate and 3'-hydroxyl group of the sugar, to the monomer level, into 5' mononucleotides.  
- Nuclease P1 can act under a wide range of conditions, including a broad range of temperatures and pH making it a frequently used enzyme for DNA structure studies.  
- The enzyme has an optimum pH, depending on the substrate, ranging from pH 4.5/5.0 - 8.0.  
- The optimal temperature is approximately 70°C, but for a long incubation period, a temperature below 60° C is more appropriate. |
Chapter Five

5.2 Materials and Methods

5.2.1 Source of chemical reagents

As described in section 3.2.1

5.2.2 Electrophoretic analysis of nuclease stability of PO and PS oligonucleotides to five commercially available nucleases

Enzymes studies: DNase I, Snake Venom Phosphodiesterase (SVPD), Benzonase®, Nuclease S1 and Nuclease P1 were obtained from Sigma-Aldrich, Dublin, Ireland. DNase I (552 units/mg) was dissolved in a reaction buffer consisting of 10mM Tris-HCl (pH 7.5), 2.5 mM MgCl₂, 0.5 mM CaCl₂ to a final stock solution of 138000KU/ml. A stock solution of SVPD (Phosphodiesterase I from Crotalus adamanteus venom Type VI, ≥0.01 unit/mg solid) (5U/ml) was made up in a reaction buffer comprising of 50 mM Tris-HCl (pH 8.5), 10 mM MgCl₂. A stock solution of Benzonase® (250000U/ml) was made up in a reaction buffer which consisted of 50 mM Tris-HCl (pH 8.0), 5 mM MgCl₂. Nuclease S1 (344100U/ml) was diluted in reaction buffer which comprised of 30 mM Sodium acetate (pH 4.6), 50 mM NaCl, 1 mM ZnCl₂ and 5% (v/v) glycerol. Nuclease P1 was dissolved in resuspension buffer containing 50 mM sodium acetate, pH 6.0 to a final stock solution of 10000U/ml. P1 nuclease cleavage experiments were carried out in a buffer consisting of 100 mM Tris-HCl (pH 7.2), 1 mM ZnCl₂.

Equal volumes (10 µl) of nuclease dilutions were added to 20 µM PO or PS oligonucleotides, to a final reaction volume of 20 µl, and incubated in a water bath at 37°C. The experimental conditions employed for each nuclease are summarised in table 5.4. Three controls were employed in this study, a substrate control, prepared by mixing 10 µl oligonucleotide with 10 µl purified water, an additional substrate control containing equal volumes of oligonucleotide and the enzyme reaction buffer and an enzyme control prepared by mixing 10 µl enzyme dilution with 10 µl of the relevant enzyme reaction buffer. The mixtures were vortexed and incubated at 37°C. Aliquots of the nuclease stability reactions were removed after 60 minutes, quenched by addition to an equal volume of formamide gel loading buffer containing tracking dye and heat inactivated for 5 minutes at 95°C. All reactions were carried out in triplicate and the cleavage products were resolved on a 25% denaturing PAGE followed by staining with Stains-all as described in section (3.2.6) or stored at -20°C for further analysis.
Table 5.4 Experimental conditions employed in the analysis of the effect of nucleases on PO and PS oligonucleotides.

Enzymes were incubated with oligonucleotides at 37°C for 60 minutes in all cases. U/ml = Units per millilitre. KU/ml = kilo units per millilitre.

<table>
<thead>
<tr>
<th>Name of enzyme</th>
<th>Reaction buffer conditions</th>
<th>Final treatment enzyme concentration (U/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNase I</td>
<td>10mM Tris-HCl (pH 7.5), 2.5 mM MgCl₂, 0.5 mM CaCl₂</td>
<td>0.069, 0.138, 0.69, 1.38, 6.9, 13.8, 69, 138, 690, 1380, 6900, 13800, 34500 and 69000 KU/ml</td>
</tr>
<tr>
<td>SVPD</td>
<td>50 mM Tris-HCl (pH 8.5), 10 mM MgCl₂</td>
<td>2.5x 10⁻⁶, 0.5 x 10⁻³, 2.5x 10⁻³, 0.5 x 10⁻², 2.5x 10⁻², 0.5 x 10⁻¹, 2.5x 10⁻¹, 0.5, 1, 2.5</td>
</tr>
<tr>
<td>Benzonase®</td>
<td>50 mM Tris-HCl (pH 8.0), 5 mM MgCl₂</td>
<td>0.25, 1.25, 2.5, 12.5, 25, 125, 250, 1250, 2500, 12500, 250000 and 250000</td>
</tr>
<tr>
<td>Nuclease S1</td>
<td>30 mM Sodium acetate (pH 4.6), 50 mM NaCl, 1 mM ZnCl₂ and 5% (v/v) glycerol</td>
<td>0.3441, 1.7205, 3.441, 17.205, 34.41, 172.05, 344.1, 1720.5 and 3441</td>
</tr>
<tr>
<td>Nuclease P1</td>
<td>100 mM Tris-HCl (pH 7.2), 1 mM ZnCl₂</td>
<td>0.01, 0.05, 0.1, 0.5, 1, 5, 10 and 50</td>
</tr>
</tbody>
</table>

5.2.3 Electrophoretic and chromatographic analysis of PO and PS oligonucleotides stability to nuclease P1

The action of the most effective nuclease at degrading the PS oligonucleotide (nuclease P1) was assessed using a higher concentration of oligonucleotide. 10 µl PO or PS oligonucleotides (25 µM final reaction concentration) were incubated with equal volumes of nuclease P1 ranging in concentration from 0.01, 0.05, 0.1, 0.5, 1, 5 and 10 U/ml (final reaction concentration) to a final volume of 20 µl. For IP-RP LC analysis, equal volumes of 30 µl substrate were added to 30 µl enzyme dilution. The mixtures were briefly vortexed and incubated in a water bath for 60 minutes at 37°C. Sample aliquots were removed and the digestion products were analysed and quantified by IP-RP LC (section 3.2.4) or 25% denaturing PAGE (sections 5.2.2 and 3.2.6).

5.2.4 Statistical analysis

Data analysis, including calculation of mean and standard deviation was conducted using Excel 2010 (Microsoft, USA).
5.3 Results and discussion

5.3.1 Electrophoretic analysis of nuclease stability of PS oligonucleotides to DNase I (bovine pancreas)

Figure 5.3 presents a representative PAGE profile of the results obtained following the treatment of PO and PS oligonucleotides with a progressive increase in DNase I concentration ranging from 0.069 – 138 KU/ml for PO oligonucleotide (figure 5.3a) and 138 – 69000 KU/ml for PS oligonucleotides (figure 5.3b) as indicted in the figure legend. Three controls were performed in all subsequent analysis with the various nuclease. They allow us to 1), have a control intact oligonucleotide band to compare subsequent degradation, 2), determine the enzyme buffer is not responsible for the degradation of the oligonucleotide and 3), investigate if the enzyme preparation alone would stain, at such treatment concentrations, and thus interfere with subsequent analysis.

Figure 5.3 Electrophoretic analysis of the optimisation and comparison of the effect of DNase I required for the degradation of a). unmodified PO oligonucleotides and b). modified (PS) oligonucleotides.

The oligonucleotides were incubated with varying concentrations of DNase I for 60 minutes at 37ºC. 
**Gel a).** C1: Oligonucleotide control (10 µM) (in water), C2: Oligonucleotide control (10 µM) (made in DNase I buffer), C3: DNase I only (138 KU/ml). **Lane 1-8:** Oligonucleotide incubated with increasing concentrations of DNase I (0.069, 0.138, 0.69, 1.38, 6.9, 13.8, 69 and 138 KU/ml, final treatment concentration).

**Gel b).** C1: oligonucleotide control (10 µM) (in water), C2: Oligonucleotide control (10 µM) (made in DNase I buffer), C3: DNase I only (69000 KU/ml). **Lane 1-6:** Oligonucleotide incubated with increasing concentrations of DNase I (138, 690, 1380, 6900, 13800 and 69000 KU/ml, final treatment concentration). Oligonucleotides were visualised upon staining the gel with Stains-all.

Two distinct bands were produced by both the oligonucleotide made up in water (C1) and the oligonucleotide made up in enzyme buffer (C2). DNase I alone at lower concentrations did not produce any bands, however, at a higher concentration, as was required for the
treatment of PS oligonucleotides, DNase I was detected by Stains-all which can detect both nucleic acids and proteins. Therefore, the smearing effect observed in lane C3 and lanes 5 and 6 (figure 5.3 (b)) likely represent both protein and nucleic acid impurities present in the commercial preparation of (crude) DNase I.

The band corresponding to that of the untreated oligonucleotide (C1 and C2) progressively decreased in intensity with increasing enzyme concentration following incubation at 37°C with both oligonucleotide chemistries. From the results presented in figure 5.3 (a), following treatment of the PO oligonucleotide, no significant degradation was observed with a low concentration of 0.069 and 0.138 KU/ml (lane 1 and 2). However, slight degradation of the unmodified phosphodiester oligonucleotide was detected as smaller breakdown bands following treatment with 0.69 KU/ml (lane 3) and to a greater extent with 1.38 KU/ml (lane 4). Following PAGE analysis in triplicate it was established that the oligonucleotide was completely degraded following treatment with 6.9 KU/ml (lane 5) where the band corresponding to the intact oligonucleotide was absent with the appearance of smaller breakdown products that moved further down the gel. Digestion of PO oligonucleotides revealed a ladder of digestion products where degradation products in the central region of the ladder were not observed. This is likely due to the endonucleolytic nature of DNase I which display a distributive mode of action and cleave the internal phosphodiester bonds of an oligonucleotide (Brnakova and Godany, 2003; Lorenz, 2008).

In contrast to treatment of the PO oligonucleotide a significant increase in DNase I concentration was required to give rise to complete degradation of the PS oligonucleotides. With a progressive increase in enzyme concentration the band corresponding to that of the intact PS oligonucleotide decreased in intensity. A final treatment concentration of 69000 KU/ml (lane 6) was required to degrade the PS oligonucleotide to less than the limit of detection of the analytical procedure.

Relative nuclease resistance/stability was calculated by dividing the observed enzyme concentration required for degradation of the PS oligonucleotide by the enzyme concentration required for the degradation of the unmodified oligonucleotide after 60 minutes incubation. A substantial 1:10,000 fold increase of DNase I concentration was required to degrade the PS oligonucleotide in relation to the unmodified PO oligonucleotide of identical length and sequence. Unlike the gel profile observed following treatment of the PO oligonucleotide (figure 5.3 (a)) where the band corresponding to the PO oligonucleotide
disappeared with the appearance of smaller degradation products, no degradation products were observed following treatment of PS oligonucleotide. This may be due to the presence of very high enzyme concentrations which are strongly bound to PS oligonucleotides and form larger degradation products with lower electromobility. Malik and Roy, (2008) state that PS oligonucleotides ‘are sticky’ binding to enzymes, proteases and proteins.

5.3.2 Electrophoretic analysis of nuclease stability of PS oligonucleotides to Snake Venom Phosphodiesterase (SVPD) (*Crotalus adamanteus*)

The extent of degradation of both unmodified and modified PS oligonucleotides by the exonucleolytic action of SVPD was analysed and semi-quantified by gel electrophoretic analysis. Figure 5.4 (a and b) displays a representative PAGE profile of the results obtained upon treatment with increasing concentrations of SVPD on both oligonucleotide chemistries at 37°C for 60 minutes. No significant degradation was observed following treatment with final reaction concentrations of 2.5 x 10⁻⁶, 0.5 x 10⁻⁵, 2.5 x 10⁻⁵, 0.5 x 10⁻⁴ U/ml of SVPD (lanes 1-4). At a concentration of 2.5 x 10⁻⁴ U/ml SVPD (lane 5) initial degradation was observed by the appearance of three smaller breakdown products and to a greater extent following treatment with 0.5 x 10⁻³ U/ml (lane 6) as detected by the complete disappearance of the intact PO oligonucleotide band and the appearance of smaller cleavage products. The degradation of PO oligonucleotides by SVPD follows the course expected for exonucleolytic action resulting in a ladder like formation of breakdown products which ran lower down the gel and differs somewhat from that of DNase I, a typical endonuclease (Williams *et al.* 1961). This is indicative of the mode of action of an exonuclease that cleaves the phosphodiester backbone sequentially starting from one or both ends of the chain (Lorenz, 2008).
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Figure 5.4 Electrophoretic analysis of the optimisation and comparison of the effect of SVPD required for the degradation of a). unmodified PO oligonucleotides and b). modified (PS) oligonucleotides.

The oligonucleotides were incubated with varying concentrations of SVPD for 60 minutes at 37°C.

**Gel a).**
- **C1**: Oligonucleotide control (10 µM) (in water),
- **C2**: Oligonucleotide control (10 µM) (made in SVPD buffer),
- **C3**: SVPD only (138 KU/ml).
- Lane 1-8: Oligonucleotide incubated with increasing concentrations of SVPD (2.5 x 10^{-6}, 0.5 x 10^{-5}, 2.5 x 10^{-4}, 0.5 x 10^{-3}, 2.5 x 10^{-3}, 0.5 x 10^{-2}, and 0.5 x 10^{-2} U/ml, final treatment concentration).

**Gel b).**
- **C1**: oligonucleotide control (10 µM) (in water),
- **C2**: Oligonucleotide control (10 µM) (made in SVPD buffer),
- **C3**: SVPD only (69000 KU/ml).
- Lane 1-8: Oligonucleotide incubated with increasing concentrations of SVPD (0.5 X 10^{-2}, 2.5 X 10^{-2}, 0.5 X 10^{-1}, 2.5 X 10^{-1}, 0.5 and 1.25 U/ml, final treatment concentration).

Oligonucleotides were visualised upon staining the gel with Stains-all. 

_____ = Low molecular weight product.

In order to obtain complete degradation of the modified PS oligonucleotide significantly greater quantities of SVPD was required as illustrated in figure 5.4 (b). Complete degradation of the modified oligonucleotide, to the limit of detection, was observed following treatment with 0.5 U/ml SVPD (lane 7). A 1000 fold greater concentration of SVPD was required to degrade the PS oligonucleotide in contrast to its unmodified PO counterpart. In contrast to the degradation pattern evidenced by the PO oligonucleotide, there is only faint evidence of the appearance of degradation products. It appears that upon treatment with a higher concentration of SVPD that the degradation products appear further up the gel as larger compounds. This may be due to increase in protein/PS oligonucleotide interaction and binding, a characteristic property of PS oligonucleotides.
5.3.3 Electrophoretic analysis of nuclease stability of PS oligonucleotides to Benzonase® (*Serratia marcescens*)

The nuclease stability of the 21-mer PO and PS oligonucleotides was investigated using Benzonase® (final reaction concentrations of 0.25, 1.25, 2.5, 12.5, 25, 125, 250 and 1250 U/ml) by gel electrophoresis. Figure 5.5 (a and b) display the profiles of nuclease digestion of the DNA as detected by a decrease in intensity of the intact oligonucleotide band caused by the action of the endonuclease. At enzyme concentrations of 125 and 250 U/ml (lane 6 and 7) degradation of the PO oligonucleotide was observed by the slight decrease in magnitude of the main band and the increase in appearance of smaller degradation bands that ran further down the gel. Complete degradation of the PO oligonucleotide, to the limit of detection, was observed at an enzyme concentration of 1250 U/ml (lane 8).

![Figure 5.5 Electrophoretic analysis of the optimisation and comparison of the effect of Benzonase® required for the degradation of a). unmodified PO oligonucleotides and b). modified PS oligonucleotides.](image)

The oligonucleotides were incubated with varying concentrations of Benzonase® for 60 minutes at 37°C. **Gel a.** C1: Oligonucleotide control (10 µM) (in water), C2: Oligonucleotide control (10 µM) (made in Benzonase® buffer), C3: Benzonase® only (1250 U/ml). **Lane 1-8:** Oligonucleotide incubated with increasing concentrations of Benzonase® (0.25, 1.25, 2.5, 12.5, 25, 125, 250 and 1250 U/ml, final treatment concentration).

**Gel b.** C1: oligonucleotide control (10 µM) (in water), C2: Oligonucleotide control (10 µM) (made in Benzonase® buffer), C3: Benzonase® only (125000 U/ml). **Lane 1-9:** Oligonucleotide incubated with increasing concentrations of Benzonase® (12.5, 25, 125, 250, 1250, 2500, 12500, 25000 and 125000 U/ml, final treatment concentration). Oligonucleotides were visualised upon staining the gel with Stains-all. " = Low molecular weight product.

Significantly higher Benzonase® concentrations were required (>200 fold) to give rise to almost complete degradation of the PS oligonucleotide. With increasing Benzonase® concentration subsequent degradation was observed by the decrease in intensity of the main intact PS oligonucleotide band concurrent with the appearance of smaller breakdown products (figure 5.5 (b)). Maximum degradation was observed following incubation with
125000 U/ml (lane 9) which gave rise to almost complete degradation of the modified oligonucleotide. However, analysis at a higher enzyme concentration could not be carried as this was the most concentrate of the commercially available nuclease preparation. Following incubation at this enzyme concentration, with a 2 fold decrease in oligonucleotide concentration, (data not shown) a faint band was still present indicating that a 2-fold higher concentration of Benzonase® (250000 U/ml) was still unable to fully degrade the modified PS oligonucleotide.

5.3.4 Electrophoretic analysis of nuclease stability of PS oligonucleotides to Nuclease S1 (*Aspergillus oryzae*)

The PO oligonucleotide was subjected to treatment with nuclease S1 at concentrations ranging from of 0.3441 to 1720.5 U/ml at 37°C over a period of 60 minutes. Results obtained following electrophoretic analysis are illustrated in figure 5.6 (a). It should be noted that when incubated, with nuclease buffer alone at pH 4.6 (C2), no degradation of the oligonucleotide, due to depurination, was observed in the acidic medium of the enzyme reaction buffer. The pH for the standard assay, pH 4.6, was selected higher than the optimum to reduce the likelihood of non-enzymatic nucleic acid breakage and depurination at low pH values (Gerhartz, 1990; Vogt, 1973). Therefore, degradation observed with increased levels of nuclease S1 was directly due to the action of the enzyme. Initial degradation was observed by the faint appearance of smaller decomposition products upon treatment of the unmodified oligonucleotide, for 60 minutes, with 1.7205 U/ml (lane 2). The rate of degradation accelerated following incubation with increased concentrations of 3.441, 17.205 and 34.41 U/ml nuclease S1 (lanes 3, 4 and 5) as highlighted by the sequential decrease in band intensity of the intact PO oligonucleotide and the increased presence of degradation product bands. Complete degradation, to the limit of detection, was observed following treatment at an enzyme concentration of 172.05 U/ml (lane 6).
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Figure 5.6 Electrophoretic analysis of the optimisation and comparison of the effect of nuclease S1 required for the degradation of a) unmodified PO oligonucleotides and b) modified (PS) oligonucleotides.

The oligonucleotides were incubated with varying concentrations of nuclease S1 for 60 minutes at 37°C.

**Gel a.** C1: Oligonucleotide control (10 µM) (in water), C2: Oligonucleotide control (10 µM) (made in nuclease S1 buffer), C3: nuclease S1 only (1720.5 U/ml). **Lane 1-8:** Oligonucleotide incubated with increasing concentrations of nuclease S1 (0.3441, 1.7205, 3.441, 17.205, 34.41, 172.05, 344.1 and 1720.5 U/ml, final treatment concentration).

**Gel b.** C1: Oligonucleotide control (10 µM) (in water), C2: Oligonucleotide control (10 µM) (made in nuclease S1 buffer), C3: nuclease S1 only (3441 U/ml). **Lane 1-9:** Oligonucleotide incubated with increasing concentrations of nuclease S1 (0.3441, 1.7205, 3.441, 17.205, 34.41, 172.05, 344.1, 1720.5 and 3441 U/ml, final treatment concentration). Oligonucleotides were visualised upon staining the gel with Stains-all.

= Low molecular weight product.

The modified PS oligonucleotide was subjected to similar treatment with nuclease S1 as presented in figure 5.6 (b). PS oligonucleotides exhibit moderately higher stability against nuclease S1 than PO oligonucleotides. Treatment with increasing concentration of nuclease S1 had the effect of increasing the rate of degradation of the modified oligonucleotide over a period of 60 minutes. Digestion of PS oligonucleotide generated a ladder of oligonucleotides of shorter lengths. Complete degradation was observed following incubation with 1720.5 U/ml nuclease S1 (lane 8). In contrast to the three nucleases previous discussed, the concentration of enzyme required for complete degradation of the PS oligonucleotide in relation to its unmodified counterpart was as little as 10 fold.

5.3.5 Electrophoretic analysis of nuclease stability of PS oligonucleotides to Nuclease P1 (*Penicillium citrinum*)

Both oligonucleotide chemistries, PO and PS, were subjected to treatment with Nuclease P1 at concentrations ranging from 0.01 to 50 U/ml at 37°C over a period of 60 minutes. Figure 5.7 (a and b) illustrates the results obtained via PAGE analysis following incubation of the unmodified and modified oligonucleotide with increasing concentrations of Nuclease P1. Treatment with increasing concentration of Nuclease P1 has the effect of decreasing the
stability of both oligonucleotide chemistries as observed by the decrease in intensity of the main intact control band and the occurrence of a chain of smaller breakdown bands than ran further down the gel. Nuclease P1, possessing both endo and exonucleolytic activity (Koziolkiewicz et al. 1995), completely degraded the PO oligonucleotide at an enzyme concentration of 1 U/ml (lane 5).

A similar trend in degradation was observed upon treatment of the modified PS oligonucleotide with Nuclease P1 (figure 5.7 (b)). It is evident that the reaction rate increased due to the higher nuclease concentration. Visible degradation products, produced by the action of the endo/exonuclease, which migrate further down the gel, were evident upon treatment of the PS oligonucleotide with nuclease P1 concentrations of greater than 0.05 U/ml. In contrast to the unmodified PO oligonucleotides a 5 fold increase in nuclease P1 (5 U/ml) (lane 6) was required to give rise to complete degradation of the PS oligonucleotide.
5.3.6 Enzyme-based oligonucleotide degradation as assessed electrophoretically - general discussion

Resistance of an antisense oligonucleotide towards destructive nuclease activity is crucial for any therapeutic activity to occur (DeDionisio and Lloyd, 1996). Numerous studies have been carried out to investigate the stability of various oligonucleotide analogues towards human nucleases. Methods used previously have been limited as such studies were carried out to demonstrate the superior nuclease stability of PS oligonucleotides as therapeutics agents and thus such analyses investigated the ratio of half-life degradation of PS oligonucleotides in contrast to the unmodified substrates as a function of time at a given concentration. Unlike those studies, the aim of this research was to establish the optimum enzyme concentration to fully degrade PS oligonucleotides in waste product streams in a time frame of 60 minutes. Five specific nucleases (DNase I, SVPD, Benzonase®, nuclease S1 and Nuclease P1) were chosen, for comparative digestion studies. The most effective nuclease for treating antisense oligonucleotide waste was chosen based on the ratio of quantity of nuclease required to degrade the PS oligonucleotide in relation to the more unstable unmodified PO oligonucleotide, of identical length and sequence over a period of 60 minutes. Relative nuclease resistance/stability was calculated by dividing the observed enzyme concentration required for complete degradation of the PS oligonucleotide by the enzyme concentration required for complete degradation of the unmodified PO oligonucleotide after 60 minutes incubation. Results obtained are summarised in table 5.5. Also outlined in the table is the diaselectivity of the selected enzymes. It is essential to note that the definition of enzyme activity, for each selected enzyme, is different. Therefore, the different enzymes cannot be compared to each other on the basis of activity units quoted. Additionally, each enzyme reaction occurred in different reaction conditions (pH/buffer composition) and this is why the extent of hydrolysis of PS versus PO oligonucleotides was applied as the measure of individual enzyme efficiency.
Table 5.5 Electrophoretic determination and comparison of the effectiveness of five specific nucleases at degrading unmodified PO oligonucleotide versus PS oligonucleotides of identical sequence.

Ratio* = Concentration of nuclease required for complete degradation of PS oligonucleotide versus concentration of nuclease required for complete degradation of PO oligonucleotide.

U/ml = Units per millilitre.
KU/ml = Kilo units per millilitre.

<table>
<thead>
<tr>
<th>Name of nuclease</th>
<th>Stereo-specificity (from literature)</th>
<th>Final reaction concentration of enzyme (U/ml) required to degrade PO/PS oligonucleotide after 60 mins (PAGE)</th>
<th>Ratio*</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNase I (Bovine pancreas)</td>
<td>Not identified</td>
<td>Unmodified PO oligonucleotide: 6.9 KU/ml; PS oligonucleotide: 69000 KU/ml</td>
<td>1:10,000</td>
</tr>
<tr>
<td>SVPD (Snake Venom Phosphodiesterase) (Crotalus adamanteus)</td>
<td>Rp</td>
<td>Unmodified PO oligonucleotide: 0.5x10^-5 U/ml; PS oligonucleotide: 0.5 U/ml</td>
<td>1:1,000</td>
</tr>
<tr>
<td>Benzonase® (Serratia marcescens)</td>
<td>Rp</td>
<td>Unmodified PO oligonucleotide: 1250 U/ml; PS oligonucleotide: &gt;250000 U/ml</td>
<td>&gt;1:200</td>
</tr>
<tr>
<td>Nuclease S1 (Aspergillus oryzae)</td>
<td>Sp</td>
<td>Unmodified PO oligonucleotide: 172.05 U/ml; PS oligonucleotide: 1720.5 U/ml</td>
<td>1:10</td>
</tr>
<tr>
<td>Nuclease P1 (Penicillium citrinum)</td>
<td>Sp</td>
<td>Unmodified PO oligonucleotide: 1 U/ml; PS oligonucleotide: 5 U/ml</td>
<td>1:5</td>
</tr>
</tbody>
</table>

PS oligonucleotides are more resistant to hydrolysis by nucleases than unmodified oligonucleotides (Ito et al. 2003; Stein et al. 1988; Kozikiewicz et al. 1997; Wei et al. 2008; Cook, 2001; Aboul-Fadl, 2005; Vosberg and Eckstein, 1982; Wójceik et al. 2007). The PS modification imparts significant stabilisation against degradation by nucleases (Engels and Parsch, 2004). De Mesmaeker et al. (1995) concur by stating that the replacement of the phosphodiester bond by these modified sulfur-containing backbones, demonstrates a significantly improved resistance towards nucleases. Eckstein, (2002), has reported that polynucleotides that contain PS internucleotide linkages demonstrated degradation by nucleases at a much slower rate than the natural congeners. The sulfurization of the internucleotide bond significantly decreases enzymatic degradation of endo-and exonucleases including 5’ to 3’ and 3’ to 5’ DNA POL 1 exonuclease, nucleases P1 and S1, RNases, serum nucleases and snake venom phosphodiesterase (Sigma-Aldrich Co., 2009b).

Analysis with all five nucleases demonstrated that PS oligonucleotides are more nuclease resistant than the PO oligonucleotides with varying degrees. This is in agreement with Stein and coworkers (1988) who stated that the stability of PS oligonucleotides against purified nucleases varies significantly depending on oligonucleotide sequence and the type of nuclease tested (Monia et al. 1996). In several cases, such modified compounds are essentially resistant to the action of a given nuclease, whereas in other cases only a small effect may be noted (Spritzer and Eckstein, 1988) as was observed in this study. Of the five
commercially available nucleases used, all exhibited distinct disparities in their efficacy and rate of degradation with PS oligonucleotides ranging from 1:10,000 to 1:5 fold increase in enzyme concentration. Based on the results obtained, under the experimental conditions used, nuclease P1 presents as the most effective nuclease at degrading PS oligonucleotides in contrast to the unmodified oligonucleotides. Qualitative results suggest that the next most effective was nuclease S1, Benzonase®, SVPD and DNase I respectively. The concentration of enzyme required to degrade PS oligonucleotide relative to their unmodified counterparts was 5 times (nuclease P1), 10 times (nuclease S1), >200 times (Benzonase®), 1,000 times (SVPD), 10,000 times (DNase I) greater for each of the respective nucleases.

Consistent with the scientific literature, degradation of the PS oligonucleotide with DNase I, SVPD and Benzonase® occurred to a lesser extent. A 10,000 fold increase in DNase I concentration was required to effectively give rise to complete degradation of the PS oligonucleotide. This corroborates previous independent reports indicating that PS oligonucleotides are stable towards the degradative effects of DNase I. This parallels results obtained by Spitzer and Eckstein, (1988) who established that a tetramer containing a single phosphorothioate group, in the centre of the molecule, of either configuration, were resistant to the action of DNase I in addition to the action of staphylococcal nuclease and DNase II. Similar results were obtained by Cummins et al. (1996), where under conditions that resulted in 80% degradation of the full length unmodified oligonucleotides, the modified PS oligonucleotide was undegraded. Under experimental conditions they found that uniformly modified PS oligonucleotides were completely stable to degradation by DNase I (Cummins et al. 1996).

Treatment with SVPD which results in hydrolysis of 3’-terminal internucleotide phosphorothioates of the Rp configuration (Wilk and Stec, 1995; Yu et al. 2000; Burgers et al. 1979) was more effective than DNase I at degrading PS oligonucleotides. This may be due to its exonucleolytic mode of action in contrast to DNase I which is an endonuclease. Tang et al. (1993) established that PS oligonucleotides were more stable than PO oligonucleotides to the exonucleolytic effect of SVPD but they were however degraded after prolonged periods of incubation. The differences observed from unmodified oligonucleotides were quite dramatic requiring a 1000 fold increase in SVPD concentration to generate complete degradation of the modified oligonucleotide. Stein et al. (1988) established that the ratio of $t_{1/2}$ PS oligonucleotide / $t_{1/2}$ PO oligonucleotide was as much as 4625 for the 28-mer oligonucleotides and 443 for the shorter 15-mer oligonucleotides. Potter
et al. (1983a) stated that SVPD hydrolyses unmodified DNA 100-fold faster than phosphorothioates. Preforming the SVPD assay at an enzyme concentration of $5 \times 10^{-3}$ U/ml no degradation of the PS oligonucleotide was detected whilst treatment with a 10 fold higher concentration of $5 \times 10^{-2}$ U/ml SVPD reaction conditions Cummins et al. (1995) demonstrated that 2’deoxy phosphorothioate analogs were 350 fold more stable than the 2’deoxy phosphodiester control. Comparably, under similar experimental conditions, no degradation of the PS oligonucleotide was observed after 60 minutes incubation at both enzyme concentrations. Complete degradation of the PS oligonucleotide was detected following incubation with 0.5 U/ml SVPD (final reaction concentration). Moreover, Griffey et al. (1996); Metelev and Agrawal, (2006); McKay et al. (1996); Bruin et al. (1995), Yamakawa, (1998), found that PS oligonucleotides were significantly more resistant than PO oligonucleotides to SVPD.

A >200 fold increase in Benzonase® concentration was required to give rise to almost complete degradation of PS oligonucleotide in relation to the unmodified form. Under experimental conditions, complete degradation of the PS oligonucleotide was not observed. Similar results were obtained by Freidhoff et al. (1996) who established that the phosphorothioate substituted pentadeoxynucleotides were not or were only barely cleaved at the site of modification. Furthermore, an additional reason why complete degradation to monomeric level was not observed may be due to the fact that for relatively good cleavage activity the substrate should contain at least five phosphate residues. The rate of DNA cleavage by the Serratia nuclease significantly and progressively decreases with oligodeoxynucleotides with a chain length <5 (Friedhoff et al. 1996).

The most effective nucleases at degrading the modified PS oligonucleotide were nuclease S1 and nuclease P1 whereby a 10 fold and 5 fold increase in enzyme concentration, than that required to degrade the unmodified PO oligonucleotide, gave rise to complete degradation of the PS oligonucleotides to the limit of detection. Stein et al. (1988), who studied the DNase sensitivity of several oligodeoxynucleotides to nuclease P1, SVPD, nuclease S1, also established that nuclease S1 and nuclease P1 were more effective at degrading PS oligonucleotides than SVPD, whereby nuclease S1 and P1 digestion proceeded at a 2-45 time slower for the PS oligonucleotide than for the unmodified oligonucleotides. For the 15-mer oligonucleotide Stein et al. (1988) established that the ratio of $t_{1/2}$ PS oligonucleotide / $t_{1/2}$ PO oligonucleotide was 9 for nuclease S1 and 4 for nuclease P1. This parallels the nuclease sensitivity observed in this study, whereby a 10 and 5 fold increase in enzyme concentration
of nuclease S1 and P1 respectively was required to produce complete degradation of the PS oligonucleotides. Cummins et al. (1995) also established that PS oligonucleotides were more resistant to SVPD in contrast to nuclease S1.

The disparity in nuclease sensitivities of PS oligonucleotides to DNase I, SVPD, Benzonase®, Nuclease S1 and nuclease P1 may be due to their diastereomer preference. The in vitro nuclease stability of PS oligonucleotides is in the order [Sp] > [Sp-Rp-Sp] > stereo-random > [Rp]. The [Sp] PS-oligonucleotide is more stable than [Rp] PS oligonucleotide. The stereo-random PS-oligonucleotide has a stability intermediate to the stability of [Sp] and [Rp] PS oligonucleotides (Yu et al. 2000). Therefore, the Sp PS oligonucleotides display greater stability against nucleases, than the stereo-random an Rp PS oligonucleotides (Stein, 1996; Eckstein, 2000). As stated previously, SVPD and Serratia marcescens nuclease preferentially recognise and hydrolyse phosphorothioates of Rp configurations, whilst nuclease P1 and nuclease S1 preferentially hydrolyse internucleotide Sp. This therefore, may explain why both nuclease S1 and nuclease P1, which are SP stereospecific nucleases, were more effective at degrading PS oligonucleotides in relation to their unmodified counterparts than DNase I, SVPD and Benzonase®.

Additionally, Desai and Shankar, (2003), have reported that the majority of extracellular single-strand specific nucleases display an associated phosphomonoesterase activity. It is reported that nuclease S1, nuclease P1, mung bean, pea seed and potato tuber nucleases have an associated 3'- phosphomonoesterase activity (Desai and Shankar, 2003; Romier et al. 1998; Maekawa et al. 1991). This additional beneficial activity may give rise to greater degradation and may also explain the decrease in concentration of enzyme required to give rise to complete degradation of PS oligonucleotides.

Although many similarities exist between nucleases S1 and P1 (both enzymes are heat-stable glycoproteins of similar molecular masses, have closely related three-dimensional structures, with 49.3% sequence identity, are single-stranded polynucleotide-specific enzymes and require Zn$^{2+}$ for stabilization and activation), there are quantitative differences between their catalytic properties (Potter et al. 1983b; Maekawa et al. 1991; Romier et al. 1998). A distinct advantage of nuclease P1 over nuclease S1 is that firstly it is slightly more effective at degrading PS oligonucleotides relative to unmodified PO oligonucleotide and secondly, nuclease P1 displays a broad pH range (5.0-8.0) (Furuichi and Miura, 1975; Fujimoto et al. 1974b) in contrast to nuclease S1 which is active only at low pH (4.0-4.3) (Gerhartz, 1990;
Vogt, 1973; Esteban et al. 1992). In this study, nuclease P1 was diluted in a reaction buffer with a pH of 7.2. Additionally, nuclease P1 has the advantage of being commercially available in crude form, easy to prepare, very stable, and completely specific for single strands. Moreover, its application in the food industry (Guo-Qing, 2006; Shi et al. 2010; Suh et al. 1987a; Suh et al. 1987b) implies that it can be prepared inexpensively and in large quantities.

The stability of the phosphorothioate esters is interesting as it is unanticipated to its extent from a purely chemical point of view. The structure of PS oligonucleotides is not significantly different from that of the parent species (Eckstein, 2000). In addition to the diastereomeric selective manner in which nucleases interact with PS oligonucleotides a further argument, for this unexpected stability, is that the sulphur cannot be accommodated in the transition state due to its moderately larger van de Waals radius than that of oxygen (Eckstein, 2002). Despite the fact that the replacement of oxygen with sulphur is considered a conservative modification, this example shows that the small difference in Van der Waal’s radius of 1.44 versus 1.85 Å and the slight lengthening of the P-S bond can be sufficient to disturb the fine balance of the arrangement of functional groups in the active site of an enzyme (Eckstein, 2000). It is believed that the replacement with the larger sulphur atom prevents the backbone from sitting in the active site of the enzyme and as a result provides enzymatic stability. Which of the diastereomers will be resistant to enzymatic hydrolysis will be dependent on the architecture of a given enzyme (Eckstein, 2000; Romier et al. 1998; Burgers and Eckstein, 1979).
5.3.7 Quantitative analysis of the degradation of PO and PS oligonucleotides by nuclease P1.

Following initial analysis via semi-quantitative PAGE, to establish the most effective commercially available nuclease at degrading PS oligonucleotides in relation to their unmodified counterparts, nuclease P1 presented as the most effective with only a 5 fold increase in enzyme concentration required to degrade the PS oligonucleotide in contrast to its more unstable unmodified counterpart. Consequently, further quantitative analysis was carried out using the chosen nuclease to investigate its efficacy at degrading oligonucleotides at the higher substrate concentration of 50 µM. Analysis was carried out by IP-RP LC and PAGE as described in section 5.2.3.

To verify quantitatively the results from PAGE analysis and to monitor the degradation of the oligonucleotides in a quantitative manner the digestion of PS oligonucleotides with nuclease P1 were followed using IP-RP LC. Figure 5.8 presents a graph illustrating the concentration dependant hydrolysis of both oligonucleotide chemistries following incubation with varying concentrations of nuclease P1 for 60 minutes at 37°C. Upon treatment of the unmodified PO oligonucleotides a significant increase in degradation from 23.3% to 72.0% was observed over a narrow enzyme concentration range of between 0.01 to 0.1 U/ml. Following treatment with an increased concentration of 0.5 U/ml the extent of degradation increased to 98.2% where it reached a plateau generating 99.2 and 100.0% degradation with subsequent enzyme concentrations of 1 and 5 U/ml respectively. Contrary to results discussed in section 5.3.6, a 5 fold decrease in enzyme concentration was required for the complete degradation of PO oligonucleotide as evidenced by HPLC analysis.
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Figure 5.8 Graph illustrating the optimisation and comparison of nuclease P1 concentrations required for the degradation of unmodified PO oligonucleotides and modified PS oligonucleotides for 60 minutes at 37°C as assessed by IP-RP LC.

The PO/PS oligonucleotide was incubated with Nuclease P1 dilutions ranging in concentration from 0.01, 0.05, 0.1, 0.5, 1, 5 and 10 U/ml. Percentage degradation of oligonucleotides = % intact oligonucleotide after treatment with the indicated enzyme concentration relative to the untreated oligonucleotide. Each value represents mean ± SD (n=3).

In contrast to hydrolysis of PO oligonucleotides the extent of degradation of the modified PS oligonucleotide was slow at concentrations between 0.01 and 0.1 U/ml, degrading the PS oligonucleotide by less than 5% under such conditions. Upon treatment with 0.5 U/ml, which gave rise to complete degradation of the PO oligonucleotide, modest degradation of 13.6% of the PS oligonucleotide was observed which increased to 63.3% following incubation with a 2 fold increase in enzyme concentration. Maximum degradation of 99.0% was detected via HPLC following treatment with 5 U/ml comparable with results obtained by PAGE (figure 5.7 (b) and 5.11 (b)). Under the experimental conditions analysed, an increase in the concentration of oligonucleotide without changing the composition of the medium, did not significantly decrease the extent of hydrolysis by nuclease P1.

The oligonucleotides and their degradation fragments were quantified directly by HPLC. A HPLC elution profile of the PO oligonucleotide, prior to and after complete hydrolysis by nuclease P1, is shown in figure 5.9. Incubation of the unmodified PO oligonucleotide with an optimum nuclease P1 concentration of 0.5 U/ml had the effect of decreasing the main sample peak by 98.2% with the appearance of smaller degradation products that eluted, due to their decreased size, with an earlier retention. Using the HPLC method, the digestion products of the unmodified PO oligonucleotide, derived from nuclease P1, were well separated. These products are almost certainly shorter oligonucleotides of varying length and...
nucleotide monomers. The overall profile of products is consistent with the profile predicted at near complete hydrolysis of an oligonucleotide.

![Diagram](image)

**Figure 5.9 Chromatographic results of the effect of nuclease P1 on PO oligonucleotides.**

The reaction was performed in the absence (untreated PS oligonucleotide) a), and presence of b). 0.5 U/ml nuclease P1 after a period of 60 minutes at 37°C.

![Decrease in starting intact PO oligonucleotide material](image)

Similarly, the effect of nuclease P1 on PS oligonucleotides was determined by HPLC and representative chromatograms prior to and after treatment are presented in figure 5.10. Treatment of the PS oligonucleotides with an optimum treatment nuclease P1 concentration of 5 U/ml resulted in 99.0% degradation of the intact PS oligonucleotide. The chromatographic profile is one of a ladder of oligonucleotide that has been sequentially degraded into shorter oligonucleotides of varying lengths. The resolution of degradation products are not as pronounced as those following treatment of the PO oligonucleotide. The disparity in the HPLC profile may be the result of the stereoselective nature of nuclease P1 digestion mechanism. Similar results were obtained via PAGE analysis as presented in figure 5.11 (a and b) which display results comparable to those obtained with a lower oligonucleotide concentration as discussed in section 5.3.5.
Figure 5.10 Chromatographic results of the effect of nuclease P1 on PS oligonucleotides.

The reaction was performed in the absence (untreated PS oligonucleotide) a). and presence of b). 5 U/ml nuclease P1 after a period of 60 minutes at 37°C.

$\downarrow$ = Decrease in starting intact PS oligonucleotide material.
5.3.8 The potential benefits of enzymatic treatment over physicochemical treatments at industrial scale

After analysis of the effectiveness of five commercially available nucleases at degrading PS oligonucleotides in relation to unmodified PO oligonucleotides nuclease P1 presented as the most effective nuclease. Effective degradation (99.0%) was obtained following treatment with 5 U/ml nuclease P1. This is significantly better or comparable with previously reported treatment methods via physical or chemical means (Chapter Three and Four) thus, making it possible to replace the conventional and environmentally harmful chemical processes.

Enzymes have become very important in industry due to their many favourable properties. Treatment of oligonucleotide waste streams via enzymatic methods may present as a more favourable option as such methods display high substrate specificity, low toxicity, biodegradability, low energy consumption, rapid and efficient action at low concentrations under mild pH values and temperatures thus resulting in low manufacturing cost, less waste and less energy consumption (Sanchez and Demain, 2011; Jayaraman, 2010; Jaeger, 2004). The use of naturally occurring nucleases poses a favourable advantage over the use of additional chemical solutions to the production process as they are environmentally friendly, and do not give rise to the release of toxic breakdown products into the environment. Enzymes can operate over a wide pH and temperature range, for example, nuclease P1 operates over a pH range of 5.0 to 8.0 and temperatures in the range of 37°C. This is in
sharp contrast to thermal waste treatment which is conducted at very high temperatures and therefore has high energy consumption. As enzymes generally operate under mild pH conditions, waste neutralisation prior to further waste treatment would not be required and corrosion within reaction vessels at large scale is reduced. Additionally, more traditional chemical treatments often produce undesirable side-effects and waste disposal issues (Jayaraman, 2010).

Nuclease P1 is presently one of the most widely used single-strand DNA specific nucleases in molecular biology (Naik and Raghavan, 2008). Nuclease P1 has displayed its effective application at large scale in the production of 5’-nucleotides in the food and pharmaceutical industry (Guo-Qing, 2006; Shi et al. 2010; Suh et al. 1987a). Nuclease p1 is a preferred choice taking into account economic constraints and ease to large-scale production (Guo-Qing, 2006).

Nuclease P1 is obtained from the fungal source of *Penicillium citrinum* (Kuninaka et al. 1961) and as such concerns in relation to the possibility of contamination with transmissible spongiform encephalopathy (TSE)-inducing agents which are associated with the use of enzymes derived from bovine sources (as was the case with DNase I) are eliminated (Capaldi and Scozzari, 2007). Additionally, microbial enzymes, which replaced many animal and plant enzymes, can be produced economically on a large scale due to inexpensive media and short fermentation (Sanchez and Demain, 2011).

Successful application of enzymatic processes in the chemical industry is however, predominantly governed by cost competitiveness. Biotechnology applications of enzymes are restricted by the cost of their production and stabilisation. As they are soluble, their recovery from the mixture of substrates and products for their reuse is not economically practical and this makes the already expensive enzymatic process even more costly (Reddy and Shankar, 1993). The majority of the analytically important nucleases, especially restriction endonucleases, are very expensive (Reddy and Shankar, 1993) as is the case with nuclease P1 (section 6.3.8). However, with the advent of immobilised enzyme technology, attempts are being undertaken to replace conventional enzymatic reactions with immobilised enzymes, as immobilisation presents many advantageous properties (Reddy and Shankar, 1993). Enzyme immobilization offers an advantageous and useful tool for industrial enzyme applications for the following reasons, (a) it allows reuse of enzymes, (b) it is ideal for continuous operations, (c) it generally improves enzyme stability, (d) the product is enzyme-
free and (e) it reduces effluent disposal problems (Sanchez and Demain, 2011; Shi et al. 2010). Due to the extensive application of nucleases, several attempts have been made to achieve highly active and stable immobilised preparations suitable for various biotechnology and food applications (Reddy and Shankar, 1993; Rokugawa et al. 1979; Rokugawa et al. 1980; Suh et al. 1987a; Shi et al. 2010). Characterisations of the immobilized enzyme demonstrated that the optimum temperature, optimum pH, and storage stability are considerably higher than those of free enzyme which made the immobilized enzyme better adapted to the change of temperature and pH and more conducive to industrial production (Shi et al. 2010). Therefore, immobilisation of nuclease P1 could be advantageous for treating PS oligonucleotide containing waste streams to reduce the overall cost of the treatment process. The application of gene technology now also presents with a more cost effective method of producing commercial enzymes.

There are, however, a number of practical problems associated with the use of enzymes at industrial scale. Enzymes are very sensitive to reaction conditions, for example, a number of components can affect the activity of nuclease P1 as does high pH (> pH 10.0). Enzymatic treatment is substrate specific and will not result in removal of a broad range of components from a waste stream. However, as a pre-treatment step, due to its specificity, it can achieve degradation of individual components thereby improving the overall waste treatment process. In addition to this, enzymatic activities often require lengthy optimization to obtain the ideal incubation time and optimum pH and temperature for optimum activity. Conversely, physicochemical treatment of PS oligonucleotides offers a distinct advantage as it is very quick, effective, efficient, and highly sensitive, less than 60 minutes application.
5.4 Concluding remarks

Enzyme production is one of the fastest growing sectors in industrial biotechnology, and imminent enzyme-based technologies, could hold the solution to new commercial opportunities and break-through technologies (Jayaraman, 2010). The objective of this study was to (1) evaluate the application and effectiveness of five specific nucleases (Benzonase®, DNase 1, nuclease S1, nuclease P1 and Snake Venom Phosphodiesterase (SVPD)) at degrading PS oligonucleotides and (2) determine the optimal operation parameters when used to treat PS oligonucleotide containing waste streams. Based on a comparison of nuclease sensitivities, using an unmodified antisense phosphodiester oligonucleotide of identical length and sequence, nuclease P1, which can act over a broad range of pH and temperatures, presented as the most effective nuclease and could potentially be used to treat PS oligonucleotide containing waste streams, rendering them free of active drug product thereby alleviating fears concerning the release of such DNA. Treatment via enzymatic methods may present as a favourable option as such methods display high substrate specificity, low toxicity, biodegradability, low energy consumption, rapid and efficient action at low concentrations under mild pH values and temperatures, thus resulting in low manufacturing cost, less waste and less energy consumption (Sanchez and Demain, 2011; Jayaraman, 2010; Jaeger, 2004). Additionally, nuclease P1 presents as a treatment method that could remove nucleic acids from biochemical preparations that are used either for pharmaceutical purposes (absence of genetic material) or as reagents in molecular biology. This enzyme represents an effective biochemical method for removing DNA and RNA in the laboratory and in industrial-scale processes.

6.1 Introduction
The occurrence of evolving or newly identified contaminants in our water resources is of constant concern for the health and safety of the general public. The existing water and waste water treatment plants have been designed for the most effective treatment and removal of contaminants and eutrophicating pollution loads, most specifically those which are stipulated in the existing regulations (Bolong et al. 2009). However, the occurrence of the new “unregulated” potential contaminants such as antisense oligonucleotides may necessitate more advanced treatment. The existing established water treatment plants were not designed for these newly identified contaminants and thus are presenting as a threat to the water supply network as a high portion of compounds and their metabolites could escape and enter the environment via sewage effluents (Bolong et al. 2009). This chapter aims to discuss the optimum treatment method for waste streams containing antisense oligonucleotides and to evaluate the most applicable process method to degrade/remove antisense drugs from waste streams as a ‘safety net’ to prevent and circumvent the accidental release of active antisense product into the environment.

6.1.1 Selection of the six optimum treatment methods
In this study, the main strategies adapted for the degradation and removal of antisense oligonucleotides include physical heat treatment, acidic and basic pH at ambient room temperature and in combination with elevated temperature (Chapter Three), chemical treatment with soft metal ions and oxidising agents (Chapter Four) and enzymatic treatment with commercially available nucleases (Chapter Five). These methods are summarised in table 6.1.

As stated previously, it is reported that a sequence as small as four bp intact oligonucleotides can have an effect of activating RNase H activity thus preventing the production of a specific gene. As a consequence of this, treatment by either physical, chemical or enzymatic methods that results in chemical instability, degradation of the oligonucleotide backbone, desulfurization, alteration or deletion of the base sequence (depurination) is required. The mode of action/pathway of degradation and the main degradation products are depicted in figure 6.1.
Table 6.1 Summary list of experimental parameters investigated in Chapter Three, Four and Five.

<table>
<thead>
<tr>
<th>Physical</th>
<th>Chemical</th>
<th>Enzymatic</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Temperature</strong>&lt;br&gt;• RT - 100°C over periods up to eight hours&lt;br&gt;<strong>Steam sterilisation</strong>&lt;br&gt;(autoclaving conditions 121°C and 15 psi for 15 minutes)</td>
<td><strong>Acids</strong>&lt;br&gt;• Hydrochloric acid&lt;br&gt;• Sulfuric acid&lt;br&gt;• Nitric acid</td>
<td><strong>Commercially available nucleases</strong>&lt;br&gt;• DNase I&lt;br&gt;• Snake Venom Phosphodiesterase (SVPD)&lt;br&gt;• Benzonase®&lt;br&gt;• Nuclease S1&lt;br&gt;• Nuclease P1</td>
</tr>
<tr>
<td><strong>Soft metal ions</strong>&lt;br&gt;• Silver nitrate</td>
<td><strong>Oxidising agents</strong>&lt;br&gt;• Iodine&lt;br&gt;• Potassium permanganate&lt;br&gt;• Potassium dichromate&lt;br&gt;• Sodium hypochlorite&lt;br&gt;• Peracetic acid&lt;br&gt;• Hydrogen peroxide&lt;br&gt;• Virkon®</td>
<td></td>
</tr>
</tbody>
</table>

Finding the most suitable treatment process for the degradation of PS oligonucleotides necessitates an understanding of the specific process variables. Based on the methods outlined in the previous chapters (table 6.1) six optimum treatments were chosen. The comparison criteria used in this assessment was efficacy (percentage degradation), desulfurization, energy required, cost, versatility and potential advantages and disadvantages of the treatment process. Based upon the concentration of reagents required, treatment duration, economic considerations and potential environmental impacts, the six optimum treatments include treatment with low pH (0.5 M HCl) at 40°C, low pH (0.5 M H₂SO₄) at 40°C, iodine (25 mM), acidic potassium dichromate (0.125 M), nuclease P1 (5 U/ml) and steam sterilisation.
Figure 6.1 The main pathways of degradation and subsequent degradation products of the 21-mer all PS oligonucleotide following treatment with elevated temperature, pH (low and high), oxidizing agents and nucleases (adapted from information obtained from Rental et al. 2005).

*a* base sequence of Vitravene™.
6.1.2 Generation of an actual simulated waste stream environment.

Prior to this, all experimental degradation analysis was carried out in purified water. To ensure that each of the proposed systems would be suitable on an industrial scale, the chosen optimised treatment methodologies were analysed, for their efficacy, in a simulated AX purification waste stream.

In Chapter Two, the manufacture of antisense oligonucleotides with particular emphasis on PS oligonucleotides from solid phase synthesis through to the generation of API (active pharmaceutical ingredient) was described. It was concluded that the majority of oligonucleotide related material, up to and including 50%, may be lost to waste following purification. Based on both personal communication and an extensive review of the literature, it was concluded that reversed phase HPLC and most specifically anion exchange chromatography, at present, represent the two most commonly utilized standard purification techniques for the manufacture of therapeutic antisense oligonucleotides under cGMP conditions (Lajmi et al. 2004).

As stated previously, there is limited information in the scientific literature in relation to the purification of antisense oligonucleotides at large commercialisation stage, as this information is confidential to the companies involved. The main rationale for the chosen case study purification method for waste stream analysis was to simulate a waste stream that would be similar to conditions carried out on an industrial scale based on the following criteria:

- How up to date/current the information was?
- Level of detail given, to accurately generate a waste stream.
- Source of information.
- Scale of the purification process.

The GE Healthcare pilot-scale methodology for the purification of 150 gram crude phosphorothioate 20-mer oligonucleotide on SOURCE 30Q packed FineLine 200L column was chosen as the case study (GE Healthcare, 2010) (figure 6.3) as this method was also discussed by Deshmukh et al. 2001 and continues to be heavily referenced in the scientific literature (Sanghvi and Schulte, 2004). In addition to this, it represents one of the largest scale purifications of antisense oligonucleotides published to date.
6.1.3 Assessment of optimum treatment methods under an actual simulated waste stream environment.

A thorough assessment of each treatment process requires experimental analysis (in simulated AX waste stream), process modelling, environmental and cost analysis prior to determining if the methodology would be feasible and suitable on a large-scale with a high degree of efficiency. Evaluation of treatment methods should be carried out to ensure that the resultant degradation of the antisense drugs is sufficient and has reached suitable levels for the subsequent disposal of such material. Additionally, the desired attributes should include the following:

- Be fast acting (less than 60 minutes).
- Give rise to significant degradation (> 75%).
- Demonstrate activity against a wide range of (if not all) antisense agents.
- Efficacy in the presence of waste stream components.
- Safe use.
- Low or no toxicity, irritancy, mutagenicity, or carcinogenicity.
- Lack of unwanted or toxic residues.
- Stability (stable even when exposed to light, heat, or other environmental factors), yet ability to be readily broken down in the environment.
- Environmental friendliness.
- Lack of damage to surfaces or areas (compatibility).
- Be inexpensive and easy to obtain and use.
- Not have an unpleasant odour.

As such, all of the optimum treatment methods were undertaken experimentally (in simulated waste product streams) and assessed under the following headings to establish the most suitable method of treating antisense oligonucleotide containing waste streams prior to their release into the environment.

- Effectiveness in simulated waste stream conditions.
- Versatility for use on additional antisense modifications.
- Feasibility and practicality of the process.
- Potential environmental impact.
- Advantages and limitations of the process.
- Cost.
The organisation of this chapter follows the scheme outlined in the flow diagram in figure 6.2. Each parameter is elucidated in the methods and materials section.

Figure 6.2 Flow diagram of the organization of the methodology carried out to establish the most favourable and optimum method for the treatment of oligonucleotide containing waste streams.
6.2 Materials and Methods

6.2.1 Source of chemical reagents
The chemicals used in this study were of analytical reagent grade and were obtained as described in section 3.2.1, 4.2.1 and 5.2.1, unless otherwise stated.

6.2.2 Preparation of waste stream solution
The AX waste stream was generated in the lab based on the purification methodology outlined by Amersham Biosciences, 2002 (now GE Healthcare) (Figure 6.3).

Two eluents were used for the process, Eluent A: 20 mM NaOH and Eluent B: 20 mM NaOH + 2.5 M NaCl. Based on the calculations of eluent required per litre for the purification procedure an AX waste stream solution was formulated containing a proportionate quantity of each of the above mentioned eluents (table 6.2). The calculations were based on the overall purification gradient methodology as detailed in figure 6.3.

Figure 6.3 Pilot-scale methodology for the AX purification of 150 gram crude 20-mer phosphorothioate oligonucleotide on SOURCE 30Q packed FineLine 200L column (Amersham Biosciences, 2002).

Optimised elution conditions for purifying a 20-mer PS oligonucleotide. The steps and gradient are expressed in mS/cm units in place of the percentage of eluent B. The blue line illustrates the crude oligonucleotide profile. The brown line denotes the optimised gradient pilot-scale methodology for purification.

Details of the gradient method:
- **Sample:** 20-mer phosphorothioate oligonucleotide
- **Sample load:** 24 mg crude oligonucleotide/ml medium
- **Column:** FineLINE 200L (packed to 21.3 cm bed height)
- **Medium:** SOURCE 30Q
- **Eluent A:** 20 mM NaOH
- **Eluent B:** 20 mM NaOH plus 2.5 M NaCl
- **Flow velocity:** 250 cm/h
- **System:** BioProcess system, 6 mm

Gradient programme:
- Gradient Linear gradient to a conductivity of 33 mS/cm, 3 CV
- Hold at 33 mS/cm, 5 CV
- Linear gradient from 33 to 80 mS/cm, 8.6 CV
- Step to and hold at 140 mS/cm 2 CV
- Step to Eluent B, 2 CV
Table 6.2 Calculations based on information from Pilot-scale purification of 150 gram crude phosphorothioate 20-mer oligonucleotide to generate an AX purification waste stream.

<table>
<thead>
<tr>
<th>Gradient Step</th>
<th>Volume Eluent B (ml)</th>
<th>Volume Eluent A (ml)</th>
<th>Total volume (ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Step 1: Linear gradient to a conductivity of 33 mS/cm., 3 CV.</td>
<td>1960.2</td>
<td>18439.8</td>
<td>20400.0</td>
</tr>
<tr>
<td>Step 2: Hold at 33 mS/cm, 5 CV</td>
<td>6232.2</td>
<td>27767.8</td>
<td>34000.0</td>
</tr>
<tr>
<td>Step 3: Linear gradient from 33–80 mS/cm, 8.6 CV.</td>
<td>18486.6</td>
<td>39933.4</td>
<td>58480.0</td>
</tr>
<tr>
<td>Step 4: Step to and hold at 140 mS/cm, 2CV</td>
<td>10578.0</td>
<td>3022.0</td>
<td>13600.0</td>
</tr>
<tr>
<td>Step 5: Step to eluent B, 2 CV</td>
<td>13600.0</td>
<td>0.0</td>
<td>13600.0</td>
</tr>
<tr>
<td>Total Volume (ml)</td>
<td>50857.0</td>
<td>89223.0</td>
<td>140080.0</td>
</tr>
<tr>
<td>Total volume (L)</td>
<td>50.9</td>
<td>89.2</td>
<td>140.1</td>
</tr>
</tbody>
</table>

6.2.3 Investigation of PS oligonucleotide stability in AX waste stream

The stability of PS oligonucleotides in the waste stream generated in section 6.2.2, from the purification process, was investigated. A stock solution of PS oligonucleotide was prepared to a 1:40 dilution in AX waste stream solution to a final concentration of 25 µM. All mixtures were vortexed briefly and allowed to stand at ambient room temperature over the period of analysis. Sample aliquots were removed after 0, 1, 2, 4 and 5 days and subsequently analysed via 25% PAGE and IP RP-LC as described in section 3.2.4 and 3.2.6.

6.2.4 Investigation of the optimum treatment methods to degrade PS oligonucleotides in water

PS oligonucleotide degradation reactions (final reaction volume = 60 µl) were performed with a 21-mer PS oligonucleotide made up in water with the five optimum treatment methods, hydrochloric acid, sulphuric acid, iodine, potassium dichromate and nuclease P1. Degradation reactions were initiated by the addition of equal volumes (30 µl) of freshly prepared treatment solutions to 30 µl PS oligonucleotide (final concentration 25 µM) as outlined in table 6.3. The reactions were performed over the specified period of analysis and subsequently analysed by IP-RP LC in triplicate.
Table 6.3 Experimental conditions used in the analysis of the effects of the optimum treatment methods on PS oligonucleotides.

R.T = Ambient Room Temperature.

<table>
<thead>
<tr>
<th>Chemical reagent</th>
<th>Reagent concentration (Final reaction conc.)</th>
<th>Length of incubation (min)</th>
<th>Temperature (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hydrochloric acid (HCl)</td>
<td>0.5 M</td>
<td>30</td>
<td>40</td>
</tr>
<tr>
<td>Sulfuric acid (H$_2$SO$_4$)</td>
<td>0.5 M</td>
<td>30</td>
<td>40</td>
</tr>
<tr>
<td>Iodine (I$_2$)</td>
<td>25 mM</td>
<td>5</td>
<td>R.T</td>
</tr>
<tr>
<td>Acidic potassium dichromate (K$_2$Cr$_2$O$_7$ / H$_2$SO$_4$)</td>
<td>0.125 M</td>
<td>60</td>
<td>R.T</td>
</tr>
<tr>
<td>Nuclease P1</td>
<td>5 U/ml</td>
<td>60</td>
<td>37</td>
</tr>
</tbody>
</table>

### 6.2.5 Investigation of the optimum treatment methods to degrade PS oligonucleotides in AX waste stream

The PS oligonucleotide was mixed with AX purification waste stream solution to produce a 1:20 dilution of 50 µM PS oligonucleotide. Equal volumes (30 µl) of 50 µM PS oligonucleotide (in waste stream) were added to equal volumes of each of the five optimum treatment solutions as outlined in table 6.3. Each micro tube was vortexed briefly. The samples were allowed to stand for the specified period of analysis (table 6.3) and were subsequently analysed via IP-RP LC in triplicate. A control containing equal volumes of 50 µM PS oligonucleotide (in waste stream) and water, in the place of the treatment reagents, were prepared and analysed in a similar manner.

### 6.2.6 Investigation of the efficacy of steam sterilisation for the degradation of PS oligonucleotides in water or AX waste stream.

A 1:20 dilution of PS oligonucleotide, to a final concentration of 25 µM, was made up in either water or AX waste stream solution. The PS oligonucleotide was also made up in a 1:20 dilution of the respective components of the waste stream; 20 mM sodium hydroxide and 0.9 M NaCl. The samples were gently vortexed and subjected to steam sterilisation at 121°C and 15 psi for 15 minutes. Once the cycle was complete, the samples were immediately placed on ice or stored at -20°C for further analysis via IP-RP LC and PAGE in triplicate.

### 6.2.7 Versatility studies of the five optimum treatment methodologies

The application of using the five chosen optimum treatment methods on unmodified PO oligonucleotide (made up in water) was carried out as described in section 6.2.4 and then subjected to analysis via IP-RP LC in triplicate.
6.2.8 pH analysis of the five optimum treatment methodologies

The pH of the five optimum treatments was recorded in the presence and absence of the AX waste stream. After the addition of equal volumes of treatment agent to either water or AX waste stream the solutions were mixed thoroughly with the aid of a magnetic stirrer. An Orion pH meter (Thermo Scientific) was used to determine the respective pH of the solutions.

6.2.9 Process model analysis of the five optimum treatment methodologies

The five optimum treatment processes for the degradation of PS oligonucleotides were diagrammatically represented using the process modelling software Super Pro Designer® 6.0. (Intelligen, Europe Inc., Thessaloniki, Greece). An assessment of further waste stream treatment requirements post breakdown of the oligonucleotides was carried out for each of the five optimum oligonucleotide treatment processes.

6.2.10 Cost analysis of the five optimum treatment methodologies

The cost of treatment, in AX waste streams, using the derived optimum treatment conditions for the degradation of PS oligonucleotides was investigated. Four laboratory suppliers, Sigma, VWR Fisher Scientific and Lennox were chosen and the costs of the respective treatments were recorded and compared accordingly.

6.2.11 Statistical analysis

Data analysis, including calculation of mean, standard deviation and linear regression was conducted using Excel 2010 (Microsoft, USA).
6.3 Results and discussion

6.3.1 Simulation of model purification waste stream

The AX waste stream was generated in the lab based on the purification methodology outlined in figure 6.3. Two eluents were used for the process, Eluent A: 20 mM NaOH and Eluent B: 20 mM NaOH + 2.5 M NaCl. Based on calculations of eluent required per litre, for the purification procedure, an AX waste stream solution was formulated containing a proportionate quantity of each of the above mentioned eluents (table 6.2). The calculations were based on the overall purification gradient methodology and as a result may contain a higher concentration of salt than would actually present in a typical waste stream. Collection and removal of fractions for analysis and further work up steps were not taken into account in this case study. Additionally, this AX waste stream represents a model of a typical purification waste stream. Each pharmaceutical company, using similar buffers, would most likely have optimised the system to their requirements.

6.3.2 Stability of PS oligonucleotides in simulated AX purification waste stream

Prior to assessing the effect of the optimum treatment methods in the simulated waste stream it was necessary to establish the stability of PS oligonucleotides in the waste stream for a prolonged period of time. As waste streams contain components such as salts and bases which may protect, degrade or modify the PS oligonucleotide, an experiment was undertaken to determine the stability of PS oligonucleotides within this waste stream as described in section 6.2.3. A period of 5 days was chosen as it is likely that such waste would be taken to the plant’s treatment plant prior to five days. The stability of the PS oligonucleotides in the AX waste stream was assessed over a period of 0, 1, 2, 4 and 5 days by both IP-RP LC and PAGE. Figure 6.4 illustrates the relationship between peak area and length of incubation of the PS oligonucleotide in the AX waste stream. The peak area of the PS oligonucleotide remained constant over the 5 day period of analysis. It was established that the PS oligonucleotides are stable in the purification waste stream over a period of up to and including 5 days and therefore would require suitable treatment for the removal of such wastes.
Figure 6.4 The stability of PS oligonucleotides in the AX purification waste stream over a period of 5 days as assessed by IP-RP LC.

Each value represents mean ± SD (n=3).

Similar findings were confirmed via PAGE analysis. Figure 6.5 presents the electrophoretic analysis of the stability of the PS oligonucleotide over a period of 5 days in the simulated waste stream. Similar to results obtained by HPLC, the band did not diminish in size over the period of analysis and there was no visible evidence or appearance of degradation products in contrast to the control sample band.

Figure 6.5 Electrophoretic analysis of the stability of PS oligonucleotides in the AX purification waste stream over a period of 5 days.

Oligonucleotides were visualised upon staining the gel with Stains-all.

C1 = PS oligonucleotide (25μM) only.

C2 = AX purification waste stream only.
6.3.3 Effectiveness of the treatment process in simulated waste stream conditions

**Optimum Treatment 1: Hydrochloric acid (HCl)**

The first optimum treatment chosen was degradation of the oligonucleotides using low pH at 40°C. From previous analysis carried out in Chapter Three, it was established that treatment with 0.5 M HCl at 40°C for 30 minutes gave maximum degradation of 84.9% under the experimental conditions analysed. The PS oligonucleotide was made up in both purified water (control analysis) and in AX waste stream (1:20 dilution) as described in section 6.2.4 and 6.2.5. The PS oligonucleotides in both conditions were treated with 0.5 M HCl at 40°C for 30 minutes and analysed in triplicate via IP-RP LC. Figure 6.6 illustrates the results obtained under AX waste stream conditions. The influence of the acid had the effect of degrading the PS oligonucleotide, made up in water, by 94.3%, a slight increase on results obtained in earlier studies. Despite the composition of the AX waste stream, which contains sodium hydroxide, the basic nature of the buffer (pH 12.1) did not hinder the effectiveness of HCl and degraded the oligonucleotide by 92.9%. This is primarily due to the fact that the concentration of the sodium hydroxide is low, (20 mM) in contrast to the concentration of the HCl (0.5 M). When the two solutions were incubated in equal volumes the sodium hydroxide did not alter the pH of the 0.5 M HCl (pH 0.3).

**Optimum Treatment 2: Sulphuric acid (H₂SO₄)**

Treatment, under similar conditions, with the industrial acid sulphuric acid, in previous studies, gave similar results to treatment with hydrochloric acid. Maximum degradation of 85.0% was observed following incubation of the PS oligonucleotide with 0.5 M H₂SO₄ at 40 °C after 30 minutes (Chapter Three). Following further analysis to establish if this method would be suitable in an industrial process, the experiment was carried out in a simulated AX waste stream environment. Similar to treatment with hydrochloric acid, it was established that the waste stream components did not hinder the efficacy of 0.5 M H₂SO₄ degrading the PS oligonucleotides by 92.4% in simulated AX waste stream in contrast to treatment of the PS oligonucleotide made up in water which gave rise to 93.3% degradation (figure 6.6).

**Optimum Treatment 3: Iodine (I₂)**

Iodine was a very effective and efficient treatment process at degrading the PS oligonucleotides where maximum degradation of 91.6% was obtained following 1 minute incubation at ambient room temperature (Chapter Four). In addition to structural degradation of the oligonucleotide a sharp shift in retention time and change in band colour
was observed upon treatment with increasing concentration of iodine solution which is indicative of desulfurization. To establish the effectiveness of this optimum treatment method in the waste stream, a time point of 5 minutes was chosen. Similar to the effects of the two acids, the waste stream components did not impair the efficacy of iodine at significantly degrading the PS oligonucleotide by 86.7% as illustrated in figure 6.6.

**Optimum Treatment 4: Potassium dichromate (K₂Cr₂O₇)**

The fourth optimum treatment was the effect of acidic potassium dichromate on PS oligonucleotides. Previous results obtained established that this treatment process was one of the most effective chemical methods at degrading the PS oligonucleotides leading to maximum degradation of 94.7% following treatment with 0.125 M K₂Cr₂O₇ in 0.025 M H₂SO₄ for 60 minutes (Chapter Four). Treatment of the PS oligonucleotides, made up in the simulated waste stream, resulted in 88.3% degradation, marginally less, in comparison to treatment of the PS oligonucleotides made up in water which gave rise to 95.9% degradation (figure 6.6).

**Optimum Treatment 5: Nuclease P1**

The final optimum treatment chosen was the most efficient nuclease at degrading the PS oligonucleotides as described in Chapter Five. In this study, nuclease P1 presented as the most effective nuclease degrading the PS oligonucleotides by 99.0% at a concentration of 5 U/ml. To establish if this method would be suitable on an industrial scale this treatment process was carried out using PS oligonucleotides made up in AX waste stream. Enzymes are very sensitive to reaction conditions and this can affect the overall activity of the nuclease. The pH of a medium can have a dramatic effect on the activity of an enzyme as can increased salt concentration. Enzymes can generally operate over a broad pH range, for example nuclease P1 operates over a pH range of 5.0-8.0 (Furuichi and Miura, 1975; Fujimoto et al. 1974b). The composition of the waste stream components and the pH of this solution, pH 12.1, did not impede the activity of the enzyme at the optimum treatment concentration of 5 U/ml. This may be due to the fact that the nuclease buffer decreased the pH of the waste stream solution to pH 8.4. Nuclease P1 effectively degraded the PS oligonucleotide by 99.5% when made up in purified water. When the oligonucleotide was made up in the AX waste stream a marginal decrease of 2.6% was observed whereby significant degradation of 96.9% was detected under such experimental conditions (figure 6.6). When the oligonucleotide (made up in waste stream) was incubated with the nuclease buffer, in the absence of the enzyme, no degradation was observed over the period of
This therefore infers that cleavage of the PS oligonucleotide backbone was due to the action of the nuclease at a concentration of 5 U/ml.

**Figure 6.6 The stability of PS oligonucleotide in a simulated AX waste stream solution following optimum treatments as assessed by IP-RP LC.**

Percentage degradation of PS oligonucleotides = % intact PS oligonucleotide after treatment for the indicated time relative to the untreated PS oligonucleotide. Each value represents mean ± SD (n=3).

**Optimum treatment 1:** Hydrochloric acid (HCl) (0.5M) at 40°C.
**Optimum treatment 2:** Sulfuric acid (H₂SO₄) (0.5M) at 40°C.
**Optimum treatment 3:** Iodine (I₂) (25mM).
**Optimum treatment 4:** Acidic potassium dichromate (K₂Cr₂O₇/H₂SO₄) (0.125M/0.025M).
**Optimum treatment 5:** Nuclease P1 (5U/ml).

### 6.3.4 Effects of steam sterilisation upon oligonucleotide stability in AX waste stream

One of the experimental parameters investigated in this study was steam sterilisation which gave significant decomposition of 77.8% of the modified PS oligonucleotide (Chapter Three). When this treatment process was carried out again, under similar conditions, comparable results of 73.3% were obtained when the oligonucleotide was made up in water as can be seen in figure 6.7. Despite these significant degradation results, when the PS oligonucleotide was diluted in the simulated waste stream, the components of the waste stream impeded the mechanism of action of stream sterilisation and no significant breakdown (0.1%) of the intact oligonucleotide was detected (figure 6.7 and 6.8). This is most likely due to the high salt concentration present in the waste stream which would impart greater structural stability to the oligonucleotide backbone. Nucleic acids are highly charged polyanions, possessing one negative charge per phosphate (Bloomfield *et al.* 2000). It is highly recognised that monovalent and divalent salts protect DNA against melting at high temperatures (thermo-denaturation) by screening the negative charges of the phosphate...
groups (Marguet and Forterre, 1998). Ionic compounds for example buffers and salts interact with macromolecules via specific or non-specific binding. A salt may increase thermal stability depending on the type of interaction (Parkins and Lashmar, 2000). A study by Marguet and Forterre, (1998) demonstrated that physiological concentrations of KCl and MgCl₂ protect both single and double stranded DNA against heat-induced cleavage and depurination. Moreover, DNA thermodegradation was greatly reduced in the presence of physiological concentrations of either monovalent (50-500 mM KCl, NaCl) or divalent salts (1-25 mM MgCl₂) (Marguet and Forterre, 1998).

![Figure 6.7 The stability of PS oligonucleotide to steam sterilization made up in water, NaOH, NaCl and simulated waste stream as assessed by IP-RP LC.](image)

Percentage degradation of PS oligonucleotides = % intact PS oligonucleotide after treatment for the indicated time relative to the untreated PS oligonucleotide. Each value represents mean ± SD (n=3).

To further investigate this observation the PS oligonucleotide was made up in both components of the waste stream, 20 mM NaOH and 0.9 M NaCl (the equivalent concentration of NaCl in the waste stream). The findings from this study were quite interesting and unexpected from a theoretical point of view. It would have been anticipated that the high salt concentration would have provided additional stability to the PS oligonucleotide. However, when the oligonucleotide was made up solely in the salt solution it resulted in 44.6% degradation following steam sterilisation. The degree of degradation may have been greater as additional breakdown products may have been masked and therefore not fully resolved. When the oligonucleotide was incubated with only 20 mM NaOH it was found to exert a protective effect on the PS oligonucleotides during the steam sterilisation process where little if any (0.2%) decrease in peak area was detected via HPLC. Therefore, it can be deduced that the weak basic solution of 20 mM NaOH gave additional
stability to the PS oligonucleotide and the salt content to a lesser degree. The presence of sodium hydroxide may to some degree competitively protect the oligonucleotide from thermal damage.

These results were further confirmed by the semi-quantitative analysis of PAGE. Electrophoretic results are presented in figure 6.8, which illustrates the effect of stream sterilisation on the PS oligonucleotide made up in purified water, NaOH, NaCl, or AX waste stream.

![Figure 6.8 Electrophoretic analysis of the stability of PS oligonucleotides to stream sterilization made up in water, NaOH, NaCl and simulated waste stream.](image)

Oligonucleotides were visualised upon staining the gel with Stains-all.
- = Prior to steam sterilization.
+ = After steam sterilization.

The band corresponding to that of the intact oligonucleotide completely disappeared following steam sterilisation when the oligonucleotide was made up in purified water and NaCl. The degradation product bands appear further down the gel when made up in water in contrast to the sample of oligonucleotide made up in salt where they appear larger, and higher up the gel which would indicate that more intact oligonucleotides, of larger base pair length, remained which is consistent with the results obtained following HPLC analysis. The effect of steam sterilisation of the oligonucleotide, made up in either 20 mM NaOH or AX waste stream, did not change the appearance, shape or intensity of the bands. Additionally, no degradation bands were evident lower down the gel. As this method is not effective in the simulated AX waste stream, only five optimum treatments will be referred to from here on in.
6.3.5 Versatility studies of the five optimum treatment methodologies

An important parameter that would reflect the effectiveness of a treatment process, for potential industrial application, would be the versatility of the methodology i.e. would this method be suitable for the treatment of other antisense oligonucleotides of varying modifications? (Section 1.4.1). Due to availability of resources, these studies were only carried out using unmodified phosphodiester oligonucleotides which are used in aptamer technology (Sinha et al. 2006). However, findings from the literature will be used to extrapolate likely effects upon additional modified oligonucleotides. As outlined in section 6.2.7, unmodified PO oligonucleotides were subjected to each of the optimum treatment processes. However, in the case of low acidic pH, only one acid (HCl) was used. The oligonucleotides were not subjected to treatment with H₂SO₄ as it was anticipated that similar results would be achieved following incubation with HCl as was determined in Chapter Three. Figure 6.9 and table 6.4 illustrate the results obtained following versatility studies and the application of optimum treatment methods on unmodified phosphodiester oligonucleotides.

![Figure 6.9 The stability of PO oligonucleotide following treatment with optimum treatments of 0.5 M HCl at 80°C, iodine, potassium dichromate and nuclease P1 as assessed by IP-RP LC.](image)

Percentage degradation of PO oligonucleotides = % intact PO oligonucleotide after treatment for the indicated time relative to the untreated PO oligonucleotide. Each value represents mean ± SD (n=3).

The unmodified PO oligonucleotide was subjected to treatment with low acidic pH, (hydrochloric acid) over a range of pH and temperatures as described and outlined in Chapter Three. It was established from these studies that maximum degradation (70.9%) of the PO oligonucleotides was obtained following treatment with 0.5 M HCl at 80°C. Similarly, the PO oligonucleotides were subjected to treatment with nuclease P1 as discussed in Chapter
Five. Significant degradation (98.2%) of the unmodified PO oligonucleotides was evidenced via HPLC analysis following treatment with 0.5 U/ml nuclease P1. Both iodine and potassium dichromate were ineffective (<10%) at degrading the PO oligonucleotides at the optimum treatment conditions for PS oligonucleotides (figure 6.9 and table 6.5). Iodine solutions are routinely used in the oxidative step of oligonucleotide synthesis and under such oxidative conditions have not been reported to have any deleterious effect on either phosphodiester or the more sensitive phosphotriester linkages (Vyle et al. 1992).

Table 6.4 Versatility studies – The stability of PO and PS oligonucleotides to the proposed optimum treatments.

<table>
<thead>
<tr>
<th>Treatment conditions</th>
<th>Unmodified PO oligonucleotide (% deg.)</th>
<th>Modified PS oligonucleotide (% deg. in AX W/S)</th>
<th>Versatility</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low pH (HCl)</td>
<td>Maximum degradation at 80ºC 70.9%</td>
<td>Maximum degradation at 40ºC 92.8% (HCl) 92.4% (H₂SO₄)</td>
<td>Yes</td>
</tr>
<tr>
<td>Iodine</td>
<td>Minimal (&lt;10%) 9.6%</td>
<td>86.7%</td>
<td>No</td>
</tr>
<tr>
<td>Potassium dichromate</td>
<td>Minimal (&lt;10%) 8.5%</td>
<td>88.3%</td>
<td>No</td>
</tr>
<tr>
<td>Nuclease P1</td>
<td>Yes 98.2%</td>
<td>96.9</td>
<td>Yes</td>
</tr>
</tbody>
</table>

From the five optimum treatments that were chosen it was concluded that treatment with low pH or nuclease P1 would be an effective treatment process that could be applied to the treatment of antisense oligonucleotide of varying modifications. Despite the fact the studies were not carried out over a wide range of oligonucleotide modifications, it has been demonstrated and reported in the literature that such treatments could effectively degrade RNA and 2nd generation oligonucleotides (Bahr et al. 2009). Nuclease P1 is a sugar non-specific nuclease which can act on both DNA and RNA (Gangadhara et al. 2008; Summerton and Weller, 1997; Fujimoto et al. 1975; Maekawa et al. 1991; Falcone and Box, 1997) and therefore may be applicable to treat and remove further modifications of antisense molecules. Moreover, in the majority of cases, the substrates specificity of a nuclease is dependent on the composition of the enzyme utilised in the reaction, with high concentrations giving rise to less specific cleavages. As a result an increase in concentration will give rise to a broader range of substrate specificity.

It is widely accepted that RNA and DNA can be degraded by acids. Bahr et al. (2009) demonstrated that treatment of RNA oligonucleotides (siRNA), both single and double stranded, with strong acids at pH 1-2 gave rise to rapid hydrolysis of the phosphodiester
bonds at the 5’-position of ribose for sequencing purposes. They also established that treatment with low pH was applicable for additional RNA modifications such as an oligonucleotide with phosphorothioate backbone and of one containing 2′-methoxyribose modifications (Bahr et al. 2009).

6.3.6 pH analysis of the final optimum treatment methodologies

pH is one of the waste water parameters that must to be managed and controlled (Noor et al. 2004). The pH values of the optimum treatment processes are presented in table 6.5. These values represent each respective optimum treatment incubated and mixed with equal volumes of AX waste stream in the absence of PS oligonucleotides. It is likely that the oligonucleotide material would have little effect on the final documented pH value. The pH of the AX waste stream alone was 12.1 and as such would have required additional neutralisation treatment to bring the pH of the influent stream to near neutrality. Due to the nature of removal treatment process, the resultant waste water following treatment with hydrochloric acid, sulphuric acid and acidic potassium dichromate are strongly acidic with pH values of 0.3, 0.3 and 1.6 respectively. In such cases the control of the pH value to legislative and environmental standard is crucial. The acceptable pH range for discharge is normally between 6 and 8 or 6 to 9 standard units (S.U.) but can also be between 5.0 to 11.0 depending on the source of discharge (EPA, 2008a; Currenta GmbH & Co. OHG, 2011; Bergemann et al. 1995). Treatment with either iodine or nuclease P1 may also require the addition of an acid to slightly lower the pH of the effluent stream.

Table 6.5 pH analysis of the five optimum treatment methodologies.

<table>
<thead>
<tr>
<th>Treatment method</th>
<th>pH value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Waste stream only</td>
<td>12.1 ± 0.0</td>
</tr>
<tr>
<td>Optimum treatment 1 (HCl at 40°C)</td>
<td>0.3 ± 0.0</td>
</tr>
<tr>
<td>Optimum treatment 2 (H₂SO₄ at 40°C)</td>
<td>0.3 ± 0.0</td>
</tr>
<tr>
<td>Optimum treatment 3 (iodine)</td>
<td>9.9 ± 0.0</td>
</tr>
<tr>
<td>Optimum treatment 4 (potassium dichromate)</td>
<td>1.6 ± 0.0</td>
</tr>
<tr>
<td>Optimum treatment 5 (nuclease P1)</td>
<td>8.4 ± 0.0</td>
</tr>
</tbody>
</table>

Each value represents mean ± SD (n=3).
The principal technology used by industry to treat highly acidic or highly basic waste solutions is neutralization of waste water with neutralising alkalis or acids for safe discharge to municipal sewer systems or to streams and to rivers (Noor et al. 2004). The most commonly employed chemicals are hydrochloric acid (HCl), sulfuric acid (H2SO4), and sodium hydroxide (NaOH) (Currenta GmbH & Co. OHG, 2011). There are several purposes for the application of neutralization which include, protection of human health and the environment including fish and other living organisms in streams and rivers, protection of the biological treatment process integrated at most publicly owned treatment works (POTW) and protection of the waste water infrastructure from corrosion, most specifically from acidic waste water (Currenta GmbH & Co. OHG, 2011; Noor et al. 2004).

6.3.7 Simulation of treatment processes, potential environmental impact and advantages and limitations of each treatment process.

The treatment process for each of the five optimum treatments were simulated using the process modelling software SuperPro Designer® 6.0 as detailed in section 6.2.9 for the treatment of a modest scale of 1 L AX purification waste stream containing PS oligonucleotide waste of a concentration of 50 µM. Each process diagram describes the treatment process requirements and additional post treatment steps if required. After the degradation treatment regeneration process, the water containing these chemical wastes must be suitably treated, if required, and appropriately disposed of. Keeping with the BAT (Best Available Techniques) objective which was introduced as a key principle in the IPPC Directive, 96/61/EC and incorporated into Irish law by the Protection of the Environment Act 2003, emphasis should be placed on pollution prevention techniques instead of end-of-pipe treatment. Issues pertaining to the use of low-waste technology, the consumption and nature of the raw materials used in the process and their energy efficiency, the use of less hazardous substances and the nature, effects and volume of the emissions concerned should be taken into consideration (EPA, 2008a).

Optimum Treatment 1 and 2: Hydrochloric acid/Sulfuric acid

Figure 6.10 and figure 6.11 illustrates the treatment process of 1 L AX purification waste stream containing oligonucleotide related wastes using low pH (HCl) or (H2SO4) under the influence of low heat of 40°C. Once the oligonucleotides have been incubated for 30 minutes with HCl or H2SO4 they are effectively degraded (92.8 and 92.4%) and the composition of the waste stream consists of degraded oligonucleotide in an acidic waste
water solution containing HCl, NaCl/Na₂SO₄ and NaOH. Due to the nature of the resultant waste stream, the waste water is strongly acidic (pH 0.3) and cannot be sent directly to the waste water treatment plant.

![Simulated process diagram](image)

*Figure 6.10 Simulated process diagram generated using SuperPro Designer® 6.0 presenting the treatment process of 1 L AX purification waste stream containing oligonucleotide related wastes with low acidic pH (HCl at 40°C).*

![Simulated process diagram](image)

*Figure 6.11 Simulated process diagram generated using SuperPro Designer® 6.0 presenting the treatment process of 1 L AX purification waste stream containing oligonucleotide related wastes with low acidic pH (H₂SO₄ at 40°C).*

The only post treatment step that would be required is to neutralise this highly acidic waste prior to release to the waste water treatment plant. This can be easily carried out by the addition of an equimolar solution of sodium hydroxide. This is a commonly employed treatment process carried out in many waste treatment plants to ensure that the pH of the waste stream influent is of neutral pH between 6 and 9 (EPA, 2008a). The outcome of this is an acceptable pH for discharge with additional increases in salt loads to the sewer system (Noor et al. 2004). The resultant waste products are sodium hydroxide, water, degraded
oligonucleotide and sodium salts of either chlorides or sulphates. The inorganic salt content of the waste stream could have a negative impact on the biosphere of receiving water (EPA, 2008b). The IPPC licencing limits and compliance (Micro-Bio (Ireland) LTD), for chloride and sulphate are 70,000 and 5000 mg/l respectively (EPA, 2007). Based on the calculations of the final resultant concentration of NaCl and Na₂SO₄ it was established that the chloride content of 16,840 mg/l is well within the limits of constraint in contrast to the levels of sulphate which are 5-fold greater than the IPPC licencing limits. This infers that the application of hydrochloric acid would be more favourable than sulphuric acid as no additional desalting step would be required prior to release to waste stream. If the inorganic salt load is in excess of the required limits high salt contents may require a desalting step by either membrane filtration or extraction (European Commission, 2006). The respective advantages and limitations for the proposed application of low pH (HCl/H₂SO₄) at 40°C are presented in table 6.6.

Table 6.6 The potential advantages and limitations of the application of low acidic pH to treat PS oligonucleotide containing waste streams.

<table>
<thead>
<tr>
<th>Advantages</th>
<th>Limitations</th>
</tr>
</thead>
<tbody>
<tr>
<td>Widely available.</td>
<td>Requirement of energy (40°C), however this is quite modest.</td>
</tr>
<tr>
<td>Easy to prepare.</td>
<td>Energy requirement would increase cost of process.</td>
</tr>
<tr>
<td>Fast (30 minutes), effective degradation (92.8% (HCl) and 92.4% (H₂SO₄)) of the PS oligonucleotides at relatively low concentrations in simulated AX purification waste stream.</td>
<td>Requirement of post neutralisation step</td>
</tr>
<tr>
<td>Waste stream products neutralised to give benign waste products of salt and water.</td>
<td>Corrosive.</td>
</tr>
<tr>
<td>Could potentially be used for the degradation of siRNA and various modifications of antisense therapeutics.</td>
<td></td>
</tr>
<tr>
<td>HCl and H₂SO₄ inexpensive.</td>
<td></td>
</tr>
</tbody>
</table>

Optimum Treatment 3: Iodine

Figure 6.12 represents the simulated process of the third treatment method, upon addition of equal volumes of 1 L 50 mM iodine, for 5 minutes at ambient room temperature. The resultant waste solution consists of decomposed PS oligonucleotide, iodine (25mM), NaCl and NaOH. Once the reaction has occurred in less than 5 minutes it is likely, due to the concentration of iodine used, that this waste stream solution would be suitable for release into the waste water treatment plant.
Iodine is categorised as a water hazard class 1. This infers that it is only slightly harmful when dissolved in water. Environmental risks may vary among iodine substances (Lenntech B.V, 2011a). If the levels of iodine are above the permitted concentration for release to waste water the iodine could be removed by means of active carbon (Lenntech B.V, 2011a; Yang et al. 1993). However, it may be the case that concentrations and chemical speciation are such that adsorption is not economical for this purpose. Additionally, the European Commission (2006) have described a methodology for the removal of iodine compounds from waste water by nanofiltration. Following the production of X-ray contrast media which contain around 1000 ppm iodine, wash-waters were collected separately and removed from the waste water stream via a 2-stage nanofiltration. In the initial stage, the iodine compounds were concentrated up to 60 g/l, followed by the second stage which ensures a permeate concentration lower than 1 ppm (European Commission, 2006). In another embodiment by the European Commission (2006), they describe a method for the recovery of iodine from waste streams. Iodine can be recovered as copper iodide (CuI) by pH adjustment, oxidation with NaSO₃, addition of CuSO₄ and filtration of the precipitate. This allows recovery of material that may be of value for reuse/resale in preference to emission to water (European Commission, 2006). The potential advantages and limitations are summarised in table 6.7.
Table 6.7 The potential advantages and limitations of the application of iodine to treat PS oligonucleotide containing waste streams.

Table compiled with information obtained from McDonnell, 2007; McDonnell and Russell, 1999.

<table>
<thead>
<tr>
<th>Advantages</th>
<th>Limitations</th>
</tr>
</thead>
<tbody>
<tr>
<td>Widely available.</td>
<td>Specificity for phosphorothioate modification.</td>
</tr>
<tr>
<td>Easy to prepare.</td>
<td>Generates brown stains on surfaces.</td>
</tr>
<tr>
<td>Fast (5 minutes), effective decomposition (86.7%) of the PS oligonucleotides at relatively low concentrations in simulated AX purification waste stream.</td>
<td>Generally unstable and surface incompatibility with some metals (corrosion) and plastic surfaces.</td>
</tr>
<tr>
<td>May have some short-lived persistent activity, remaining after application to provide residual degradation activity.</td>
<td>Gives water a slight straw colour at high levels.</td>
</tr>
<tr>
<td>Does not require energy.</td>
<td>More expensive than HCl and H$_2$SO$_4$ and K$_2$Cr$_2$O$_7$</td>
</tr>
</tbody>
</table>

Some of these limitations, outlined above, can be overcome and reduced by the development of iodophors (“iodine carriers” or “iodine-releasing agents”). Povidone-iodine and poloxamer-iodine are the most widely used in both disinfectants and antiseptics. Iodophors are complexes of iodine and a solubilizing agent or carrier, which acts as a reservoir of the active “free” iodine (Gottardi, 1991). Iodophors are generally water soluble and nonstaining, have little or no odour, and increase the stability of iodine in solution (McDonnell, 2007).

**Optimum Treatment 4: Potassium dichromate**

Figure 6.13 displays the process simulation for the treatment of PS oligonucleotide containing waste stream with acidic potassium dichromate. Despite the fact that this chemical treatment was an effective treatment process degrading the intact oligonucleotide by 95.9 and 88.3% in both water and the AX purification waste stream solution respectively such treatment generates an excess of hexavalent chromium in the waste stream solution and has significant drawbacks in terms of environmental impact. Chromium, which is on the top priority list of toxic pollutants identified by the US EPA, is an extremely toxic substance (Fournier and Meyer, 1975; Babel and Kurniawan, 2004). The health hazards related to chromium exposure are determined by its oxidation state. Chromium exists predominately in two valent states in nature: hexavalent chromium [chromium (VI)] and trivalent chromium [chromium (III)] (Bagchi et al. 2002). Chromium (VI) and chromium (III) compounds differ in their environmental and health effects. Owing to its high solubility, Cr (VI) is extremely toxic to living organisms compared to Cr (III) (Selvaraj et al. 2003). Chromium (VI) compounds are categorised as water hazard class 3 (Lenntech B.V, 2011b). It generates stoichiometric amounts of heavy-metal waste and Cr (VI) is a proven toxin, carcinogen and mutagen (de Flora, 2000; Bagchi et al. 2002; Babel and Kurniawan, 2004; Kawanishni et al. 1986). Chromium (VI) induced acute and chronic toxicity, carcinogenicity, genotoxicity,
neurotoxicity, dermatoxotoxicity, immunotoxicity and general environmental toxicity have been widely demonstrated (von Burg and Liu, 1993). Soluble and insoluble hexavalent chromium salts have proven to induce morphological and neoplastic transformation and mutagenicity in the human and murine cells (Bagchi et al. 2002). A large number of studies established that chromium (VI) induces oxidative stress, DNA damage, altered gene expression and apoptotic cell death upon exposure of cells to chromium (VI) (Bagchi et al. 2002; de Flora, 2000). This therefore, suggests that treatment of waste water prior to discharge is required to avoid these harmful effects and to employ appropriate recovery and/or abatement techniques for residues.

Numerous techniques may be used to treat metal contaminated water, such as electrochemical precipitation, dialysis (Fournier and Meyer, 1975), ion-exchange (Renjaraj et al. 2001), reverse osmosis (Benito and Ruiz, 2002) and ultrafiltration (Yurlova et al. 2002). Fournier and Meyer, (1975) state that the most commonly employed method for treating aqueous solutions containing hexavalent chromium is by chemical reduction to trivalent chromium followed by precipitation of the reduced chromium with alkali (figure 6.13). The reducing agent is usually sulphur dioxide, a derivative such as a sulphite or a bisulfite, or ferrous sulphate. Sodium bisulfite has been employed as an alternate material for the reduction of hexavalent chromium to avoid the hazards and difficulties associated with SO2 (Fournier and Meyer, 1975). A major disadvantage with precipitation is sludge production.

Ion-exchange is deemed to be a better alternative technique for this purpose (figure 6.14). However, owing to its high operational cost, it may not be economically appealing (Babel and Kurniawan, 2004).

Figure 6.13 Simulated process diagram generated using SuperPro Designer® 6.0 presenting the treatment process of 1 L AX purification waste stream containing oligonucleotide related wastes with acidic potassium dichromate at ambient room temperature followed by ion-exchange remediation.
This chemical reagent would require addition treatment steps to remove the chromium (VI) from the waste stream adding to the cost of the process and overall feasibility of using this as a potential treatment process to treat PS oligonucleotide containing waste streams on an industrial scale. The respective advantages and limitations of the application of acidic potassium dichromate are summarised in table 6.8.

Table 6.8 The potential advantages and limitations of the application of acidic potassium dichromate to treat PS oligonucleotide containing waste streams.
Table compiled with information obtained from Fournier and Meyer, 1975; Babel and Kurniawan, 2004; de Flora, 2000; Bagchi et al. 2002; Kawanishi et al. 1986.

<table>
<thead>
<tr>
<th>Advantages</th>
<th>Limitations</th>
</tr>
</thead>
<tbody>
<tr>
<td>• Widely available.</td>
<td>• Chromium is an extremely toxic substance.</td>
</tr>
<tr>
<td>• Fast (60 minutes), effective decomposition (88.3%) of the PS oligonucleotides at relatively low concentrations in simulated AX purification waste stream.</td>
<td>• Generates stoichiometric amounts of heavy-metal waste and Cr (VI), a proven toxin, carcinogen and mutagen.</td>
</tr>
<tr>
<td></td>
<td>• Requirement of additional step for the removal and conversion of Cr (VII) to Cr(III) followed by removal or recycle of Cr(III) prior to discharge to avoid these deleterious effects.</td>
</tr>
<tr>
<td></td>
<td>• More expensive than HCl and H$_2$SO$_4$.</td>
</tr>
</tbody>
</table>

Optimum Treatment 5: Nuclease P1

Figures 6.14 and 6.15 illustrate the simulated SuperPro Designer® diagram for the treatment of PS oligonucleotides via enzymatic means using nuclease P1 from *Penicillium citrinum*. Treatment with nuclease P1 presents as a very effective method for treating oligonucleotide waste streams. In addition to yielding the most significant degradation of 96.9% in the AX waste stream solution, treatment via enzymatic methods is an environmentally friendly removal method. As such, there is no requirement for a post treatment step as the resultant solution comprises of degraded PS oligonucleotides, trace quantities of zinc chloride and benign biodegradable products (figure 6.14).

![Figure 6.14 Simulated process diagram generated using SuperPro Designer® 6.0 presenting the treatment process of 1 L AX purification waste stream containing oligonucleotide related wastes with nuclease P1.](image-url)
One limitation of the application of nuclease P1 is that enzymes are very expensive. Despite this, much research has been carried out on the application of immobilization of nucleases most specifically nuclease P1 with successful results (section 5.3.7) (figure 6.15). This could dramatically reduce the cost limitations of this process as the enzymes could be reused over a period of time. Additional advantages and limitations are discussed in table 6.9.

![Simulated process diagram generated using SuperPro Designer® 6.0 presenting the treatment process of 1 L AX purification waste stream containing oligonucleotide related wastes with immobilized nuclease P1.](image)

**Table 6.9 The potential advantages and limitations of the application of nuclease P1 to treat PS oligonucleotide containing waste streams.**

<table>
<thead>
<tr>
<th>Advantages</th>
<th>Limitations</th>
</tr>
</thead>
<tbody>
<tr>
<td>• Fast (60 minutes), effective decomposition (96.9%) of the PS oligonucleotides in simulated AX purification waste stream.</td>
<td>• Very expensive.</td>
</tr>
<tr>
<td>• Environmentally friendly method and breakdown products</td>
<td>• pH dependant</td>
</tr>
<tr>
<td>• Nuclease P1 (non-sugar specific) works on both DNA and RNA Applicable for treatment of various modification of antisense therapeutics.</td>
<td>• Energy requirement (37 °C)</td>
</tr>
</tbody>
</table>
A summary table detailing the number of process steps required for each of the respective optimum treatment methods and the respective resultant waste products is outlined in table 6.10.

Table 6.10 The number of process steps required for each optimum treatment process and the resultant waste products.

* = Prior to removal/recovery of Cr (VI).

<table>
<thead>
<tr>
<th>Treatment method</th>
<th>Number of process steps</th>
<th>Resultant waste products</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Low acidic pH (1M HCl)</td>
<td>2</td>
<td>5 mM Sodium hydroxide</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.475 M Salt (NaCl)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Water</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Degraded PS oligonucleotide</td>
</tr>
<tr>
<td>2. Low acidic pH (1M H₂SO₄)</td>
<td>2</td>
<td>5 mM Sodium hydroxide</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.225 M Salt (NaCl)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.25 M Salt (Na₂SO₄)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Water</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Degraded PS oligonucleotide</td>
</tr>
<tr>
<td>3. Iodine (50 mM)</td>
<td>1</td>
<td>25 mM Iodine</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10 mM Sodium hydroxide</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.45 M Salt (NaCl)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Degraded partial PS/PO oligonucleotide</td>
</tr>
<tr>
<td>4. Potassium dichromate (0.25M)/Sulfuric acid (0.05M)</td>
<td>Dependent on removal/recovery method</td>
<td>0.125M Potassium dichromate</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.025 M Sulfuric acid*</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10 mM Sodium hydroxide*</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.45 M Salt (NaCl)*</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Degraded PS oligonucleotide*</td>
</tr>
<tr>
<td>5. Nuclease P1</td>
<td>1</td>
<td>50 mM Tris-HCl</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.5 mM zinc chloride</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10 mM Sodium hydroxide</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.45 M Salt (NaCl)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Degraded PS oligonucleotide</td>
</tr>
</tbody>
</table>

An assessment of the environmental impact of a process is an essential part of the design. The costs associated with the treatment and disposal of the waste generated by a particular process solution should be estimated and used as one of the key elements in the final decision on which process to select. Environmentally-unfriendly operations such as those that generate large amounts of hazardous solvents and materials should be avoided as is the case with acidic potassium dichromate. Hexavalent chromium has a very negative health and environmental impact and is extremely toxic (Lenntech B.V, 2011b). Based on the environmental assessment of each of the five optimum treatment methods, treatment with low pH and nuclease P1 present as the most environmentally friendly. Treatment with hydrochloric acid in contrast to sulphuric acid would be preferred as an additional post process step of desalting may not be required as the levels of chloride, in the effluent, are within the limits stipulated by the EPA (EPA, 2007).
6.3.8 Cost implications of the optimum treatment processes.

Successful application of waste management processes in the chemical industry is predominantly governed by cost competitiveness. Table 6.11 details the cost of each of five optimum treatment methods for the treatment of 1 L AX purification waste stream containing oligonucleotide waste of a concentration of 50 µM based on the most competitive quote from four commercial laboratory suppliers (Sigma, Lennox, Fisher Scientific and VWR). The costings are based on lab scale quotations, as opposed to bulk scale. Costings of most chemicals are significantly reduced if ordered in bulk at industrial scale.

Table 6.11 Cost analysis of the five optimum treatment methods.
Information obtained from Sigma, Lennox, Fisher Scientific and VWR, personal communication, 2011.
*To treat 1L of AX waste stream containing PS oligonucleotides 50 µM.

<table>
<thead>
<tr>
<th>Treatment method*</th>
<th>Cost (€)</th>
<th>Company Name</th>
</tr>
</thead>
<tbody>
<tr>
<td>Optimum treatment 1 (HCl at 40°C)</td>
<td>0.22</td>
<td>VWR</td>
</tr>
<tr>
<td>Optimum treatment 2 (H₂SO₄ at 40°C)</td>
<td>0.35</td>
<td>Lennox</td>
</tr>
<tr>
<td>Optimum treatment 3 (iodine solution)</td>
<td>8.87</td>
<td>Sigma-Aldrich Co.</td>
</tr>
<tr>
<td>Optimum treatment 4 (potassium dichromate)</td>
<td>0.97</td>
<td>VWR (Potassium dichromate) and Lennox (Sulfuric acid).</td>
</tr>
<tr>
<td>Optimum treatment 5 (nuclease P1)</td>
<td>10,344.00</td>
<td>Sigma-Aldrich Co. (2009)</td>
</tr>
</tbody>
</table>

It was established that treatment with low acidic pH (hydrochloric acid and sulphuric acid) presented as the most favourable option from a cost point of view. Based on costing obtained (July, 2011) hydrochloric acid presents as the most cost effective at €0.22, in contrast to €0.35 for H₂SO₄, to treat 1 L of AX waste stream containing oligonucleotide waste of a concentration of 50 µM. The next most cost effective method was potassium dichromate (€0.97) and then iodine solution (€8.87). However, the cost of iodine treatment could be dramatically reduced if the source of iodine was bought as solid iodine crystals. Nuclease P1, as predetermined, represented the most expensive treatment method (€10,344). This cost is based upon a speciality enzyme price and if produced on bulk scale by a company such as Novozymes its cost could be dramatically reduced. Bulk scale enzymes, for example proteases, are usually very cost competitive as they are added to price sensitive products such as washing detergents. Moreover, for many bulk enzyme applications, for example, bleaching of cellulose pulp with xylanases, liquefaction of starch with amylases, or use of enzymes in animal feed the cost of the enzymes must be kept low to make their use economical. The cost of extracellularly produced bulk enzyme concentrates is only US $ 10-
As stated previously, biotechnology applications of enzymes are restricted by the cost of their production and stabilisation. The majority of the analytically important nucleases, especially restriction endonucleases, are very expensive (Reddy and Shankar, 1993). However, with the advent of immobilised enzyme technology, attempts are being undertaken to replace conventional enzymatic reactions with immobilised enzymes, as immobilisation presents many advantageous properties such as reuse of enzymes and could potentially reduce the cost implications of such a treatment process (Reddy and Shankar, 1993; Sanchez and Demain, 2011; Shi et al. 2010). Additionally, the application of gene technology now also presents with a more cost effective method of producing commercial enzymes (Sanchez and Demain, 2011).

6.4 Concluding remarks

Currently, due to the increasing presence of biomolecules and compounds in the waste water streams, from pharmaceutical and biopharmaceutical production, the investigation and introduction of new technologies to degrade these molecules and to ensure complete treatment of the effluent, has become imperative. The manufacture of antisense oligonucleotides at industrial scale will carry with it the risk of accidental release into the environment. The release of active antisense products could potentially pose an environmental threat and as a result in house regulations should be established and put in place for the suitable treatment of such active products.

Physical, chemical and enzymatic methodologies were investigated in this study to effectively degrade/remove antisense drugs from waste streams as a ‘safety net’ to prevent and circumvent the accidental release of active antisense product into the environment. Six optimum treatment processes were chosen. An assessment of each process based on experimental analysis, process modelling and environmental and cost evaluation was carried out to determine which treatment process would be most effective, feasible and suitable on an industrial scale. A summary of the results obtained is outlined in table 6.12.
Table 6.12 Summary of the parameters and results obtained to determine the optimum treatment method for waste streams containing PS oligonucleotides

*To treat 1L of AX waste stream containing PS oligonucleotides 50 µM.

<table>
<thead>
<tr>
<th>Method</th>
<th>Maximum Degradation (%)</th>
<th>Desulfurization</th>
<th>Energy required</th>
<th>Cost (€)*</th>
<th>Requirement of additional removal step</th>
<th>Versatility vs. Specificity</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Optimum Treatment 1:</strong> Hydrochloric acid (0.5 M at 40°C) 30 minutes</td>
<td>92.9</td>
<td>No</td>
<td>Yes (40°C)</td>
<td>0.22</td>
<td>Yes (neutralisation)</td>
<td>Versatile</td>
</tr>
<tr>
<td><strong>Optimum Treatment 2:</strong> Sulphuric acid (0.5 M at 40°C) 30 minutes</td>
<td>92.4</td>
<td>No</td>
<td>Yes (40°C)</td>
<td>0.35</td>
<td>Yes (neutralisation)</td>
<td>Versatile</td>
</tr>
<tr>
<td><strong>Optimum Treatment 3:</strong> Iodine (25 mM) 5 minutes</td>
<td>86.7</td>
<td>Yes</td>
<td>No</td>
<td>8.87</td>
<td>No</td>
<td>Specific</td>
</tr>
<tr>
<td><strong>Optimum Treatment 4:</strong> Potassium dichromate (0.125 M K₂Cr₂O₇ in 0.025 M H₂SO₄) 60 minutes</td>
<td>88.3</td>
<td>No</td>
<td>No</td>
<td>0.97</td>
<td>Yes</td>
<td>Specific</td>
</tr>
<tr>
<td><strong>Optimum Treatment 5:</strong> Nuclease P1 (5U/ml) 60 minutes.</td>
<td>96.9</td>
<td>No</td>
<td>Yes (37°C)</td>
<td>10,344</td>
<td>No</td>
<td>Versatile</td>
</tr>
</tbody>
</table>

Exposure of the PS oligonucleotides to five of the six optimum treatment methods (low pH with HCl at 40ºC, low pH with H₂SO₄ at 40ºC, iodine, acidic potassium dichromate and nuclease P1) resulted in almost 100% degradation of the PS oligonucleotides in a simulated waste stream environment. Steam sterilisation was ineffective at degrading the PS oligonucleotides in the AX waste stream solution. Treatment with nuclease P1 was the most effective treatment process in the simulated waste stream solution degrading the PS oligonucleotide by 96.9%. The next most effective treatment method was low pH with HCl at 40ºC, low pH with H₂SO₄ at 40ºC, acidic potassium dichromate and iodine degrading the PS oligonucleotide in simulated waste stream conditions by 92.9, 92.4, 88.3 and 86.7% respectively.

Each removal option has its own advantages and limitations in degrading and removing antisense oligonucleotides. An analysis of the effectiveness of the treatment process and the cost implications were made and weighed against the environmental impacts. Nuclease P1 may present as the most environmentally compatible process of treatment as it does not generate significant quantities of hazardous materials, and thus no special safety
requirements are envisaged as they do not pose any special regulatory concern. Additionally, treatment with low acidic pH followed by a subsequent neutralisation step generates benign waste products of water and their respective salt. It is conclusively evident from these studies that the application of low pH with HCl at 40ºC or nuclease P1 appears to be technically feasible and eco-friendly for the treatment of antisense oligonucleotides. They presented as the most effective treatment methods for degrading PS oligonucleotides in simulated AX purification waste stream, confirmed their suitability for the treatment of additional antisense oligonucleotide modifications and as the most environmentally favourable methods of treatment. In contrast to treatment via enzymatic means, which would be prohibitively expensive at industrial scale, treatment with commercially available acids, which are easily assessable, are more cost effective. An additional advantage of using low acidic pH is the simplicity of the proposed system and the ability to accept a wide variation of effluent concentration.

Treatment with low pH or with nuclease P1 may also be suitable for a range of additional applications in the biotechnology industry. These methods could potentially be used as a treatment method that could remove nucleic acids from biochemical preparations that are used either for pharmaceutical purposes (absence of genetic material) or as reagents in molecular biology. They present as an effective method for removing DNA and RNA in the laboratory and in industrial-scale processes.
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