



Mesoporous silica as a protective matrix for nisin

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Declaration

I declare that this thesis is entirely my own work and has not been previously submitted to this or any other university.

Sarah Mallen

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Contents

Declaration	i
Acknowledgements	ii
Abbreviations	vi
Symbols	viii
Abstract	ix
1. Literature review and project goals	1
1.1 Antibiotic resistance	1
1.2 Antimicrobial peptides	4
1.3 Peptide structure	6
1.4 Peptide stability	9
1.4.1 Physical stability	9
1.4.2 Chemical stability	10
1.5 Peptide analysis	13
1.6 Peptide drug delivery	15
1.6.1 Parenteral route	16
1.6.2 Oral route	20
1.6.3 Transdermal route	25
1.6.4 Alternative routes	28
1.7 Commercially available peptide drug formulations	29
1.8 Conclusions	29
1.9 Project motivation and aim	30
2. Nisin	33
2.1 Introduction	33
2.1.1 History	34
2.1.2 Structure and characterisation of nisin	35
2.1.3 Properties, current uses and limitations of nisin	37

2.1.4	Previous delivery strategies for nisin	40
2.1.5	Prospective strategies for nisin delivery	43
2.2	Experimental	45
2.2.1	Materials.....	45
2.2.2	Nisin AP composition	45
2.2.3	Activity assays	46
2.2.4	Stability testing	46
2.2.5	Estimation of molecular dimensions.....	48
2.2.6	Statistical Analysis	49
2.3	Results and discussion.....	50
2.3.1	Nisin AP composition	50
2.3.2	Activity assays	51
2.3.3	Stability testing	53
2.3.4	Estimation of molecular dimensions.....	60
2.4	Conclusions	61
3.	Mesoporous silica	63
3.1	Introduction	63
3.1.1	History.....	63
3.1.2	Synthesis and structure of MPS	64
3.1.3	Application of MPS in drug delivery	66
3.1.4	Potential use of MPS as drug delivery system for nisin.....	72
3.2	Experimental	74
3.2.1	Materials.....	74
3.2.2	MPS synthesis and characterisation	74
3.2.3	Nisin-loaded MPS	77
3.2.4	Release study.....	78
3.2.5	Proteinase K digestion study.....	79

3.3	Results	80
3.3.1	MPS synthesis and characterisation	80
3.3.2	Nisin-loaded MPS	89
3.3.3	Release study.....	95
3.3.4	Proteinase K digestion study	97
3.4	Conclusions	98
4.	Overall conclusions and future work	100
5.	References.....	102
6.	Appendix.....	139

Abbreviations

ACN	Acetonitrile
AMP	Antimicrobial peptide
AMR	Antimicrobial resistance
ATR-FTIR spectroscopy	Attenuated total reflectance-Fourier transform infrared spectroscopy
BAPNA	N _α -Benzoyl-L-arginine 4-nitroanilide
BSA	Bovine serum albumin
CFU	Colony forming units
DI	Deionised
DLS	Dynamic light scattering
EDTA	Ethylenediaminetetraacetic acid
Eq.	Equation
FDA	Food and drug administration
GI	Gastrointestinal
GRAS	Generally regarded as safe
IBS	Irritable bowel syndrome
IPA	Isopropanol
LAB	Lactic acid bacteria
LHRH	Luteinising hormone-releasing hormone
MALDI-TOF MS	Matrix-assisted laser desorption/ionisation time of flight mass spectrometry
MPS	Mesoporous silica
MRSA	Methicillin-resistant <i>Staphylococcus aureus</i>

NMR	Nuclear magnetic resonance
NOE	Nuclear Overhauser effect
PBS	Phosphate buffered saline
PLGA	Poly(lactic-co-glycolic acid)
P-XRD	Powder X-ray diffraction
R&D	Research and development
RP-HPLC	Reverse phase-high performance liquid chromatography
SDS-PAGE	Sodium dodecyl sulphate-polyagarose gel electrophoresis
SEM	Scanning electron microscopy
SGF	Simulated gastric fluid
SIF	Simulated intestinal fluid
TEM	Transmission electron microscopy
TEOS	Tetraethyl orthosilicate
TFA	Trifluoroacetic acid
TGA	Thermogravimetric analysis
VRE	Vancomycin-resistant <i>Enterococci</i>
WHO	World Health Organisation

Symbols

Å	Angstrom
Da	Dalton
ϵ	Extinction coefficient
I	Ionic strength
M_w	Molecular weight
t_r	Retention time

Abstract

Currently, the treatment of certain antimicrobial infections is hindered by the increase in resistance acquired by pathogenic species of bacteria, fungi and viruses. Thus, there is a growing need for novel antimicrobial agents which are active against resistant bacterial strains. Bacteriocins are antimicrobial peptides, some of which have shown activity against resistant strains of bacteria, e.g. MRSA. However, as peptides, they are subject to proteolytic degradation *in vivo* following administration, particularly by the oral route due to the abundance of proteases in the GI tract. Therefore, suitable protective matrices are required to prevent the degradation of bacteriocins *in vivo* and allow their antimicrobial properties to be exploited. In this work, mesoporous silica matrices with different pore size and functionality were prepared and tested for their suitability in protecting the bacteriocin nisin from protease degradation *in vitro*. Reducing the pore size of these matrices appeared to improve the protection of nisin A from digestion by the broad-spectrum protease, proteinase K. MCM-41 (pore size 28 Å, unfunctionalised) improved protection of nisin A over SBA-15 (pore size 68 Å, unfunctionalised). Increasing the hydrophobicity of silica by functionalisation also improved the protection of nisin, with MSE (pore size 60 Å, ethylene-functionalised) showing improved protection of nisin against proteinase K compared to SBA-15. Under simulated gastric conditions, a burst release was observed from unfunctionalised MCM-41 and SBA-15, which present silanol groups at the surface. The ethylene-functionalised, hydrophobic matrix, MSE, provided a more gradual, extended release of nisin showing the impact of functionalisation on the release profile of nisin from the matrices. Under simulated intestinal conditions, release from MCM-41 and MSE was not detected, while SBA-15 only released 12% of the adsorbed nisin. Although the release of nisin from MCM-41 and MSE was not detected into simulated intestinal fluid, the release media, and the suspended particles, were still active against the indicator strain, *Lactobacillus delbrueckii* subsp. *bulgaricus*. According to these results, MCM-41 may prove to be a successful matrix in partially protecting nisin, and potentially other bacteriocins, following oral administration for infections of the colon, e.g. *Clostridium difficile*. However, an enteric coating would need to be applied, to prevent premature release in the gastric environment. The slow release provided by the ethylene-functionalised MSE may be beneficial for subcutaneous implants of

therapeutic peptides to prolong their effect and reduce the need for frequent, painful injections

1. Literature review and project goals

1.1 Antibiotic resistance

When an antibiotic drug substance does not effectively inhibit a bacterial strain, that strain is said to be resistant. The threat of antimicrobial resistance (AMR) was recognised by Alexander Fleming when he accepted his Nobel Prize in 1945 *“for the discovery of penicillin and its curative effect in various infectious diseases.”* In his speech, Fleming stated that:

“The time may come when penicillin can be bought by anyone in the shops. Then there is the danger that the ignorant man may easily under dose himself, and by exposing his microbes to non-lethal quantities of the drug, make them resistant.”

In September 2016, the UN General Assembly met to discuss this threat, which is now a global health issue of great concern.[1] As predicted by Fleming over 70 years ago, the predominant cause of AMR is the overuse or misuse of antibiotics by *“the ignorant man”*, exposing pathogenic bacteria to sub-inhibitory levels of antibiotics and inducing resistance. The rise in AMR has also been attributed to the prevalence of antibiotic use in agriculture and household products.[2] This prevalence of resistance in strains such as *Streptococcus pneumoniae* and *Mycobacterium tuberculosis*, methicillin-resistant *Staphylococcus aureus* (MRSA) and multidrug resistant Gram-negative bacteria has resulted in the reduced effectiveness or ineffectiveness of certain antibiotics which can be life-threatening, particularly in the case of nosocomial infections where the patient’s immune system is compromised.[3]

The resistance of bacteria to antibiotics can be intrinsic or acquired. Intrinsic resistance occurs when the bacterial species is not naturally susceptible to the antibiotic based on its inherited features. For example, Gram-negative bacteria are intrinsically resistant to the antibiotic vancomycin which disrupts cell wall biosynthesis, since their cell wall is protected by an outer membrane.[4] Intrinsic resistance can be avoided by treating bacterial infections with appropriate antibiotics. In cases where Gram-negative bacterial infections are diagnosed or suspected, the use of antibiotics which have alternative targets to cell wall biosynthesis can be used to overcome intrinsic resistance, e.g. using fluoroquinolones which target DNA

replication.[5] The major problem associated with antibiotic resistance is the resistance which is acquired by bacteria through horizontal gene transfer and/or chromosomal DNA mutation. Horizontal gene transfer involves the transfer of resistant genes among bacteria by the absorption of naked DNA or by the transfer of genes via plasmids, bacteriophages or transposons.[6]–[9] While resistant genes in bacteria generally code for mechanisms resulting in resistance against a specific class of antibiotic, bacterial species can also develop resistance to more than one kind of antibiotic. This leads to what is known as multi-drug resistance (MDR).[10] Acquired resistance reduces the effectiveness of antibiotics towards bacterial strains, which previously showed sensitivity, and can reduce the effectiveness or result in the failure of antibiotic treatments. An example of this is the resistance acquired by the Gram-negative genus, *Enterococci*, towards fluoroquinolone through mutation of this antibiotic's target - the ribosome.[11] Besides modification of the drug target, other resistance mechanisms acquired by bacteria include drug efflux pumps, the ability to inactivate the drug (e.g. by enzymatic degradation) and the formation of biofilms.[12]

Both preventative and therapeutic approaches are being worked towards in an attempt to control AMR. As part of the preventative approach in tackling AMR, guidelines have been issued. These guidelines include the surveillance of AMR, isolation of patients with hospital-acquired resistant infections and the reduction of antibiotic use in agriculture.[3] Increasing awareness of AMR among the general public is also hugely important in tackling this problem and different initiatives are underway to educate “*the ignorant man*”. These initiatives include Antibiotic Guardian (www.antibioticguardian.com), e-Bug (www.e-bug.eu), Under the Weather (www.undertheweather.ie) and European Antibiotic Awareness Day, run by the European Centre for Disease Control (www.ecdc.europa.eu/en/EAAD). These initiatives highlight the importance of taking antibiotics only when necessary and as prescribed by a healthcare professional. Improvement in sanitation and public health worldwide, e.g. by providing access to clean water and vaccination against bacterial diseases, is also being worked towards to improve the quality of life and help reduce the prevalence of AMR.[13]

From a therapeutic perspective, research and development (R&D) is being carried out to uncover new antimicrobial agents and to exploit the antimicrobial

agents currently available by modification, formulation or combination.[14] At present, the rate of novel antibiotics reaching the market is constantly being surpassed by the rate at which certain bacteria are developing resistance.[15] Only 6 of the 204 drugs approved by the FDA over the last 5 years were antibiotics. Fidaxomicin (Difcid) developed by Optamer Pharmaceuticals was approved for the treatment of *Clostridium difficile* infections in 2011.[16] Four antibiotics were approved in 2014 - dalbavancin (Dalvance), tedizolid phosphate (Sivextro), oritavancin (Orbactiv) and a combination of ceftolozane and tazobactam (Zerbaxa).[17] The sixth of these antibiotics is a combination of ceftazidime and avibactam, launched as Avycaz in 2015.[18] Avycaz is used against Gram-positive bacteria which are resistant to macrolide antibiotics.

On a positive note, research into novel antibiotics is currently on the rise – mainly thanks to academia and small biotech companies.[19] Most antibiotics in the pipeline have reduced toxicity in comparison to current antibiotic treatments or enhanced activity against bacterial infections resistant to current treatments.[14] These novel substances include modified versions of existing antibiotics along with new types of antibiotics. Modified cephalosporins (e.g. CXA-201), macrolides (e.g. EDP-420), vancomycin (e.g. televancin), tetracyclines, oxazolidonones (e.g. razidolid, torezolid), fluoroquinolones (e.g. delafloxacin), aminoglycosides (e.g. ACHN-490, SPX 1212) and streptogramins (e.g. NXL-103) have all been synthesised with improved antimicrobial properties.[14], [20]–[22] CEM-101, a modified fluoroketolide, has shown reduced toxicity compared with telithromycin and oritavancin and is currently being developed by Cempira for the treatment of macrolide-resistant Gram-positive bacterial infections.[23] Some of the new types of antibiotics include nanoemulsions (e.g. NB-002) and porphyrin derivatives (e.g. XF-73) which target cell membranes, glycosylated macrolactams (e.g. novolactamycin) and enzymes which lyse staphylococci (e.g. lysostaphin).[14], [24]–[26] The pleuromutilins, previously only used in veterinary medicine, have also been modified to form BC-3781 which tested successfully in phase II clinical trials in humans in 2011.[27] While most of the emerging therapies show selective inhibition of Gram-positive bacteria, some of the treatments in the pipeline have also shown activity against Gram-negative bacteria. Examples include CXA-201, delafloxacin, ACHN-490, SPX 1212, D-16, L19-45 and BAL 30072.[14], [20], [28]–[30]

The pharmaceutical industry has also begun to recognise the clinical advantages of the so-called probiotics (i.e. commensal bacterial strains showing benefits to the health of the host) and the effect of the gut microbiota composition on human health.[31] Previously viewed as an “alternative” medicine, probiotics have since proven to be successful in the treatment of mastitis, in an animal model, and irritable bowel syndrome (IBS) and colon cancer, in human subjects.[32], [33] It was found that the presence of probiotics, including species from the *Lactobacillus* and *Bifidobacterium* genera, reduced the risk of colon cancer, while the presence of species from the *Clostridium* and *Bacteriodes* genera increased the risk.[34] Part of the therapeutic effect provided by probiotics has been attributed to peptides produced by these commensal strains, some of which have shown antimicrobial activity against pathogenic strains of bacteria.[35] These antimicrobial peptides, along with their benefits and limitations in treating resistant bacterial infections *in vivo*, are the topic of the following section.

1.2 Antimicrobial peptides

Peptides have proven to be highly efficacious drug substances with good selectivity for therapeutic targets. They have shown improved selectivity compared to small molecule drugs and lower production costs and less complications than biologics. To quote Fosgerau *et al*, peptides are “*the sweet spot between small molecules and biopharmaceuticals*”.[36] Peptide drugs are currently available for the treatment of multiple sclerosis (Copaxone®), prostate cancer (Lupron®), type-2 diabetes (Bydureon® and Victoza®) and osteoporosis (Forteo®) among many other diseases.[37]

In nature, peptides with antimicrobial properties are produced by the innate immune system of virtually all organisms from plants to invertebrates and mammals to simpler organisms, such as bacteria.[38]–[40] In multicellular organisms, these antimicrobial peptides (AMPs) act in both the innate immune response and the adaptive immune response by stimulating monocytes and T-cells.[41] In bacteria, these AMPs are produced to inhibit competing microbial species.[42] As of July 2016, the discovery of 2720 naturally occurring AMPs has been reported in the AMP Database (APD).[43] Apart from antimicrobial activity, some of these AMPs have also demonstrated anticancer, insecticidal, spermicidal, antioxidant and chemotactic effects. [44]–[48] AMPs are generally, cationic, amphiphilic peptides composed of

less than 100 amino acid residues.[49] The discovery of the AMP, teixobactin, was reported by Ling *et al* in 2015.[50] Teixobactin drew great attention as it could be incubated at sub-inhibitory concentrations with *Staph. aureus* for over 27 days without triggering resistance. In July 2016, Peschel *et al* published their discovery of another AMP - lugdunin. Lugdunin is produced by bacteria in the human nasal passage and demonstrated activity against both MRSA and VRE.[51] Currently pexiganan (Locilex®), a synthetic analogue of the antimicrobial peptide magainin found in amphibians, is in phase III clinical trials for wound healing in diabetic foot and other topical infections.[52] Pexiganan along with melittin have also shown anticancer properties.[53]

AMPs produced by bacteria to inhibit competing strains, known as bacteriocins, were first discovered in 1925.[54] Of the 2720 AMPs discovered to date, roughly 10% are bacteriocins.[43] Bacteriocins are ribosomally-synthesised, heat-stable peptides produced by both Gram-negative and Gram-positive bacteria.[42] However, the bacteriocins of Gram-positive lactic acid bacteria (LAB) are the only bacteriocins that have been used commercially to date as effective food preservatives.[55] While many classification systems exist for the bacteriocins of LAB, Cotter *et al* recently established a system with two major classes – those which contain lanthionine rings (lantibiotics) and those which do not.[42] Lantibiotics are bacteriocins which are post-translationally modified to include dehydrated amino acids, some of which bond to the thiol groups of cysteine residues to form lanthionine or methyl-lanthionine rings.[56] Lanthionines are classed on the basis of their structure and the enzymes involved in their post-translational modification.[57] Type-I lantibiotics (e.g. nisin A, mutacin B and subtilin) are long, flexible peptides modified by the enzymes LanB and LanC. LanB is a dehydratase, which performs the dehydration of amino acid residues while LanC, a cyclase, forms the (methyl-)lanthionine rings. Type-II lantibiotics (e.g. cinnamycin, mersacidin and actagardine) are globular, rigid peptides modified by one enzyme, LanM, which performs both the dehydration and cyclisation functions.[58] Lantibiotics exert their antibacterial activity by binding to the peptidoglycan precursor, lipid II, in the bacterial cell membrane.[59] This is the same target for glycopeptide antibiotics, such as vancomycin. However, the site on lipid II which lantibiotics bind to differs from that of glycopeptides and, therefore, vancomycin-resistant pathogens, e.g. VRE, have

shown susceptibility to lantibiotics, including microbisporicin and actagardine.[60] Apart from their antimicrobial activity against resistant strains, some lantibiotics have shown other clinical advantages, e.g. antihypertension (ancovenin), antiviral (lanthiopeptin against *Herpes simplex*), immunosuppression (mersacidin), spermicidal and anticancer (nisin).[46], [61], [62] The lantibiotics, mutacin 1140 and microbisporicin, have made it as far as late pre-clinical trials.[60], [63] Another lantibiotic, NVB302, has completed phase I clinical trials for the treatment of *Clostridium difficile* infections.[64] Lancovutide (Lantibio Inc.) has been the only bacteriocin to date to make it as far as phase II clinical trials. This bacteriocin holds promise as a new and exciting intranasal airway-rehydrating agent for the treatment of cystic fibrosis.[65]

Along with AMPs of natural origin, peptides with antimicrobial properties have also been developed by the synthetic route. These include α -helical peptides (e.g. V13K), peptides that mimic protegrin I and the synthesis of STAMPs (specifically targeted AMPs).[29], [66], [67] The improvement of solution and solid-phase synthesis of peptides using protective groups, such as Fmoc, has enabled the efficient synthesis of libraries of peptides to test for antimicrobial activity.[68]

With the rise in antibiotic resistance among pathogenic bacteria, novel antimicrobial agents are required to meet the growing need for effective drug treatments.[3] AMPs, including bacteriocins, with activity against resistant strains of bacteria have shown great potential as the next generation of effective antibiotics.[49], [63] However, the exploitation of these AMPs to date has been hindered by their susceptibility to protease degradation, rapid renal clearance and poor oral bioavailability.[69] Therefore, the modification of these AMPs or the development of suitable delivery devices is necessary to increase their stability, on the shelf and *in vivo*, and to allow these invaluable antimicrobial agents to act successfully as novel antibiotic treatments.

1.3 Peptide structure

Peptides are sequences of amino acids linked together through amide (or “peptide”) bonds. Peptides are loosely defined as poly(amino acids) with a molecular mass of less than 5 kDa, poly(amino acids) with a higher molecular mass than this

tend to be referred to as proteins. There are 20 naturally occurring amino acids (see Appendix, Table 13).

The sequence of amino acids or “residues” in a peptide is known as the primary structure. This is generally written using single letter abbreviations which begin at the amino end, the “N-terminal”, and finish at the carboxyl end, the “C-terminal”. The individual amino acids range from hydrophilic and polar (C, S, T, Y) to hydrophobic and non-polar (F, W) and from negatively charged (D, E) to positively charged (K, H, R) to uncharged (L, I, V). Therefore, it is not surprising that there is such a diverse range in the physicochemical properties and physiological effects of peptides. The substitution of even a single amino acid residue in the sequence can result in dramatic changes in the properties of a protein or peptide. For example, the protein β -haemoglobin in the red blood cells of patients with sickle cell anaemia differs from normal haemoglobin by the replacement of a glutamine residue with valine. This single point mutation results in the aggregation of haemoglobin which leads to deformation of the red blood cells to a “sickle” shape, preventing their entry into the capillaries.[70]

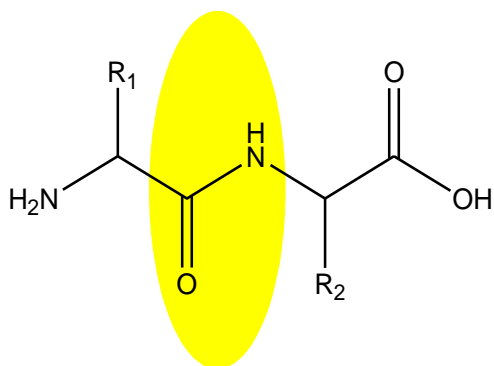


Figure 1-1. Generic dipeptide molecule (amide or "peptide" bond highlighted in yellow).

Secondary structure is not commonly found in peptides with less than 20 residues. However, secondary structure can exist in peptides with close to 50 residues which border on being proteins, e.g. insulin.[71] The secondary structure of a peptide is made up of α -helices, β -sheets, β -turns, small loops and random coils. Alpha helices are coil-like conformations (Figure 1-2). They form through a cascade of hydrogen bonding between “C=O” oxygens to “N-H” hydrogens four residues apart on the main chain of the peptide. Proline is the only amino acid that cannot hydrogen bond in this way, since the amino group is covalently bound to the side

chain. In β -sheets, hydrogen bonding occurs between the “C=O” oxygens of one segment of the peptide backbone to “N-H” hydrogens along another adjacent segment (Figure 1-2). For parallel β -sheets, hydrogen bonding occurs between segments of the peptide chain which align in the same direction. For antiparallel β -sheets, the segments of the chain align in opposite directions. Antiparallel β -sheets tend to be connected by β -turns, i.e. sharp bends which reverse the direction of the peptide chain.[72]

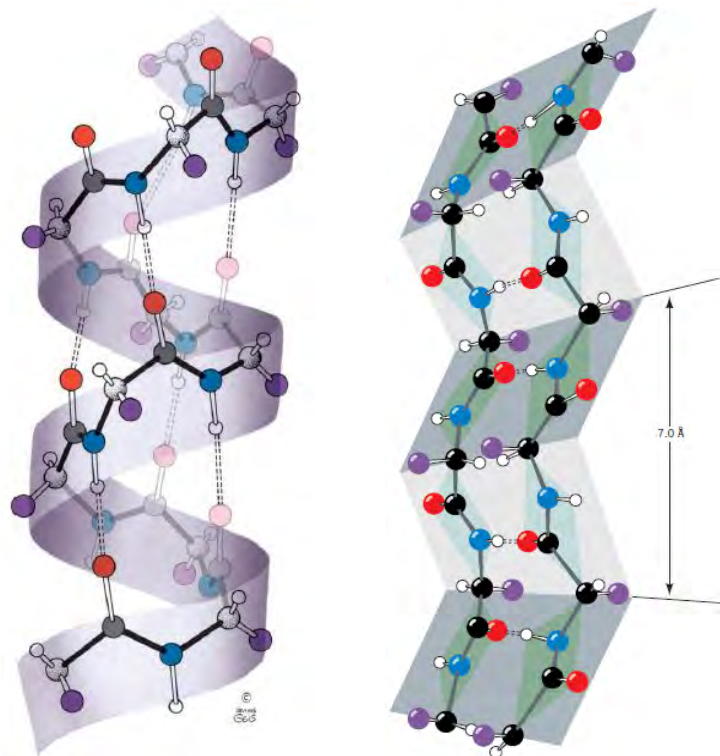


Figure 1-2. Secondary protein structure: alpha helices (left) and beta sheets (right).[73]

For proteins, further 3D folding of the chain tends to occur, stabilised by covalent (e.g. disulphide bridges) and non-covalent bonding (e.g. hydrophobic interactions, salt bridges, hydrogen bonding). This is known as the tertiary structure. The folding of the protein is guided by molecular chaperones and enzymes which facilitate disulphide bridge formation. This generally leads to dense, spherical structures with hydrophilic groups on the surface and hydrophobic groups within the sphere. The stabilisation of the protein structure by covalent and non-covalent bonds means that proteins tend to only be present in one 3D conformation. However, peptides (< 5 kDa) may have multiple conformations since they lack tertiary structure.[74]

1.4 Peptide stability

While peptide drugs show great promise in tackling a wide range of diseases, their physical, chemical and biological instability can prevent their transition from the research lab to the clinic. Physical instability of peptides can occur due to aggregation, precipitation and adsorption. Chemical instability occurs due to the formation or cleavage of covalent bonds in the peptide.[75] While chemical and physical instability tend to be discussed separately, and are split into two sections here, the two tend to be interconnected. For example, chemical degradation of peptides by deamidation tends to lead to a higher chance of peptide aggregation.[76] Ideally, peptide drug formulations should be stable for a minimum of 1.5 to 2 years.[77] Any degradation products formed must also undergo toxicity testing to ensure they do not cause adverse effects *in vivo*.

1.4.1 Physical stability

One of the main causes of physical instability in peptides is aggregation. Aggregation and its macroscopic form, precipitation, are caused by the self-association of peptides due to covalent bonding, non-covalent bonding or a combination of both.[78] Aggregation of peptides can occur due to intrinsic factors or extrinsic factors. Extrinsic factors include temperature, pH and ionic strength of the surrounding environment. Intrinsic factors are generally structural features which make the peptide more susceptible to aggregation. [70], [79]

Fibrillation is another form of aggregation that can result in peptide instability. This is when the peptide aggregates to form long fibre-like structures, known as fibrils. This form of aggregation was detected in glucagon, the 29-residue peptide responsible for glucose upregulation, when this peptide was being developed for clinical use in the treatment of diabetes.[80] To prevent aggregation, “glucagon-like” peptides (GLPs) with altered amino acid composition to increase stability have been developed for clinical use.[81] Fibrillation is also seen in Alzheimer’s disease, when fibrils (or plaques) of the protein β -amyloid are formed.[82] The formation of these β -amyloid plaques is thought to be due to an age-related decrease in the levels of protein chaperones which would normally prevent aggregation of this protein.[73]

The concentration of peptides in solution can also be a factor influencing their physical stability. High concentrations of hydrophobic peptides in aqueous solution

can form threadlike, peptide liquid crystals (PLCs) and can lead to the formation of gels. PLC formation has been observed for the LHRH (luteinising hormone-releasing hormone) analogues, detirelix and nafarelin, at concentrations above 4 and 8 mg/mL respectively. PLC formation can be prevented by using FDA-approved organic co-solvents, such as propylene glycol.[83], [84]

Physical instability due to the adsorption of peptides to surfaces can also occur. This depends largely on the primary sequence of the peptide, with hydrophobic peptides being more likely to adsorb strongly to glass and other hydrophobic surfaces. The pH and ionic strength of the solution also play a significant role in adsorption.[85], [86] Adsorption tends to be irreversible with simple washing being ineffective for removing the peptide from the surface. Albumins, e.g. human serum albumin (HSA) and bovine serum albumin (BSA), have been used to prevent adsorption of peptides onto surfaces. The albumin acts as unreactive blocking agent, preventing non-specific adsorption of proteins and peptides to surfaces.[87] However, contamination of serum albumin with blood-borne pathogens can occur and hence the use of albumin tends to be avoided.[88]

It is vital that the physical behaviour of the peptide is known if it is to be administered parenterally since aggregation can lead to an immunogenic response and the adsorption of peptides to surfaces can result in inconsistent dosing.[89] To detect peptide aggregation, dynamic light scattering (DLS), fluorescent microscopy, size exclusion-HPLC (SE-HPLC), SDS-PAGE, analytical ultracentrifugation and microflow imaging can be used.

1.4.2 Chemical stability

Chemical instability occurs when peptides are covalently modified. This can occur by various mechanisms including hydrolysis, deamidation, oxidation, β -elimination and racemization. These reactions can occur due to light exposure, the presence of moisture or oxygen, acidity or basicity of the environment or the presence of reactive species, e.g. peroxides.

Chemical hydrolysis of peptide bonds is rare due to the formation of an unstable intermediate during the reaction. However, the presence of enzymes or extremely high concentrations of acid or base can lower the activation energy of hydrolysis enabling cleavage of the peptide bond. Peptide bonds containing aspartic acid

residues have the highest susceptibility to hydrolysis, e.g. Asp-Gln, Asp-Pro, due to the formation of a more stable cyclic imide intermediate.[90]

Another hydrolysis reaction that can occur is deamidation of the amino acids, asparagine and glutamine.[91] Deamidation is the most common chemical degradation mechanism for peptides.[75] In the deamidation reaction, the asparagine or glutamine residue is hydrolysed to form aspartic acid and glutamic acid respectively. This reaction can be acid-catalysed or base-catalysed. Acid-catalysed deamidation tends to occur below pH 4, with the base-catalysed cyclic imide reaction usually occurring over pH 6. Glutamine is far less susceptible to deamidation. This decreased susceptibility has been attributed to the six-membered cyclic imide formed during base-catalysed deamidation of glutamine which is substantially less stable than the five-membered cyclic imide formed for asparagine during this reaction.[75] The amino acid sequence also appears to influence deamidation. Asparagine residues framed by amino acids with small side-chains or sequences where asparagine is followed by hydrogen bond-donating amino acids show a higher rate of deamidation.[92] Deamidation can lead to a decrease in the half-life of the peptide, reduced bioactivity, aggregation and/or increased immunogenicity. Therefore, it is important to avoid deamidation where possible. An example of a peptide pharmaceutical which undergoes deamidation is the nonapeptide oxytocin, used clinically to induce labour. This peptide undergoes deamidation below pH 2 and above pH 9 and is therefore formulated at pH 4.5.[74] Isoelectric focusing, ion exchange chromatography or RP-HPLC can be used to determine if deamidation has taken place. Altering the pH to between 3 and 6, as with oxytocin, is the main approach that has been taken to preventing the deamidation in peptide pharmaceutical formulations.

Chemical instability of peptides can also occur due to oxidation. Methionine, cysteine, histidine, tryptophan, tyrosine and phenylalanine residues have shown increased susceptibility to oxidation in comparison to other amino acids. Traces of metals, reactive oxygen species (ROS, e.g. peroxides) and light can lead to the oxidation of these amino acids.[93], [94] Atmospheric oxygen can be sufficient to oxidise methionine, yielding methionine sulfoxide.[75] Under more extreme conditions, methionine oxidises to methionine sulfone.[95] Cysteine is also easily oxidised, forming intra- or inter-chain disulphide bridges. Inter-chain disulphide

bridges can cause aggregation which can affect the immunogenicity of the peptide.[96] The aromatic amino acids tryptophan, tyrosine and phenylalanine are susceptible to photo-oxidation as they absorb light around 300 nm.[97] Oxidation of peptides can be detected by RP-HPLC, peptide analysis, fluorescence or HPLC in combination with MS. RP-HPLC can also be used to separate oxidised variants.

β -elimination and racemisation can also compromise the chemical stability of peptides. Both of these mechanisms require deprotonation of the α -carbon, a very slow process which usually requires high temperatures. For β -elimination, the proton on the α -carbon leaves and the carbanion rearranges so that a group on the β -carbon is ejected and a double bond forms. This reaction requires high temperatures and a pH of 4 to 8. An example of this is the β -elimination which occurs at the cysteine residues of lyophilised bovine insulin in the presence of water at 50 °C. Under these conditions, it is hypothesised that water acts as the nucleophile, reacting with cysteine to form dehydroalanine and thiocysteine residues. Cross-links can form between dehydroalanine and lysine residues which may result in aggregation or loss in activity due to structural changes.[98] β -elimination can be avoided to some extent by formulating and storing peptides containing cysteine residues under nitrogen.[77]

Base-catalysed racemisation of the L-enantiomers of amino acids to the D-enantiomers can also occur. While the D-amino acids have the advantage of being less susceptible to enzyme degradation, they may not show the same *in vivo* effect. Racemisation occurs most rapidly for L-aspartic acid and L-asparagine. However, it is not yet known why this preferential racemisation occurs.[75] This racemisation can be seen as a result of aging in certain proteins in the human body, particularly myelin basic protein in the brain and dentin in the teeth, and has been used in age estimation in forensic science .[99], [100]

In the presence of reducing sugars, glycation of peptides (a.k.a. the Maillard reaction) can occur. This requires a basic residue, such as lysine, which attacks the carbonyl of the reducing sugar to form a Schiff base which can rearrange to form stable products. Although this reaction usually requires high temperatures and acidic pH to occur, reducing sugars (e.g. lactose and glucose) tend to be avoided when formulating peptides.[101]

The N-terminal of peptides can act as a nucleophile, especially above pH 8. The N-terminal can react with the second carbonyl in the peptide chain to form what is known as a diketopiperazine ring. This reaction is most common for the sequence H₂N-Gly-Pro.[75] The N-terminal can also attack the carbonyl of a glutamic acid residue to form a five-membered ring, pyro-glutamate (pGlu).[102]

1.5 Peptide analysis

When working with therapeutic peptides, it is important to be able to identify the peptide, determine the concentration present and to detect the presence of aggregation or degradation. This can be carried out in a number of ways.

To determine the amino acid composition of a peptide, amino acid analysis is carried out. This involves subjecting the peptide to intensive acid hydrolysis to cleave the peptide bonds, followed by separation of the amino acids on an ion-exchange column and identification on the basis of their retention time.[103] However, acid hydrolysis can pose as a threat to the stability of certain amino acids. For example, asparagine and glutamine are hydrolysed to aspartic acid and glutamic acid respectively. Therefore it is the sum of the amide and carboxylic acid which is usually measured, i.e. asparagine and aspartic acid. Serine and threonine can also be affected by hydrolysis over extended time periods. Reducing the acid hydrolysis time to less than 24 h can help to prevent this.[104] However, for analysing valine and isoleucine, extended hydrolysis time to around 72 h is required.[105] Phenol is generally added when analysing the tyrosine content, to avoid halogenation of this amino acid by the chloride ions in the hydrochloric acid solution.[106] Tryptophan usually requires alkali hydrolysis, as this amino acid is unstable in the presence of acid.[107] Due to their susceptibility to oxidation, quantifying cysteine and methionine residues present requires pre-treatment with performic acid. This intentionally oxidises cysteine and methionine to cysteic acid and methionine sulfone respectively, which can then be quantified.[108] However, the use of performic acid destroys tyrosine and tryptophan.

While amino acid analysis provides information on the individual residues present in the peptide, it does not uncover the peptide sequence. However, the peptide sequence can be determined using Edman degradation. This method reacts phenylisothiocyanate with the peptide under acidic conditions to cleave the N-

terminal residue, forming a phenylthiohydantion with the terminal residue. This can be analysed to determine the identity of the N-terminal residue. Subsequent reaction allows the peptide to be sequenced from the N-terminal to the C-terminal.[109] Electrospray ionisation mass spectrometry (ESI-MS) and X-ray crystallography can also be used to sequence peptides, however, the latter requires crystallisation of the peptide.[110]

Analysis by NMR spectroscopy can detect modified amino acids, such as dehydroalanine and dehydrobutyrine. [111] NOE (nuclear Overhauser effect) correlations can also be used to detect connectivity within the peptide, e.g. for the lanthionine rings of the lantibiotics. 3D-NMR analysis can also provide information on the secondary structure of peptides, including β -sheets and α -helices.[112] For peptides with more than 10 amino acid residues, a magnet of 500 MHz or more is required for peak resolution.[113] However, highly concentrated solutions are required for NMR analysis. This is not always possible, due to the low quantities of peptides produced from natural sources and the mostly limited solubility of peptides. Another method that can be used to determine the secondary structure and orientation of peptides is circular dichroism (CD). Circular dichroism determines the secondary structure of the peptide based on the differences in the absorption of left-handed and right-handed circularly polarised light.[114] This can be used separately or alongside NMR analysis to determine peptide conformations.

Reverse-phase high performance liquid chromatography (RP-HPLC) is used to separate and quantify peptides. Separation is based on the peptide's affinity towards a non-polar stationary phase, usually alkyl chains containing 4, 8 or 18 carbons (C_4 , C_8 , C_{18}) bound to silica.[115] The mobile phase used starts out using a polar solvent moving towards less polar solvents as the run progresses. In general, a gradient of water to acetonitrile is used. 0.1% TFA is added for the analysis of peptides as an ionic modifier to prevent strong adsorption of peptides to the column and improve peak resolution.[116] RP-HPLC is commonly coupled with UV-vis spectrophotometry, fluorescent spectroscopy or mass spectroscopy for detection. Peptides containing the aromatic residues, phenylalanine, tyrosine and tryptophan, absorb UV light at 280 nm. In the absence of aromatic residues, the peptide bond, which absorbs at 214 nm, can be used for the UV-vis detection.[117] Fluorescent spectroscopy detects aromatic amino acids, with tryptophan exhibiting the highest

level of fluorescence followed by tyrosine and phenylalanine. For mass spectrometry detection, MALDI-TOF MS (matrix-assisted laser desorption ionisation time-of-flight mass spectrometry) is the most commonly used method for determining the mass of peptides as it uses a “soft ionisation” technique resulting little or no fragmentation of the peptide.[118] The combination of RP-HPLC with MALDI-TOF MS is also commonly used to detect degradation of peptides.

When formulating peptide drugs, aggregation must be avoided. Some of the methods used to detect the formation of peptide aggregates are electrophoresis, size exclusion chromatography (SEC), dynamic light scattering (DLS), analytical ultracentrifugation (AUC) and field-flow fractionation (FFF). Gel electrophoresis by SDS-PAGE is used to qualitatively measure peptide aggregation. For peptides of less than 30 kDa, a Tricine-Tris buffer system and lower acrylamide concentrations are used.[119] For analysing peptide aggregation quantitatively, SEC analysis is generally used which separates peptide monomers from aggregates on the basis of size using a porous gel matrix. Elution from the column occurs in order of size with the larger aggregates eluting first.[109] However, non-specific binding of aggregates to the SEC column can occur.[120] Therefore, the combination of SEC with methods such as DLS, AUC or FFF tends to be required when analysing the aggregation of therapeutic peptides.[121]

These analytical methods are among many which allow for the separation, identification, quantification and degradation studies of peptides. They are of utmost importance in the discovery of peptide drugs and in their modification and suitable formulation to allow for their delivery, which is the topic of our next chapter.

1.6 Peptide drug delivery

Peptides cover a vast range of biological roles in nature, including defence, immunity, stress, growth, homeostasis and reproduction.[122] Their diversity has translated to the potential of peptides in the treatment of infections, pain, cardiovascular disease, diabetes, cancer among other ailments.[123] An example of this is the naturally occurring peptide, exenatide, found in the venom of the Gila monster (*Heloderma suspectum*) and translated into a treatment for type-2 diabetes mellitus approved by the FDA-approved in 2005 - Byetta®.[124] Peptides also tend

to have the advantage of higher specificity compared with small molecule drugs, leading to less off-target effects and therefore lower toxicity.[125]

When it comes to drug delivery, the most convenient and acceptable route of administration is the oral route. The ease of swallowing a pill increases the compliance of patients to orally administered drug treatments. Currently, small molecule drugs rule the market due to the simplicity of their synthesis and their higher oral bioavailability. In contrast, peptide drugs involve a more complicated synthesis and have very limited oral bioavailability due to enzyme degradation and permeability issues. Due to their low oral bioavailability, most of the FDA-approved peptide drugs are administered by the parenteral route, i.e. intravenous (IV), intramuscular (IM) or subcutaneous (SC). However, this route of administration involves painful injections, leading to increased healthcare costs and decreased patient compliance. Peptides also face the threat of serum proteases at the site of injection which lead to rapid clearance from the blood. Another route which is commonly explored for peptide drug delivery is the transdermal route which, like the oral route, is non-invasive and easily administered by the patient. The transdermal route also has the added advantage over the oral route in that it avoids first pass metabolism. However, this route tends to only be suitable for small, lipophilic drug molecules required in low doses.[126] Other administration routes, such as buccal, intranasal and pulmonary, are also being investigated to bypass the high protease concentration of the GI tract and increase absorption without the pain and discomfort of frequent injections.[127]

However, regardless of the route of administration, peptides face challenges with maintaining stability and/or penetrating epithelial barriers. In this section, a brief summary is provided on the challenges faced when administering peptide drugs by the parenteral, oral, transdermal, intranasal, pulmonary and buccal routes and some of the approaches that have been taken to overcome these challenges.

1.6.1 Parenteral route

1.6.1.1 Challenges

While parenteral administration of peptides avoids both the high concentration of proteases associated with the GI tract and the need to penetrate epithelial tissue, peptide drugs administered by this route generally suffer from short

plasma half-lives in the range of a few minutes. This is due to degradation by serum proteases, neutralization by antibodies, rapid renal clearance and solubility issues.[128] IV administration, which assumes 100% bioavailability for small molecule drugs, shows decreased bioavailability for peptide drugs due to obstacles faced at the site of injection, e.g. degradation by serum proteases.[129] Peptides also face the threat of proteolytic degradation as they journey to the liver and the kidneys.[130] Therefore, frequent administration of peptide therapeutics is generally required which is inconvenient, invasive and causes pain and discomfort to the patient. Frequent parenteral administration also raises healthcare costs, since the supervision by a healthcare professional is generally required.

Like all parenterally delivered drugs, there is the risk of haemolysis, precipitation and phlebitis at the site of injection.[131] To reduce this risk, formulations must be stable, sterile and free of particulates and pyrogens. The pH and ionic strength of solutions for injection must also be considered to prevent irritation and haemolysis. For IV and IM administration, the acceptable pH range is pH 2–12, whereas for SC administration it is pH 2.7–9.0.[132] For peptide drugs, pH and ionic strength also need to be considered for peptide stability to avoid aggregation. The solubility of the peptide in the blood serum (around pH 7.4) must be taken into consideration. Peptide solubility is minimal at the isoelectric point (pI), i.e. the pH at which the peptide carries a net charge of zero. If the pI of the peptide is close to that of serum pH, insoluble aggregates of the peptide may form upon injection which can affect the activity of the peptide or result in the induction of an immune response.[89] For continuous IV infusion, a lower solubility is acceptable due to the high volume that can be administered.

Due to the rapid clearance, enzyme degradation and solubility challenges faced by peptide drugs administered via the parenteral route, there is a need to chemically modify or formulate peptides to increase their stability and prolong their therapeutic effect.

1.6.1.2 Current approaches

The approaches that have been taken to reduce the clearance rate and increase the stability of peptides following parenteral administration include peptide

engineering, conjugation to serum proteins or polymers and the use of polymer matrices or pumps to prolong the therapeutic effect of the peptide.

Peptide engineering involves making or breaking bonds in the peptide or altering the amino acid composition. This approach has been carried out to increase peptide solubility or to prevent recognition of the peptide by serum proteases. This can be carried out by chemical synthesis of peptides or the enzymatic or chemical modification of peptides from synthetic or natural sources.[133]–[135] In the chemical synthesis of peptides, one of the main approaches for increasing peptide stability is the substitution of L-amino acid with D-amino acids. The unnatural D-amino acids are generally not recognised by proteases *in vivo* and can therefore avoid degradation.[133] An example of this is the commercially available anti-diuretic desmopressin (1-deamino-[8-D-arginine]-vasopressin, DDAVP), in which L-arginine at position 8 is substituted with D-arginine to prolong the therapeutic effect.[136] However, care must be taken to ensure the activity of the peptide is preserved and that no increase in toxicity is incurred by altering the peptide sequence.[137]

Chemical modification of peptides can be carried out by conjugation to polymers or serum proteins. Conjugation is generally carried out by bonding the polymer or serum protein to the N-terminal of the peptide or to functional groups on the amino acid side-chains, e.g. phenol (tyrosine), amino (lysine), thiol (cysteine).[123] The most commonly used polymer for conjugation is polyethylene glycol (PEG). PEGylation, i.e. covalent bonding of the peptide to PEG, has the potential to shield peptides from the reticuloendothelial system (RES) and proteases. This prevents immunogenic response to the peptide and proteolytic degradation respectively. The increase in molar mass incurred by conjugation of the peptide to PEG is also an advantage, as this reduces renal clearance of the peptide.[128] PEGylation is used in the commercial parenteral peptide formulation, Omontys®, for the treatment of anaemia.[138] However, there have been reports of the clearance of PEGylated peptides by antibodies following repeated dosing and potential toxicity of metabolites.[139], [140] PEGylated peptides must also be approved by the FDA, even if the peptide itself has been approved, as they are viewed as new chemical entities[77].

Conjugation of peptides to serum albumin and the Fc region of the serum protein, immunoglobulin, has also been found to increase the half-life of the peptide by increasing the molecular weight to above the renal filtration threshold.[37] For example, the anti-HIV peptide, albuvirtide (FB006M), was modified with maleimide to induce binding to serum albumin upon injection.[141] This resulted in an increased serum half-life for the peptide without triggering an immune response. This treatment is currently in P III clinical trials as a once-weekly injection in patients where first-line treatment has failed.[142] The currently FDA-approved peptide, enfuvirtide, for the treatment of HIV requires injections twice daily. Therefore, the success of albuvirtide would hugely benefit HIV patients by reducing the costs and the pain associated with frequent injections. However, conjugation requires the presence of suitable functional groups on the peptide to form bonds with the polymer or serum protein. Good knowledge of the structure-activity relationship (SAR) between the peptide drug and its target is required so that conjugation does not interfere with the therapeutic effect of the peptide. It is also important that the bond formed is site-specific to prevent the formation of isomers.

The use of enzymes to modify peptides can reduce costs compared to chemical modification and can also improve the stereospecificity of the modified peptide. For example, NisB and NisC, a dehydratase and cyclase respectively, produced by *Lactococcus lactis* were used by Rink *et al* to increase the stability of LHRH. NisB formed the unusual dehydrated residues dehydroalanine (Dha) and dehydrobutyrine (Dhb) through dehydration of the serine and threonine residues of LHRH respectively. NisC catalysed the formation of thioether bridges resulting in cyclisation of the peptide.[143] This improved the stability of LHRH towards proteolytic degradation.

The controlled release of peptides following parenteral administration using polymeric matrices and pumps has also been used to prolong the therapeutic effect of peptides and reduce the frequency of injections.[144] The polymer of choice for matrices providing controlled release of peptides is poly(lactic-co-glycolic acid) (PLGA). PLGA was first applied to peptide delivery in 1986 for the parenteral delivery of LHRH agonist peptide (1.31 kDa) which provided controlled release of the peptide over 1 month.[77] PLGA is used in most of the commercially available slow-release peptide formulations including Lupron Depot®, Sandostatin® LAR

Depot, Decapeptyl®, Bydureon® and Zoladex® (goserelin acetate).[145] *In situ* forming peptide drug depots have also been synthesised using technologies such as Saber™ (DURECT Corporation). This technology uses a hydrophobic, viscous, fully-esterified glucose derivative known as SAIB. The peptide drug is dissolved or dispersed in a solution of SAIB mixed with solvent. Upon SC or IM injection, the solvent diffuses out leaving a viscous depot of the drug in SAIB.[146]

Hydrogels are cross-linked polymeric matrices that absorb water and swell to release their cargo. Some hydrogels absorb water in response to their environments, e.g. temperature, pH, chemicals or magnetic field.[147]–[150] Peptides, such as vasopressin, LHRH, insulin, are naturally released in the body in a pulsatile fashion. Therefore, a delivery system which responds to stimuli would be ideal. pH-sensitive cellulose hydrogels containing glucose oxidase have been tested for regulating insulin release.[151] In the presence of glucose, the glucose oxidase of the hydrogel converts glucose to gluconic acid. This reduces the pH of the local environment and triggers the release of insulin from the pH-sensitive hydrogel. Pumps which deliver drugs based on differences in osmotic or mechanical pressure have also been employed for pulsatile delivery of peptides. These pumps can be external or implantable. Omnipod® (Insulet Corporation) is an external, wirelessly-controlled patch pump for the delivery of insulin. Blood glucose levels can also be measured by this system using a built-in glucose meter.[152] The implantable pump, Alzet®, uses osmotic pressure to pump the peptide out of a SC implant. Viadur™ is a commercially available leuprolide formulation which uses Alzet® to deliver leuprolide over a 1 year period for the palliative treatment of advanced prostate cancer.[153]

1.6.2 Oral route

1.6.2.1 Challenges

Although oral administration is the preferred delivery route, peptide drugs are faced with an abundance of obstacles following administration by this route. The first port of call as the peptide travels down the GI tract is the stomach with its harsh acidic environment, which in the fasted state is on average pH 1.7.[154] The acidic pH of the stomach can lead to peptide instability, e.g. due to acid-catalysed deamidation, while larger peptides with tertiary structure can unfold, leaving the

amino acid sequence more susceptible to proteolytic degradation. The stomach also contains the aspartic protease pepsin which favours the cleavage of peptide bonds at hydrophobic residues, such as phenylalanine, leucine and methionine.[155]

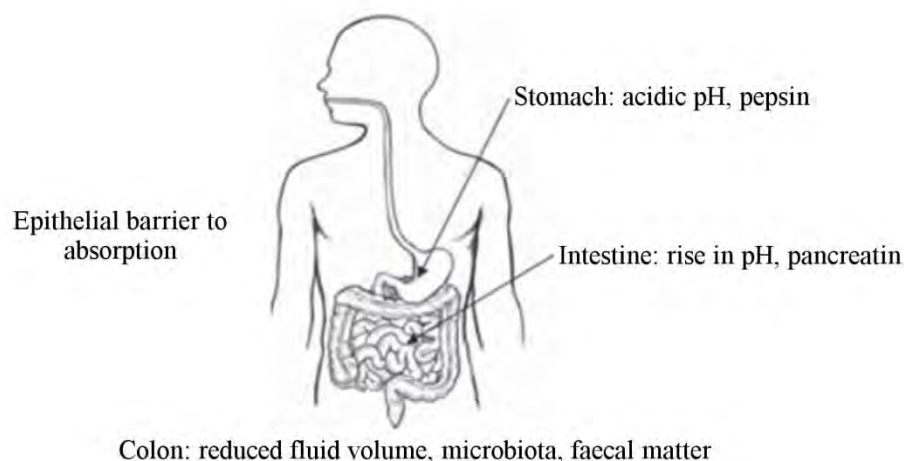


Figure 1-3. Some of the barriers faced by orally administered peptides, adapted from Smart *et al.*[156]

Should it make it through the stomach, the peptide is then faced with a sharp increase in pH as it enters the small intestine, rising from pH 1-3 to pH 6-8.[157] This rise in pH may induce aggregation or precipitation of the peptide as it transitions from the stomach to the intestine if the isoelectric point (pI) is within the intestinal pH range.[71] The intestine is also home to an abundance of proteases including serine endopeptidases, carboxypeptidases, aminopeptidases and dipeptidases. The serine endopeptidase, trypsin, is a 23-26 kDa proteolytic enzyme, produced by the pancreas and localised in the enterocytes of the small intestine. Trypsin recognises and catalyses cleavage at the carboxyl end of the basic residues, arginine, lysine and histidine.[158] Chymotrypsin induces cleavage at the carboxyl end of the aromatic residues, tyrosine, tryptophan and phenylalanine. The optimal pH for chymotrypsin is pH 7.8.[159] Carboxypeptidases catalyse cleavage of amino acids at the carboxyl end of the peptide and aminopeptidases at the amino end.[143] The presence of bile salts, which are secreted in the duodenum, can increase the susceptibility of peptides to degradation by these proteases.[160], [161] Reduced glutathione in the small intestine can also affect peptide stability by causing thiol-disulphide exchange reactions with cysteine residues.[156]

For peptide drugs intended for systemic delivery, the intestinal epithelium acts as a physical barrier against absorption. The intestinal epithelium is covered in a layer of polysaccharides, known as the glycocalyx, and heavily glycosylated proteins known as mucins.[162] The paths across the intestinal epithelium are through cells (transcellular) or between cells (paracellular).[163] Transcellular transport across the apical cell membrane by diffusion occurs only for small, hydrophobic molecules. Since peptides are generally large hydrophilic molecules, transcellular transport of peptides requires specific active transporters. However, in the absence of these specific transporters, absorption of peptides must occur by paracellular transport via tight junctions. While these tight junctions allow for the passage of hydrophilic molecules, the diameter between cells is only approximately 4 Å and therefore prevents peptide absorption.[164]

The colon is possibly the compartment of the GI tract with the least threats to peptide stability. However, while the protease concentration in the colon is much lower than in the small intestine, the gut microbiota produce enzymes, such as hydrolases and oxidases, which may affect peptide stability – particularly in the case of glycopeptides.[165] In addition, the fluid volume of the colon is low in comparison to the rest of the GI tract which may affect peptide stability. There is also the effect of faecal matter, dietary metabolites, intestinal secretions and gases, such as carbon dioxide and methane, which may affect the stability of the peptide.[166] For peptides intended for systemic delivery, the colon presents a much lower surface area than the small intestine but has a much longer transit time, approximately 52 h.[167] However, this transit time can vary from patient to patient, with disease (e.g. IBS and ulcerative colitis) also having an impact.[166]

1.6.2.2 Current approaches

The approaches that have been taken to improve the oral bioavailability of peptide drugs are focused on increasing the stability of the peptides along the GI tract and increasing the ability of peptides to penetrate the intestinal epithelium. Some of the techniques that have been applied include enteric coatings, the co-administration of protease inhibitors, colon-targeted systems, penetration enhancers and mucoadhesive polymers.

Enteric coatings are used to protect peptides as they travel through the stomach and deliver them to the intestine. These coatings make use of polymers which are stable at acidic pH but rapidly degrade or swell at intestinal pH due to ionisation.[168] Some examples of the polymers used in this regard are hydroxypropyl methylcellulose (HPMC) and the Eudragit® polymers, composed of poly(methyl methacrylate) (PMMA) and poly(methacrylate) (PMA). HPMC is used in the FDA-approved oral formulation of the peptide, linaclotide (Linzess®). This enables delivery of linaclotide to the intestine for the treatment of IBS and chronic idiopathic constipation.[169]

To decrease the degradative effects of GI proteases on peptide drugs administered by the oral route, protease inhibitors have been used.[170] Examples of inhibitors which have been co-administered with peptide drugs to increase their stability include soybean trypsin inhibitor, chymostatin, aprotinin, puramycin and bacitracin.[171], [172] In a study carried out by Werle *et al*, aprotinin was used to protect the peptide, calcitonin, from degradation by the GI proteases, trypsin and α -chymotrypsin.[172] This formulation successfully reduced calcium levels in a rat model. However, the co-administration of protease inhibitors may not be suitable for prolonged treatments of as it may disrupt the metabolism of dietary peptides and proteins *in vivo* and may lead to hypertrophy or hyperplasia of the pancreas.[171]

Penetration enhancers and mucoadhesive polymers have been used to increase absorption of peptide drugs across the intestinal epithelium. Penetration enhancers work by opening the tight junctions between epithelial cells in the small intestine, enabling paracellular transport of peptides into systemic delivery. Examples include medium chain fatty acids (MCFA), sodium caprate (C10), sodium caprylate (C8), n-(8-[2-hydroxybenzoyl]amino)caprylic acid (SNAC), (N-(5-chlorosalicyloyl)-8-aminocaprylic acid) (5-CNAC), acyl carnitines, EDTA and selected bile salts.[173] Bile salts have been used in the oral calcitonin formulation, Oracal®, developed by Tarsa Therapeutics.[174] Phase III clinical trials for this formulation began in 2009.[175] However, no further progress has been reported to date. Mucoadhesive systems work to increase absorption of peptides by increasing the contact time between the peptide and the intestinal epithelium. Examples include liposomes and polymers such as chitosan and lectins.[176]–[178] Nanoparticle formulations have also been investigated for the delivery of peptides.[179] This

generally involves encapsulation of peptides in a polymer to protect from degradation but has the added advantage of increasing the chances permeation across epithelial barriers for systemic delivery and evading the mononuclear phagocytic system due to their nano-size.[123] However, there is some concern surrounding the toxicity of nanoparticles.[180] To date, none of these efforts to increase absorption of peptides across the epithelial membrane have proven successful.

Colon-targeted systems have been developed based on transit time, pH, polysaccharides degraded by enzymes produced by the gut microbiota.[166], [168], [181] These systems take advantage of the lower protease concentration and longer transit time of the colon to increase peptide stability and chances of absorption respectively. Polymers specifically degraded by the gut microflora have been investigated to target delivery of peptides to the colon, where there is a lower abundance of proteases than the stomach or the duodenum. An example of this is the formulation of the antimicrobial peptide, nisin, using the polysaccharide, pectin.[182] Pectin is degraded by pectinases in the colon and can therefore provide colon-specific nisin delivery. However, gut microflora, transit times and pH gradients vary among patients preventing the reproducibility of colon-targeted peptide delivery.

To date, only four orally administered peptide drugs have been approved by the FDA.[183] These orally administered peptides include two which are locally delivered to the GI tract and two systemically delivered. The locally delivered peptides are vancomycin, for the treatment of *Clostridium difficile*-associated diarrhoea and staphylococcal enterocolitis, and linaclotide, for the treatment of IBS and constipation. The cyclic structure and modified amino acids of vancomycin make it relatively stable towards proteases and the formulation in a gelatin capsule further enhances this stability.[183] The FDA-approved oral linaclotide formulation (Linzess®), uses an enteric coating to bypass the stomach, as discussed above.[184] The FDA-approved oral peptide formulations for systemic delivery are the antidiuretic, desmopressin, and the immunosuppressant, cyclosporine. Both of these peptides have cyclic structures, which enhance their stability in the GI tract. The replacement of L-Arg with D-Arg at position 8 in desmopressin improves its stability by preventing recognition by proteases.[136] The lipophilicity of cyclosporine (logP = 3.64) facilitates its absorption across the intestinal wall into systemic

circulation.[185] Although desmopressin shows limited oral bioavailability, the dose required is quite low and therefore only a small amount of the peptide needs to be absorbed from the intestine into the bloodstream.

1.6.3 Transdermal route

1.6.3.1 Challenges

Topical delivery of drug molecules has been generally confined to the treatment of local infections or disorders of the skin. However, the success of transdermal patches for the systemic delivery of drugs, including nicotine (Nicorette) and testosterone, led to increased research and today 19 FDA-approved drug treatments are administered by this route.[126]

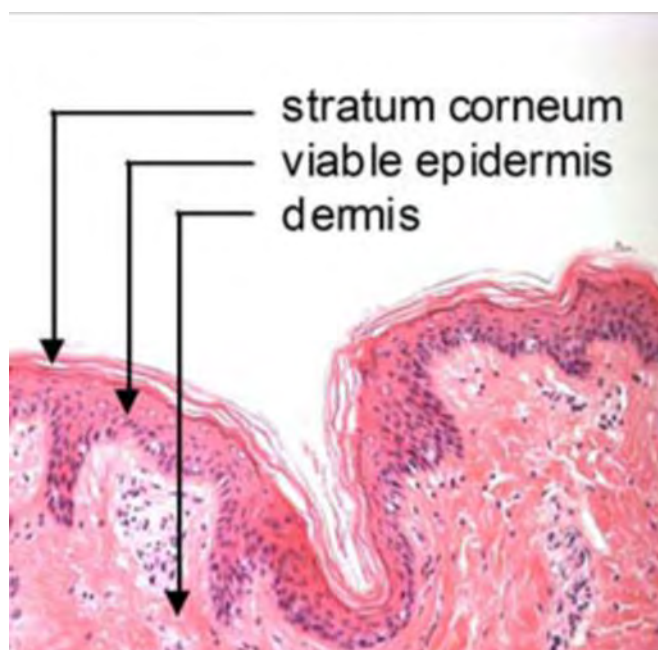


Figure 1-4. Histological image of a cross section taken from mammalian skin, adapted from Prausnitz *et al.*[126]

Administration of peptides via the transdermal route is desirable as it avoids the high abundance of proteases in the GI tract and is also relatively non-invasive in comparison to parenteral administration. However, no transdermal peptide drug formulations have been approved by the FDA so far. This is mainly due to difficulty in penetrating the skin barrier. To reach systemic circulation, peptides must penetrate the outer stratum corneum (10-20 μm) followed by the epidermis (50-100 μm) before reaching the capillary-rich dermis layer where it can be systemically absorbed

(**Error! Reference source not found.**). Also, the lipophilic nature of the skin does not naturally allow the diffusion of large, charged molecules such as peptides

1.6.3.2 Current approaches

Since the natural permeability of the skin is limited to small, lipophilic molecules, extensive measures need to be taken to allow the passage of peptides across the skin. These should reversibly disrupt the outer stratum corneum and drive the transport of peptides across the skin without causing significant injury to skin tissue.[126] The approaches which have been taken to overcome permeation issues associated with the transdermal delivery of peptides include chemical enhancement (e.g. using surfactants, fatty acids or peptides) and physical enhancement (e.g. iontophoresis, thermal ablation, microneedling and phonophoresis).

Chemical permeation enhancers, such as surfactants, fatty acids and cell-penetrating peptides (CPP) have been used to enhance permeation of the skin. These are generally amphiphilic molecules which insert themselves into the stratum corneum causing disruption and, therefore, increase permeability. An example of this is the CPP, TD-1, which has been used in a study by Chen *et al* to enhance the transdermal delivery of insulin and human growth hormone.[186] Another polyarginine-heptamer cell-penetrating peptide (CPP) has been conjugated to cyclosporine, acting as a prodrug to enable transdermal delivery of this immunosuppressant.[187] Permeation enhancers are associated with skin irritation. However, high throughput screening (HTS) methods have been used in the search for chemical combinations which enhance permeability of the skin without causing irritation.[188] Using these methods, transdermal delivery of the peptide, leuprolide, was increased in hairless rats using a combination of sodium laureth sulphate (SLS; a non-ionic surfactant) and phenyl piperazine.[189]

One of the methods for physically enhancing transdermal delivery of peptides is iontophoresis. Iontophoresis uses an electrical current ($<0.5 \text{ mA/cm}^2$) to deliver ionic drug molecules, such as peptides across the stratum corneum.[190] This method of transdermal peptide delivery allows for high control over the rate and dose of the drug delivered. The predominant mechanisms of iontophoresis are electromigration and electroosmosis.[191] Electromigration is the repulsion of the peptide by an external electrode, with the same charge as the peptide, across the skin.

This can be cathodal, pushing negatively charged peptides across the skin, or anodal, repelling positively charged peptides across the skin. The other mechanism, electrosmosis, is dependent on the pH of the carrier solution. Since the skin has an isoelectric point of approximately pH 4, peptide solutions above this pH can flow from an external anode through the skin, which acts as a cathode. The opposite applies for peptide solutions below pH 4. Under this pH, a cathode can be used to direct the flow of the solution through the skin, which under these conditions acts as an anode. However, altering the pH of peptide solutions can compromise the chemical and physical stability of peptides (Section 1.4). Patches with microprocessors which are pre-programmed or can administer drugs on-demand using iontophoresis are currently under investigation. Iontophoresis has been successfully applied in the delivery of leuprolide and calcitonin.[192], [193] However, while this method of administration seems ideal, the costs associated with these delivery systems may not be so favourable. Also, the long term implications of applying low electrical currents to increase the permeability of the skin need to be investigated.

Microneedling involves piercing the skin with very short needles to create micron-sized pores through the stratum corneum.[194] This can be carried out by subsequent topical delivery of the drug following microneedling, coating the microneedles with the drug or by synthesising biodegradable microneedles that allow for a prolonged drug release.[195] The coating of microneedles with desmopressin has been used for the transdermal delivery of this peptide.[196] Thermal ablation also forms micron-sized cavities by which the drug molecule can manoeuvre its way across the stratum corneum. However, this method uses high temperatures to rapidly vaporise water from the skin to form the pores. This approach has been applied to the transdermal delivery of human growth hormone (HGH) and interferon α -2b.[194], [197]

Ultrasound has also been used as a means for increasing the permeability of the skin to peptides and proteins in what is known as sonophoresis. High frequency, oscillating ultrasound waves are used to form bubbles, a process known as cavitation. This occurs in the medium, e.g. a hydrogel, between the ultrasound transducer and the skin which contains the drug. The bubbles oscillate and collapse to generate shock at the skin surface and disrupt the stratum corneum to increase its

permeability. This method was used by Tachibana *et al* in 1992 to deliver insulin transdermally.[198]

1.6.4 Alternative routes

1.6.4.1 Pulmonary and intranasal

The pulmonary and intranasal routes pose as attractive non-invasive alternatives to parenteral delivery. In general, drugs delivered by the pulmonary and intranasal routes are rapidly absorbed as the epithelial tissue in the nasal passage and the lungs is thin, compared to gastrointestinal epithelium, and highly vascularised. However, administration of peptides by both routes may be degraded by proteases and may experience issues penetrating the mucus and epithelial layers to reach systemic circulation. The surface area of the intranasal route is small, decreasing the chances of absorption, and the use of certain excipients to deliver drugs by this route may lead to rhinitis.[199] While the surface area for absorption in the lungs is much higher, peptides administered by the pulmonary route may be deposited before reaching the alveoli. To reach the alveoli, the particle size must be 1-3 μm . Particles above this size will most likely be deposited in the upper respiratory tract while particles below this size could potentially be exhaled.

Liposomes coated with PEG have been used in an attempt to facilitate pulmonary delivery of vasoactive intestinal peptide (VIP).[200] These liposomes showed sustained release and prolonged therapeutic effect compared to free VIP administered via the pulmonary route. An inhalable insulin formulation, Exubera®, produced by Pfizer was approved by the FDA in 2006 with particles sizes of 1-5 μm . However, the high price, bulky inhaler and failure to excel over current treatments led to its discontinued production as sales were not sufficient. In 2014, a second inhalable insulin formulation, Alfrezza®, came to the market. Alfrezza® uses fumaryl diketopeperazine microspheres, with particle sizes of 2-3 μm , to deliver insulin to the lungs.[201] The compact size of the inhaler may lead to the improved patient acceptance of this delivery system but only time will tell.

1.6.4.2 Buccal

The buccal delivery of peptides is convenient for patients, avoids first pass metabolism and is more permeable than the skin.[202] In addition, the protease activity in buccal mucosa is significantly lower than in the GI tract. However, the

surface area of buccal mucosa is quite low in comparison to the GI tract and, although permeability is higher than the transdermal or oral routes, buccal delivery still faces permeability issues.[203] Taste can also be an issue for patient compliance when delivering peptides via the oral route.

To overcome these barriers permeation enhancers, mucoadhesives and taste-masking agents have been used. Permeation enhancers and mucoadhesives employed for buccal delivery of peptide drugs are similar to those that have been tested for oral delivery, e.g. fatty acids, surfactants and bile salts.[203] Cell turnover of buccal mucosa is much slower than GI mucosa, prolonging the effect of peptide formulations using mucoadhesives administered by the buccal route.[127] Xu *et al* formulated insulin for buccal delivery using the permeation enhancers, lecithin and propane-diol. While this formulation demonstrated hypoglycaemic effect, it only showed around 30% of the bioavailability seen for subcutaneously administered insulin.[204]

1.7 Commercially available peptide drug formulations

The number of peptide formulations reaching the market is increasing. Parenteral administration remains the predominant route for peptide delivery (Table 1). However, the use of controlled release systems, mainly using PLGA encapsulation, has allowed the therapeutic effect of peptides to be prolonged and the frequency of injections reduced. The linaclotide formulation, Linzess®, was approved for oral delivery in 2012. However, this peptide is used in the treatment of irritable bowel syndrome (IBS) and does not need to be absorbed across the GI epithelium to reach systemic circulation. Further examples of commercially available peptide formulations are shown below in Table 1.

1.8 Conclusions

Peptides have shown great potential as drug treatments due to their high selectivity and efficacy. Many different approaches have been taken to improve the bioavailability of peptides following administration by the oral, transdermal, pulmonary, intranasal and buccal routes. However, due to the presence of proteases, epithelial barriers and solubility issues associated with these delivery routes, the majority of peptides continue to be administered via the parenteral route which is inconvenient and painful for the patient. Some peptide drugs, such as those listed in

Table 1, have been successfully formulated and approved by the FDA. However, there are many peptides with therapeutic properties which are still unexploited and require modification or formulation in order to allow their therapeutic properties to be unleashed.

Among these currently unexploited peptides are those with antimicrobial activity, including the bacteriocins, some of which have shown activity against resistant strains of bacteria. Antibiotic resistance among bacterial strains, such as MRSA and VRE, is on the rise and requires immediate attention from a preventative and a therapeutic perspective. The formulation of antimicrobial peptides, either alone or in combination with existing drug treatments, could enable these peptides to act as the next generation of antibiotics and provide effective treatments against resistant bugs.

1.9 Project motivation and aim

The aim of this project is to improve the bioavailability of peptides, in particular bacteriocins. Bacteriocins have shown activity against antibiotic-resistant strains of bacteria, including MRSA and VRE.[54] These peptides may prove to be the next generation of antibiotics to serve mankind against the plight of antibiotic resistance amongst pathogenic bacteria.[57] However, as peptides, bacteriocins are susceptible to physical, chemical and enzyme degradation. Therefore, applying state of the art formulation strategies to improve the stability of these novel antimicrobial peptides may provide the next generation of antibiotics active against resistant bacterial strains. In this study, nisin will be used as a model bacteriocin, due to its commercial availability and comparable size to novel bacteriocins of interest, e.g. thuricin CD and bactofencin A.[205], [206] Nisin itself has also shown interesting biomedical properties and has the potential to be transformed into an antimicrobial, anticancer or spermicidal therapy if a suitable formulation can be devised.

Table 1. Examples of commercially available peptide formulations

<i>Peptide drug</i>	<i>Company</i>	<i>Year of approval</i>	<i>Size of peptide</i>	<i>Formulation technique</i>	<i>Route of admin</i>	<i>Indication</i>
Lupron Depot® (leuprolide acetate)	Abbott Industries	1984	9-aa 1.2 kDa	PLGA microsphere suspension	Monthly IM injection	Prostate cancer/breast cancer
Zoladex® (goserelin acetate)	AstraZeneca	1987	1.3 kDa	Biodegradable PLGA implant	12-week local SC implant	Prostate cancer
Sandostatin® LAR Depot (octreotide)	Novartis Pharmaceuticals	1988	8-aa 1 kDa	PLGA microsphere suspension	Monthly IM injection	Adenoma, Acromegaly
Copaxone® (glatiramer acetate)	Teva Pharmaceutical Industries	1996	4-aa 0.62 kDa	Solution for injection	Daily SC injection	Multiple sclerosis
Decapeptyl® (triptorelin)	Allergan	2000	10-aa 1.7 kDa	PLGA microsphere suspension containing pamoate salt of peptide	IM injection every 4-24 weeks	Prostate cancer
Forteo® (teriparatide)	Eli Lilly	2002	34-aa 4.1 kDa	Solution for injection	Daily injection	Osteoporosis
Victoza® (liraglutide)	Novo Nordisk	2009	3.75 kDa	Solution for injection	Daily SC injection	T2DM

Bydureon® (exenatide)	Bristol-Myers Squibb	2012	39-aa 4.2 kDa	PLGA microsphere suspension	Weekly SC injection	T2DM
Surfaxin® (lucinactant)	Discovery Laboratories	2012	21-aa 2.5 kDa	Solution for injection	Intratracheal injection	Respiratory distress syndrome
Omontys® (peginesatide)	Affymax	2012	2x21-aa 4.9 kDa	PEGylated peptide, Solution for injection	IV or SC injection	Anaemia
Signifor® LAR (pasireotide)	Novartis	2012	6-aa 1.3 kDa	PLGA microsphere suspension	IM injection	Cushing's disease
Kyprolis® (carfilzomib)	Onyx Pharmaceuticals	2012	4-aa 0.7 kDa	Solution for injection (pH 3.5)	IV infusion	Multiple myeloma
Linzess® (linaclotide)	Ironwood Pharmaceuticals	2012	14-aa 1.5 kDa	Hard gelatin capsules containing linaclotide-coated beads	Oral administration	IBS
Gattex® (tedglutide)	NPS Pharmaceuticals	2012	33-aa 3.8 kDa	Lyophilised powder for reconstitution before injection	Daily SC injection	Short bowel syndrome
Adlyxin (lixisenatide)	Sanofi-Aventis	2016	44-aa 4.9 kDa	Solution for injection	Once daily SC injection	T2DM

*n-aa refers to the number of amino acids in the peptide sequence; T2DM: type-2 diabetes mellitus.

2. Nisin

2.1 Introduction

Nisin is a 3.4 kDa bacteriocin produced by the Gram-positive lactic acid bacteria (LAB), *Lactococcus lactis* subsp. *lactis*. Among bacteriocins, nisin is classed as a lantibiotic, due to the presence of lanthionine rings within the chemical structure (Figure 2-1). Nisin is a class I lantibiotic, on the basis that two enzymes, a dehydratase (NisB) and a cyclase (NisC), are involved in the post-translational modification of this peptide.[58] Nisin-producing *L. lactis* strains have so far been isolated from cheese, human and bovine faeces, human milk, sauerkraut and river water.[55], [207], [208] Nisin is FDA-approved as a food additive (E234) and is certified as GRAS (Generally Regarded as Safe). Nisin has shown antimicrobial activity against pathogenic bacterial strains including *Staphylococcus aureus*, *Escherichia coli*, *Streptococcus pneumoniae*, *Enterococcus faecium* and *Clostridium difficile*, indicating its potential for clinical application.[209]–[211] Along with antimicrobial activity, nisin has also shown immunomodulatory, spermicidal and anticancer activity.[46], [62], [212]

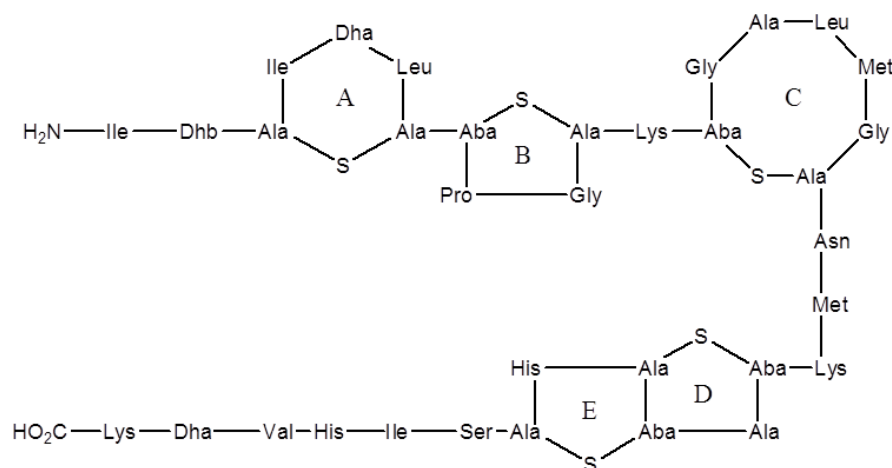


Figure 2-1. Structure of nisin A, adapted from Liu *et al.*[213]

However, these activities reported for nisin with the potential for therapeutic application have not yet translated from the laboratory to the clinic. This is mainly due to the susceptibility of nisin to protease degradation and the instability of nisin at neutral to alkaline pH. [214], [215] This chapter aims to confirm these stability issues for the Handary product Nisin AP, containing 95% nisin (5% moisture).

Studying these properties of Nisin AP will aid in the design of an appropriate oral delivery system to improve the stability, and therefore the bioavailability, of nisin and enable this bacteriocin to be applied as a treatment for bacterial infections of the GI tract, e.g. *Clostridium difficile*.

2.1.1 History

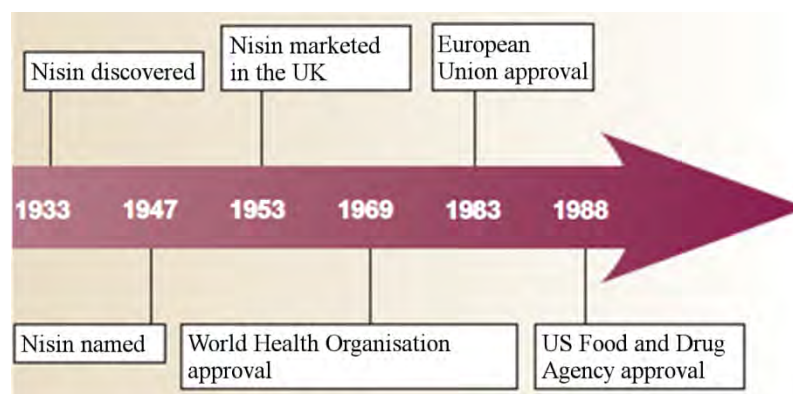


Figure 2-2. Nisin timeline, adapted from Cotter *et al.*[42]

In 1928, the same year that Alexander Fleming discovered penicillin, Rogers and Whittier noticed that the bacterial strain *Lactococcus lactis* subsp. *lactis* excreted a heat-stable, alcohol-soluble, diffusible substance which inhibited other LAB strains in milk.[216] Five years later, Whitehead *et al* determined that this antimicrobial substance was a peptide.[217] It was not until ten years later, in 1943, that this antimicrobial peptide (AMP) was concentrated for testing as an antimicrobial agent. In 1944, Mattick and Hirsch discovered that this peptide was active against certain pathogenic bacterial strains, including streptococci associated with mastitis and some species of *Bacillus* and *Clostridium*, and was therefore classed as an antibiotic.[218] In 1947, this AMP was labelled “nisin”. This came from the description of the peptide as an “N-inhibitory substance” produced by *L. lactis*, N being the serological group of the *L. lactis* strain which produced “nisin”.[219]

Initially tested for use in human or veterinary medicine, nisin was never successfully applied in these areas due to its low solubility and stability at physiological pH and susceptibility to proteolytic degradation *in vivo*.[220], [221] In 1951, Hirsch *et al* suggested that nisin be used as a food preservative.[222] Nisin was approved for this application in 1969 by the World Health Organisation (WHO) and received GRAS status from the FDA in 1988. Nisin has since been used as a food additive, E234, in the preservation of meat, dairy products, beverages, baked

goods and canned vegetables. Today, nisin is used in over 80 countries to combat food pathogens but has not yet been approved as an antibiotic treatment.[56]

2.1.2 Structure and characterisation of nisin

Nisin is a 34-residue, cationic AMP which contains lanthionine and β -methyl-lanthionine rings along with dehydrated amino acid residues. The amino acid sequence of nisin A was first resolved by Gross and Morell in 1971, using amino acid analysis (Figure 2-1).[223] The structure was subsequently confirmed by mass spectroscopy, NMR and chemical synthesis.[224]–[226] Nisin does not contain any aromatic residues and therefore does not absorb UV light at 280 nm. However, detection of nisin can be carried out at 214 nm since peptide bonds absorb UV light at this wavelength.[117], [214] Nisin is amphiphilic in nature with a hydrophobic N-terminal and a hydrophilic C-terminal.[215]

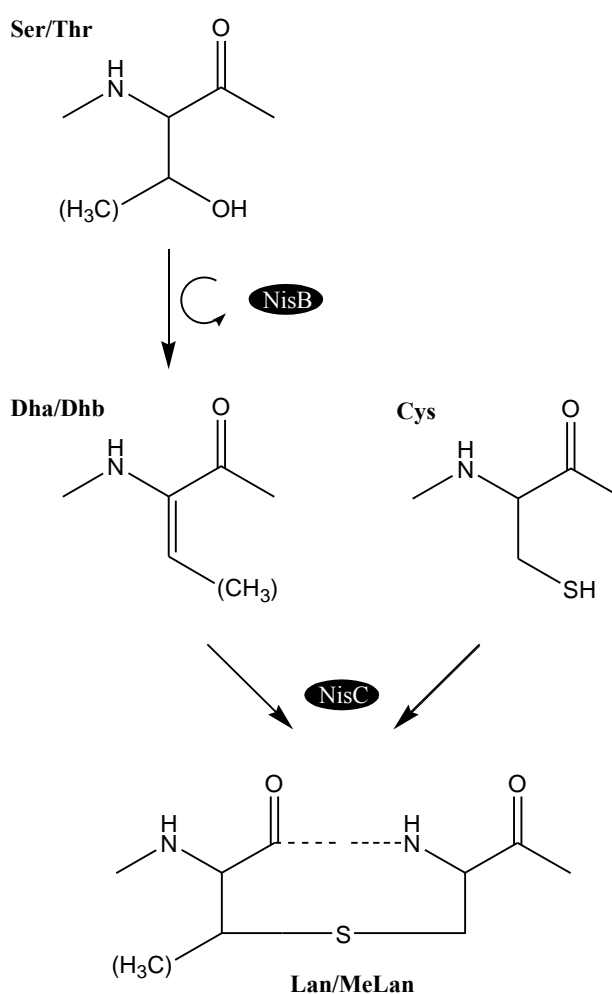


Figure 2-3. Dehydration of serine/threonine (catalysed by NisB) to form dehydroalanine/dehydrobutyrine and subsequent cyclisation (catalysed by NisC) with cysteine to form lanthionine (Lan)/ β -methyl-lanthionine (MeLan).[227]

Nisin biosynthesis involves translation of the gene coding for the nisin precursor, pre-nisin, in the ribosome. Pre-nisin, a 57-residue peptide, is then post-translationally modified to include the dehydrated residues and lanthionine and β -methyl-lanthionine rings. The leader peptide is cleaved to produce nisin, a 34-residue peptide. The dehydratase enzyme, NisB, catalyses the dehydration of serine and threonine residues, to form dehydroalanine (Dha or Δ Ala) and dehydrobutyrine (Dhb or Δ Abu) respectively (Figure 2-3).[228] These dehydrated residues provide nisin with characteristic allyl peaks when analysed by NMR.[229] A cyclase enzyme, NisC, catalyses the addition of the thiol group of cysteine residues to the α,β -double bond of Dha and Dhb to form lanthionine rings (Figure 2-1; ring A) and β -methyl lanthionine rings (Figure 2-1; rings B-E) respectively.[230] The nisin gene cluster also codes for a transporter protein, NisFEG, and a specific immunity protein, NisI, to allow *L. lactis* to release nisin from the cell and prevent autoimmunity.[231]

Eight naturally occurring variants of nisin have been discovered to date - nisin A, nisin Z, nisin Q, nisin H, nisin U, nisin U2, nisin P and nisin F. Nisin A, Z, Q and F are produced by *Lactococcus* species while nisin H, U, U2 and P are produced by species of *Streptococcus*. The sequence of nisin Z only differs from nisin A by the substitution of a histidine residue in place of an asparagine at position 27 (His/Asn₂₇).[229] Nisin Q and nisin F also contain this substitution. Nisin F contains one other substitution (Ile/Val₃₀) and nisin Q contains three additional amino acid substitutions (Ala/Val₁₄, Met/Leu₂₀, Ser/Val₃₀).[55], [232] Nisin U and U2 are produced by *Streptococcus uberans* and were the first nisin variants to be discovered which contain an aromatic residue – phenylalanine (Phe).[233] Nisin U contains 9 substitutions (Ile/Lys₄, Ala/Ile₁₅, Gly/Thr₁₈, Asn/Pro₂₀, Met/Leu₂₁, His/Gly₂₇, Ser/His₂₉, Ile/Phe₃₀, and His/Gly₃₁) and lacks the three C-terminal residues. Nisin U2 includes these changes along with Ile/Val₁. Nisin P, produced by *Streptococcus gallolyticus* subsp. *pasteurianus*, differs from nisin U2 at two positions (Phe/Ala₂₀ and Leu/Ile₂₁).[234] Lastly, nisin H, which was recently isolated by O'Connor *et al* from *Streptococcus hyointestinalis*, differs from nisin A by 5 amino acids (Ile/Phe₁, Leu/Met₆, Gly/Thr₁₈, Met/Tyr₂₁ and His/Lys₃₁).[231] This nisin variant is closer to the variants produced by lactococci (A, Z, Q and F) than any of the variants previously isolated from streptococci (U, U2 and P). However, like nisin U and U2, nisin H contains aromatic residues (Phe and Tyr) which allow it to

be analysed by UV-vis adsorption analysis at 280 nm. While all of these nisin variants have been isolated, for the purpose of this research project any further reference to nisin will refer to nisin A (Figure 2-1) unless otherwise stated.

2.1.3 Properties, current uses and limitations of nisin

As mentioned earlier in this chapter, nisin has demonstrated antimicrobial activity against Gram-positive and Gram-negative pathogens. Among these pathogens are *S. aureus* (including methicillin-resistant strains), *E. coli*, *Streptococcus pneumoniae*, *Enterococcus faecium* (including vancomycin-resistant strains), *Helicobacter pylori* and *Clostridium difficile*. [209]–[211], [235] Nisin has also demonstrated activation of the adaptive immune system, exerting immunomodulatory effects, along with spermicidal and anticancer activity. [46], [62], [212] Currently, nisin is limited to its application as a food preservative based on solubility and stability issues, although detailed *in vivo* studies have not yet been carried out to completely rule out nisin as an effective antimicrobial drug treatment. In the late 1990s and 2000s, nisin was used against outbreaks of food-borne *Listeria monocytogenes*. [69] Nisin is currently used as an antimicrobial dip or spray for sliced cheese, deli meats and cut fruits. It is also incorporated into grated cheese, minced meat and beverages and used in films for packaging meat, fish and ready-to-eat meals. [236]

The solubility of nisin is reported as being high at low pH but decreasing substantially at alkaline or neutral pH. [213], [215] This decrease in solubility is due to the isoelectric point (pI) of nisin being approximately pH 8.8. [43] At this pH, there is no net charge on nisin which causes molecules of the peptide to aggregate into multimeric forms. This can further lead to precipitation of the nisin out of solution, therefore affecting solubility. Liu *et al* showed this aggregation of nisin at high pH values using gel electrophoresis which confirmed the formation of multimeric forms of nisin following 12 h incubation at pH 11. [213]

At pH 2, nisin can be stored at 4 °C for months at a time or autoclaved without a detectable loss in antimicrobial activity. [220] Antimicrobial activity is also retained up to pH 5. [222] However, above this pH, the activity of nisin is compromised. A 30% loss in the antimicrobial activity of nisin has been reported at pH 6 and a 90% loss in activity at pH 9.5. [222] Although similar antimicrobial

activity is observed for nisin Z, this variant shows decreased solubility at low pH due to the reduced charge as a result of the substitution of a histidine for an asparagine.[215] The thioether bonds, which form the lanthionine and β -methyl-lanthionine rings, along with the methionine and histidine residues are susceptible to oxidation.[237] This may pose as a problem in the formulation of nisin, especially with regards to methionine which can be oxidised by atmospheric oxygen.[95] To prevent oxidation, the use of excipients containing trace amounts of metals or peroxides (e.g. Tween 20, Tween 80, PEG) should be avoided.[77] The variant, nisin Q, has shown a reduced susceptibility to oxidation compared with nisin A due to the reduced number of methionine residues.[55]

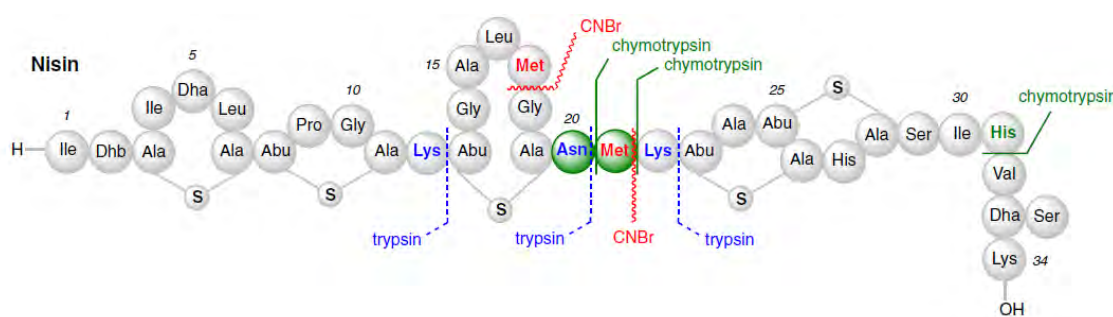


Figure 2-4. Cleavage sites of nisin by trypsin and α -chymotrypsin, as shown by Slootweg *et al.*[214]

The common degradation products reported in literature for nisin include nisin¹⁻³², Ser³³-nisin, (des- Δ Ala5)-nisin, (des- Δ Ala5)-nisin¹⁻³² and (α -X-Ala⁵)-nisin.[225], [238] These products are formed as a result of exposure to light, pH or temperature and occur at either the C-terminal or at dehydroalanine residues.[239] The dehydroalanine residues are sensitive to light-induced free-radical reactions.[213] Although no loss in antimicrobial activity is detected at low pH, the dehydroalanine residues of nisin were shown to be degraded in the presence of dilute acid, especially at high temperatures (>50 °C).[225] The degradation product, nisin¹⁻³², lacks the two C-terminal residues ($M_w \approx 3157$ Da) but despite this has shown antimicrobial activity comparable to nisin and does not appear to affect the peptide conformation.[228] For Ser³³-nisin, a serine residue is substituted in a dehydroalanine residue at position 33. This modification ($M_w \approx 3370$ Da) shows antimicrobial activity comparable to nisin A. (des- Δ Ala5)-nisin ($M_w \approx 3372$ Da) and (des- Δ Ala5)-nisin¹⁻³² ($M_w \approx 3175$ Da) have similar structures to nisin and nisin¹⁻³² respectively apart from a loss of the dehydroalanine residue at position 5. However,

the loss of this dehydroalanine residue results in the cleavage of ring A (Figure 2-1) and a decrease in antimicrobial activity by more than a factor of 100 compared to nisin A. This shows the importance of ring A in the antimicrobial activity of nisin. All the rings (A to E) of nisin were found to be crucial for its antimicrobial activity. Removal of the five C-terminal residues resulted in a 10-fold decrease in the potency of nisin. Removal of the C-terminal 14-residues, including rings D and E, led to a 100-fold decrease in potency. Cleavage or removal of ring C essentially abolished nisin activity. The nisin fragment (1-12), generated by cleavage at ring C (e.g. by trypsin), is not only inactive, but actually antagonises nisin activity.[240]

In addition to chemical and physical instability, nisin has also shown susceptibility to proteolytic degradation. In previous studies, nisin remained stable towards the gastric enzyme, pepsin, but was degraded by the intestinal enzymes, trypsin and α -chymotrypsin.[214], [217], [223] After 64 h incubation with trypsin, cleavage of nisin at Lys₁₂, Lys₂₂, His₂₈, His₃₁ and Asn₂₀ was detected by mass spectrometry following separation by RP-HPLC. In the case of α -chymotrypsin, cleavage was detected at His₃₁, Asn₂₀ and Val₃₂. [214] The bacterial protease, thermolysin, also demonstrated proteolytic activity against nisin over a prolonged period of exposure.[240]

Nisin has multiple mechanisms of action including the formation of pores in the bacterial cell membrane, the inhibition of cell wall biosynthesis and the prevention of spore outgrowth for certain *Clostridium* and *Bacillus* species.[240]–[243] To inhibit cell wall biosynthesis, the N-terminal isoleucine of nisin binds to the pyrophosphate groups of lipid II, the precursor to the peptidoglycan. Lipid II also acts as a docking station for nisin so that the C-terminus of nisin can insert itself into the bacterial cytoplasmic membrane to form pores. The resulting pores are quite stable and composed of 8 nisin and 4 lipid II molecules.[243] The mechanism behind the prevention of spore outgrowth has been attributed to the dehydrated residues which are thought to act as Michael acceptors, causing the modification of sulfhydryl groups present in the envelopes of germinated spores.[240], [242]

Nisin has shown negative interactions with the drugs, vancomycin and chloramphenicol, *in vitro*. Vancomycin, which also targets lipid II, appeared to block the membrane leakage ability of nisin.[209] However, vancomycin-resistant

enterococci, in which the structure of lipid II is modified, have still shown sensitivity to nisin. The fact that bacterial resistance against vancomycin does not result in resistance against nisin means that these two antimicrobials most likely have different binding sites on lipid II. Also, while lipid II is the target for the antimicrobial action of vancomycin, it is a docking site for nisin. Chloramphenicol and nisin were found to have an antagonistic effect on each other, when studied *in vitro*. [244] Nisin has also demonstrated synergistic effects with antibiotics such as ramoplanin and ranoloxin. This may prove useful for combination therapies to treat infection in the future. [244], [245]

2.1.4 Previous delivery strategies for nisin

In 1998, Goldberg *et al* demonstrated the excellent activity of nisin against the pathogenic bacterium *Streptococcus pneumoniae* in *in vitro* studies. However, when applied to a mouse model, nisin was degraded within 3 h. [246] Nisin has also demonstrated antimicrobial activity against other pathogenic bacteria, such as *Helicobacter pylori*, [247] *Clostridium difficile* [248] and *S. aureus*. [220] However, due to its poor *in vivo* stability, peptide engineering or suitable formulation is necessary if nisin is to be successfully translated into an antibiotic drug treatment. The strategies that have been employed in the effort to bring nisin into a clinic setting are listed in Table 2. AMBI/Astra completed phase I clinical trials with nisin in combination with omeprazole against *Helicobacter pylori* infections in 1996. However, this formulation of nisin has not progressed any further as an antimicrobial treatment since, nor has any other nisin formulation made it as far along the path towards FDA approval. [249]

Due to its widespread application in food preservation, many attempts have been made to improve the solubility and stability of nisin to prevent bacterial spoilage and the growth of food-borne pathogens. Formulation strategies that have been tested for nisin as a food preservative include nisin-containing films for antimicrobial packaging and the encapsulation of nisin into polymeric particulate systems for incorporation into food products. The nisin-containing films which have been synthesised use polymers such as low-density polyethylene (LDPE), zein, hydroxypropyl methylcellulose (HPMC), starch, poly (ethylene-co-vinyl acetate), polyvinylidene and nylon. [250]–[253] The encapsulation of nisin into polymeric particles has been carried out using polymers such as alginate, chitosan-carageenan,

lecithin and co-polymers of alginate-chitosan-pluronic.[254]–[257] Peptide engineering has also been carried out to try and improve the solubility and stability of nisin. Rollema *et al* substituted dehydroalanine for dehydrobutyrine at position 5 and added lysine residues to the sequence to improve the aqueous solubility of nisin.[215]

In 1997, Valenta *et al* first attempted to formulate nisin as an antibiotic therapy for the topical treatment of atopic dermatitis as a result of *S. aureus* infection.[258] Hydrogels made of hydroxyethyl cellulose were used as the carriers for nisin. This formulation only showed a slight reduction in antimicrobial activity after 5 weeks storage at room temperature. However, the effectiveness of this formulation *in vivo* for the treatment of atopic dermatitis was never tested. In 2007, Ugurlu *et al* attempted to formulate nisin for colon-targeted delivery following oral administration. They used a tablet with a core which contained nisin, mannitol, lactose and PVP K30 coated with HPMC/pectin in varying ratios of pectin. The aim was that nisin would be released in the colon in response to pectinolytic enzymes produced by the gut microflora. The delivery was studied *in vitro* for 2 h at pH 3.3, simulating the gastric environment, and 8 h at pH 6.8, simulating intestinal pH. After 6 h, approximating arrival at the colon, a pectinolytic enzyme was added. The coatings containing pectin alone degraded too rapidly, however, the addition of HPMC to the coatings slowed pectin degradation and showed promising results for colon-specific nisin delivery following oral administration.

Five years later, Van Staden *et al* incorporated nisin F into calcium phosphate bone cement.[259] This formulation released 72% of the loaded nisin over the first 12 h, followed by a 5% release over the following 108 h. The calcium phosphate formulations of nisin were also tested *in vivo* by implanting the material into a mouse model infected with *S. aureus*. The incorporation of nisin into the bone cement proved successful in preventing *S. aureus* colonisation on the implant. Tests also concluded that this formulation was not haemolytic and may prove useful in preventing *S. aureus* colonisation associated with calcium phosphate bone implants. In 2013, Yamakami *et al* encapsulated nisin into liposomes intended for the prevention of dental cavities.[260] The hope was that nisin would suppress the formation of glucan biofilms of *Streptococcus mutans* (i.e. plaque) on teeth and prevent cavity formation. The study showed the release of nisin over 6 h at pH 7

from the liposomes, prolonging the antimicrobial effect of nisin against *S. mutans* at this pH. This nisin formulation may prove useful in toothpastes to prevent the formation of *S. mutans* plaques and subsequent cavity formation.

In 2015, Jiang *et al* electrospun nisin-containing, nanofibrous membranes.[261] These membranes were intended for use as antimicrobial wound dressings. The nanofibrous membranes were composed of a combination of phosphorylated soybean protein isolate (PSPI), poly(lactic acid) (PLA) and zirconium dioxide (ZrO₂). An increase in the PSPI content resulted in an increased diameter of the nanofibers and an increased degradation rate. Incorporation of nisin showed no change in the size or morphology of the nanofibres. This formulation provided controlled release of nisin at pH 7.2, with retained activity against *S. aureus*. An initial burst release was observed over the first 7 h, followed by a gradual release up to 3 days. These results look promising for the topical delivery of nisin to treat surface wounds. Also in 2015, Correia *et al* incorporated nisin into PLGA by incubating the polymeric matrices with nisin.[262] This formulation provided delivery of nisin over more than 2 weeks at pH 4.5 and pH 7.4, with higher antimicrobial activity at pH 4.5. However, further studies need to be carried out to determine if this formulation would be suitable in a clinical application.

While there have been a few attempts at delivering nisin as an antibiotic, none as of yet have been successfully translated into drug therapies. The clinical trials carried out by AMBI/Astra in 1996 have presumably been abandoned as there have been no signs of the Phase II trials that were scheduled to start in 1997.[249] AMBI/Astra also received approval to start Phase I clinical trials for the use of nisin to treat *Clostridium difficile* and VRE infections but did not follow through. Research has shown that the formulation of nisin may enable its use in biomedical implants, dental hygiene products, wound healing applications and as an orally administered drug to treat infections in the colon.[182], [258]–[263] However, there is still a lot of work that needs to be done if this bacteriocin is to reach the market as a pharmaceutical formulation.

Table 2. Nisin formulations for biomedical applications reported in literature.

Formulation technique	Intended application	Reference
Hydroxyethyl cellulose hydrogel	Atopic dermatitis (topical delivery)	Valenta, 1996 [258]
Encapsulation in pectin/HPMC	Colon-specific delivery (oral delivery)	Ugurlu, 2007 [182]
Calcium phosphate	Prevention of biofilm formation on bone implants	Van Staden, 2012 [259]
Liposomal encapsulation	Prevention of dental cavities	Yamakami, 2013 [260]
Adsorption onto PEO	Antibacterial coatings for medical devices	Dill, 2013 [263]
Electrospun SPI/PLA/ZrO ₂ fibres	Wound healing (topical delivery)	Jiang, 2015 [261]
PLGA adsorption	Wound healing (topical delivery)	Correia, 2015 [262]

2.1.5 Prospective strategies for nisin delivery

In this chapter, the antimicrobial activity of Nisin AP and the effect of pH and the presence of proteases on the stability of Nisin AP were evaluated. This was carried out to predict which techniques and conditions would be most suitable in formulating nisin as an antibiotic treatment, preferably for oral administration targeting infections located within the GI tract. The stability of nisin was tested in a range of buffers to determine a suitable pH for formulation and to determine the stability in simulated gastrointestinal fluids to mimic *in vivo* conditions. The antimicrobial activity of nisin against *Lactobacillus delbrueckii* subsp. *bulgaricus*, *Listeria innocua*, *Staphylococcus epidermis* and *Escherichia coli* was tested in order to select a suitable indicator strain to determine the effectiveness of nisin

formulations in preserving the antimicrobial activity of the peptide.[214] The effect of proteases present in the gastrointestinal tract on the stability and activity of nisin as well as proteinase K, a broad spectrum protease, was also studied so that formulations could be tested for their effectiveness in protecting nisin from protease degradation.

2.2 Experimental

2.2.1 Materials

Nisin AP (95% from *Lactococcus lactis* in sauerkraut) was obtained from Handary, Belgium. Trifluoroacetic acid (TFA, $\geq 99\%$), acetonitrile (ACN, $\geq 99.9\%$), sodium taurocholate (NaTc, $\geq 95\%$), potassium phosphate monobasic (KH_2PO_4 , $\geq 99\%$), magnesium chloride (MgCl_2 , $\geq 98\%$) and L- α -lecithin ($\geq 99\%$, from egg yolk) potassium chloride (KCl, $\geq 99\%$), hydrochloric acid (HCl, 36.5-38%), acetic acid (99.8-100.5%), sodium citrate dihydrate ($\geq 99.0\%$), citric acid monohydrate ($\geq 99.0\%$), calcium chloride hexahydrate (98%), de Man, Rogosa and Sharpe (MRS) agar, MRS broth, Brain Heart Infusion (BHI) agar, BHI broth, Luria-Bertani (LB) broth, trypsin (from porcine pancreas) and proteinase K were all obtained from Sigma Aldrich Ireland Ltd. Bovine serum albumin (BSA, 96-100%), sodium bicarbonate (NaHCO_3) and Tris base ($\geq 99.8\%$) were purchased from Fisher Scientific Ireland. Sodium acetate and α -chymotrypsin (from bovine pancreas) were purchased from VWR International Ltd (Ireland). DI water was obtained from an Elga PURELAB System. *L. innocua* (DSM 20649), *S. epidermis* and *E. coli* were purchased from ATCC. *Lb. bulgaricus* (LMG6901) was a gift from Teagasc, Moorepark.

2.2.2 Nisin AP composition

Nisin AP (95%) from Handary was analysed by RP-HPLC using a similar method to Slootweg et al.[214] Analytical RP-HPLC was carried out on a Supelco LC-304 column (C_4 , 25 cm x 4.6 mm, 5 μm) using an Agilent 1260 Infinity system with UV-vis detection operating at 214 nm. The mobile phase consisted of 0.1% TFA (buffer A) and ACN containing 0.1% TFA (buffer B) using a gradient of 75:25 to 55:45 (A/B) over 35 min.

MALDI-TOF MS analysis was carried out following separation by liquid chromatography. 50 μl of Nisin AP solution (1 mg/mL in Milli Q water) was separated on a Kinetix LC-HPLC column (150 x 4.6 mm, 2.6 μm , 100 \AA). A gradient of 28:72 to 57:43 (A/B) over 35 min was used with fractions collected every minute. MALDI-TOF MS analysis was carried out on fractions of interest using an Axima TOF2 MALDI TOF mass spectrometer (Shimadzu Biotech, Manchester, UK). 0.5 μL matrix solution (α -cyano-4-hydroxycinnamic acid; 10 mg/mL in buffer A) was

deposited onto the targets for 5 s, then removed. The residual solution was allowed to air-dry and 0.5 μ L of the fractions of interest were deposited onto the coated targets. 0.5 μ L of matrix solution was added on top of the deposited sample and allowed to air-dry. The sample was analysed in positive-ion reflectron mode.

2.2.3 Activity assays

Overnight cultures of *Lb. bulgaricus*, *L. innocua*, *E. coli* and *S. epidermis* were grown up. The cultures were diluted to $OD_{595\text{ nm}} = 0.1$ with broth before incubating at 37 °C for 24 h with nisin solutions over a range of concentrations made up in 0.1% TFA. Each nisin concentration along with controls and blanks were tested in triplicate in a 96-well plate. The antimicrobial activity of Nisin AP against the bacterial strains was monitored by measuring the absorbance at 595 nm over the 24 h at 0.5 h intervals.

2.2.4 Stability testing

2.2.4.1 Impact of pH on nisin stability

To observe the effect of pH on nisin stability by RP-HPLC, nisin solutions (0.5 mg/mL) were made up in buffer A (pH 2), citrate buffer (100 mM, pH 4) and Tris acetate buffer (25 mM NaOAc, 5 mM Tris, 5 mM CaCl_2 ; pH adjusted with 100 mM acetic acid to pH 7.01; buffer composition taken from Sloatweg *et al.*).[214] The samples were stirred for 30 min to dissolve the nisin and placed on a rocker at 37 °C. After 0, 5 and 24 h, 0.5 mL aliquots (x3) of each sample were removed, filtered (0.2 μ M PES) and analysed by RP-HPLC to determine the stability of nisin at these pH values.

2.2.4.2 Impact of simulated gastrointestinal fluids on nisin stability

Simulated intestinal fluid (SIF, 6.8 mM KCl, 0.8 mM KH_2PO_4 , 85 mM NaHCO_3 , 38.4 mM MgCl_2 , 0.6 mM $\text{CaCl}_2(\text{H}_2\text{O})_6$, 15 mM HCl) was made up according to Minekus *et al.*.[264] Simulated gastric fluid (SGF, 80 μ M NaTc, 20 μ M L- α -lecithin, 34.2 mM NaCl, 25 mM HCl) was made up based on the fasted state simulated gastric fluid in a paper by Vertzoni *et al* without pepsin.[154]

To try and simulate the stability of nisin along the GI tract, nisin (0.5 mg/mL) was made up in simulated gastric fluid (SGF) and simulated intestinal fluid (SIF).

The solutions were left rocking at 37 °C and 1 mL of each sample was taken after 1, 5, 24, 72 and 96 h for RP-HPLC analysis.

2.2.4.3 Trypsin activity assay

Trypsin was tested for its activity against the substrate L-BAPNA to determine its catalytic activity of trypsin for further assays. L-BAPNA (1 mg/mL) was made up in DI water, stirring at 60 °C until dissolved. 2.5 mg trypsin was added to 20 mL 1 M HCl and stirred for 2-3 min. 0.45 g NaCl was added to 50 mL DI water. 533 µL Tris/HCl buffer (100 mM, pH 8.12) was added to 67 µL each of the trypsin and NaCl solutions in a quartz cuvette and inverted to mix. The cuvette was incubated at 25 °C for 5-10 min in a UV-vis spectrophotometer, to allow the temperature to equilibrate. 330 µL L-BAPNA solution was added, the cuvette inverted and the change in absorbance at 405 nm (ΔA_{405nm}) read over 10 min at 0.1 min intervals. This was repeated twice.

The above method was repeated in Tris acetate buffer (as described above, pH 7.01) and acetate buffer (100 mM, pH 4.06). Trypsin activity in each buffer was calculated using the following Eq. (1).

$$\text{Trypsin activity } \left(\frac{\text{U}}{\text{mg}} \right) = \frac{(\Delta A_{405nm})(V)*1000}{\epsilon cl} \quad (1)$$

Where V is the total volume added to the cuvette, ϵ is the extinction coefficient of p-nitroanilide, c is the quantity of trypsin (mg) and l is the path length (1 cm).

2.2.4.4 Impact of proteases on nisin stability

Nisin (0.2 mg/mL), trypsin (10 mg/mL) and α -chymotrypsin (2.5 mg/mL) solutions were made up in Tris acetate buffer (as described above). 0.5 mL each of the trypsin and nisin solutions was combined in an Eppendorf tube. In another Eppendorf tube, 0.5 mL each of the α -chymotrypsin and nisin solutions was combined. 0.5 mL nisin solution was added to 0.5 mL buffer as a control. The tubes were placed on a rocker at 37 °C for 2 h. After this time, the samples were centrifuged and the supernatants analysed by RP-HPLC.

2.2.4.5 Impact of prolonged trypsin exposure on nisin stability

Trypsin was added to nisin in a ratio of 2:1 (w/w) in Tris acetate buffer (as described above). This was carried out in triplicate. The tubes were placed on a rocker at 37 °C for 24 h.

After 24 h, the samples were acidified to pH 4 using 0.1 M HCl. 100 µL of each sample was added to 3 mL of an overnight culture of *Lb. bulgaricus* culture in 15 mL tubes and incubated at 37 °C for 2 h (5% CO₂). After 2 h, samples were diluted and spread on MRS agar (3 plates per dilution). The plates were incubated overnight at 37 °C (5% CO₂). The colony-forming units (CFU) on the plates containing 30-300 colonies were counted.

The remaining acidified samples were centrifuged (5000 RPM, 15 min) and the supernatants analysed by RP-HPLC.

2.2.4.6 Impact of proteinase K on nisin stability

Proteinase K (0.1 mg/mL) was added to nisin (0.5 mg/mL) in Tris.HCl buffer (200 mM, pH 7.5). A proteinase K control and a nisin control were made up in buffer. The samples were placed on a rocker at 37 °C. After 24 h, the samples were acidified to pH 2 using 1 M HCl. The acidified samples were analysed by RP-HPLC and tested against *Lb. bulgaricus* (as described for the trypsin samples). Following separation by liquid chromatography, MALDI-TOF MS analysis (as described above, Section 2.2.2) was carried out on fractions of interest.

2.2.5 Estimation of molecular dimensions

Files corresponding to the structures of trypsin (5FXL), α-chymotrypsin (2CHA) and proteinase K (5B1D) were downloaded from the Protein Database (<http://www.rcsb.org/pdb>). Since the structure for nisin A was not available alone, the structure of the nisin-lipid II complex (1WCO), as previously determined by solution NMR, was downloaded and modified to exclude lipid II.[243] Using PyMOL, the three dimensions of the enzymes and nisin were measured using the Measuring Wizard function.

2.2.6 Statistical Analysis

Comparisons were done with a one way analysis of variance (ANOVA) tests to determine significant differences in data, followed by Bonferroni post tests using OriginPro 8. Means and standard deviations were calculated using Excel.

2.3 Results and discussion

2.3.1 Nisin AP composition

RP-HPLC analysis of the as-received Nisin AP showed the presence of three components with retention times (t_r) of 9, 10 and 12 min (Figure 2-5). MALDI-TOF MS analysis of the fractions collected from a semi-preparative RP-HPLC run confirmed the molar masses (M_w) of the three components as 3370, 3352 and 3154 Da, in order of elution (Figure 2-5, inset).

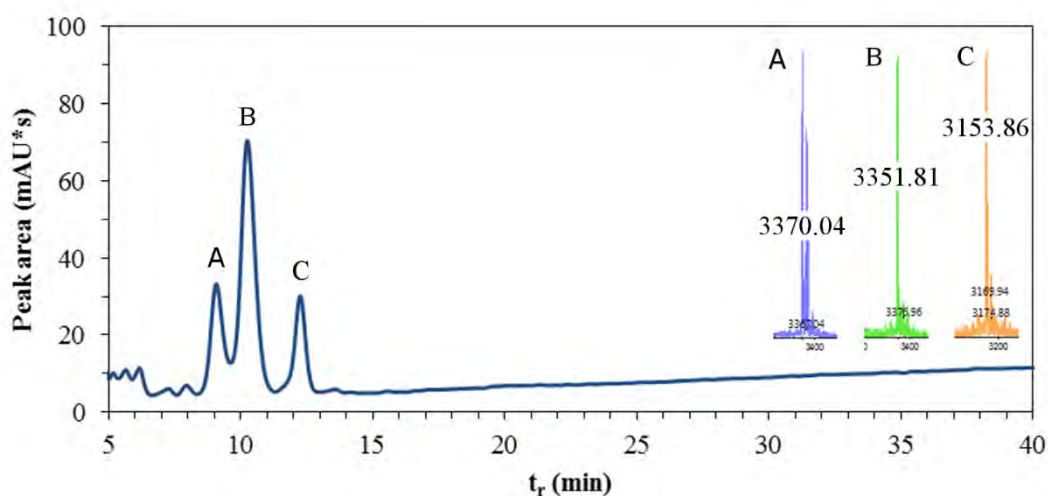


Figure 2-5. RP-HPLC chromatogram of Nisin AP (0.5 mg/mL in 0.1% TFA) with MALDI-TOF mass spectra corresponding to each component (inset).

The molar mass of the middle peak (**B**, t_r = 10 min) corresponded to that of nisin A (M_w = 3352 Da). The first component to elute (**A**, t_r = 9 min) was identified as Ser³³-nisin (M_w = 3370 Da), a degradation product reported by Rollema *et al* in which a serine residue replaces a dehydroalanine (Dha) residue at position 33.[238] This was previously reported as a minor component in commercial formulations of nisin (approximately 2.5% nisin).[214], [240] The decrease in retention time for this component is due to the more hydrophilic nature of serine compared to dehydroalanine, resulting in fewer interactions with the non-polar stationary phase (C₄). The final component to elute (**C**, t_r = 12 min) was identified as the nisin fragment (1-32) (nisin¹⁻³², M_w = 3154 Da), in which both the dehydroalanine and the lysine at the C-terminal are cleaved, as reported by Lian *et al*. [228] The structures of these components are shown below in Figure 2-6.

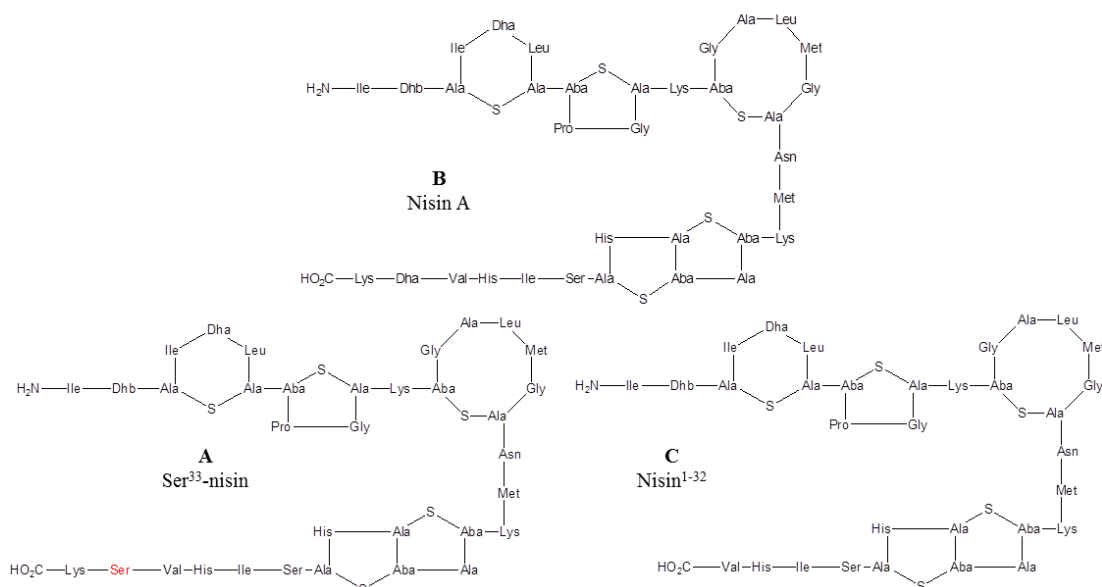


Figure 2-6. Structures of the components of Nisin AP, as identified using MALDI-TOF MS analysis of RP-HPLC fraction.

2.3.2 Activity assays

Nisin AP demonstrated activity against all three Gram-positive strains tested, *L. innocua*, *S. epidermidis* and *Lb. bulgaricus*. 325 and 650 µg/mL Nisin AP was sufficient to inhibit 50% growth of *S. epidermidis* and *L. innocua* respectively after 24 h at 37 °C, compared to controls (Figure 2-7, Figure 2-8). *L. innocua* was chosen as it is a non-pathogenic indicator for the foodborne *L. monocytogenes*. *S. epidermidis* was chosen as a non-pathogenic indicator strain for *S. aureus*. *Lb. bulgaricus* was the most sensitive to nisin activity of the four bacterial strains tested. Even at concentrations as low as 9 µg/mL, Nisin AP completely inhibited *Lb. bulgaricus* (Figure 2-9). The sensitivity of this lactic acid bacterial (LAB) strain to nisin is due to its close relation to the nisin-producing LAB, *Lactococcus lactis*.^[55]

As previously reported, *E. coli* did not show susceptibility to the antimicrobial activity of nisin. This is because the outer membrane of this Gram-negative bacterial species prevents nisin from binding to lipid II and forming pores in the cytoplasmic membrane.^[265] However, in the presence of chelators, e.g. EDTA, antimicrobial activity of nisin against Gram-negative bacteria has been demonstrated.^[266]

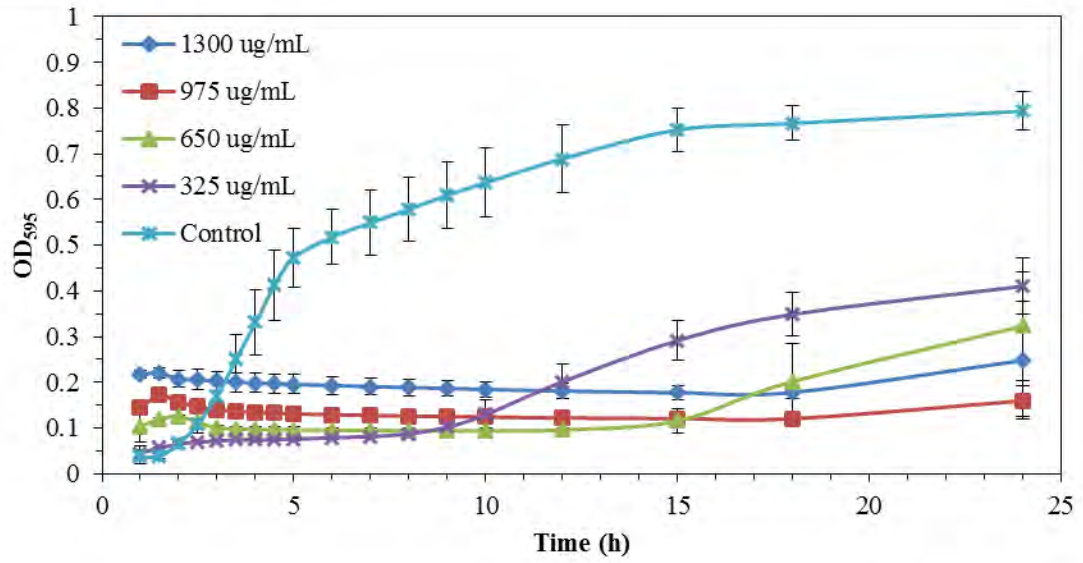


Figure 2-7. Antimicrobial activity of nisin against *S. epidermidis* over 24 h at 37 °C (y-error bars present, indicating standard deviation among six replicate wells).

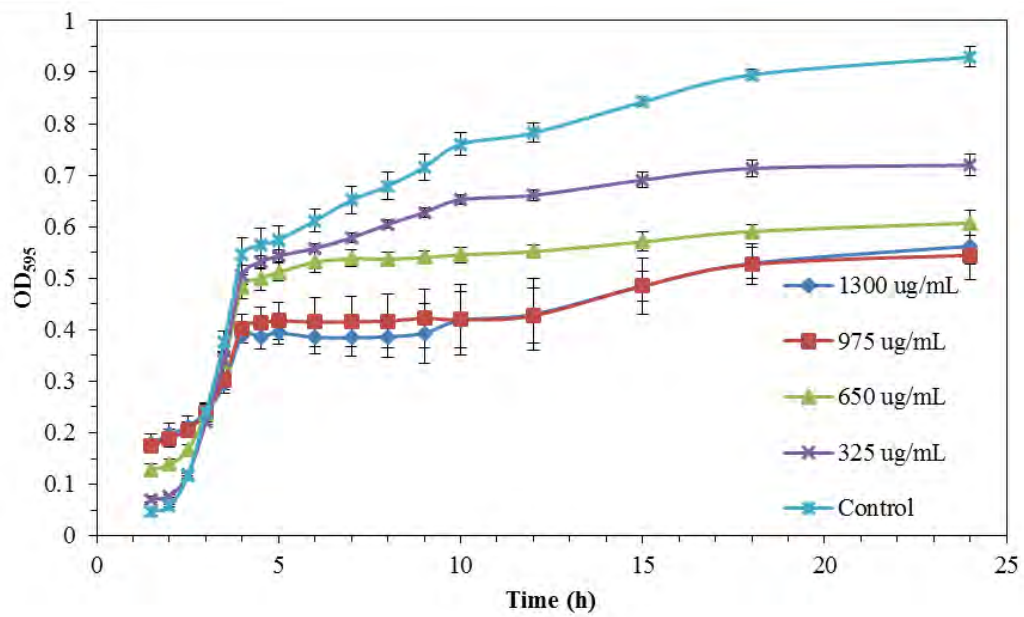


Figure 2-8. Antimicrobial activity of nisin against *L. innocua* over 24 h at 37 °C (y-error bars present, indicating standard deviation among six replicates).

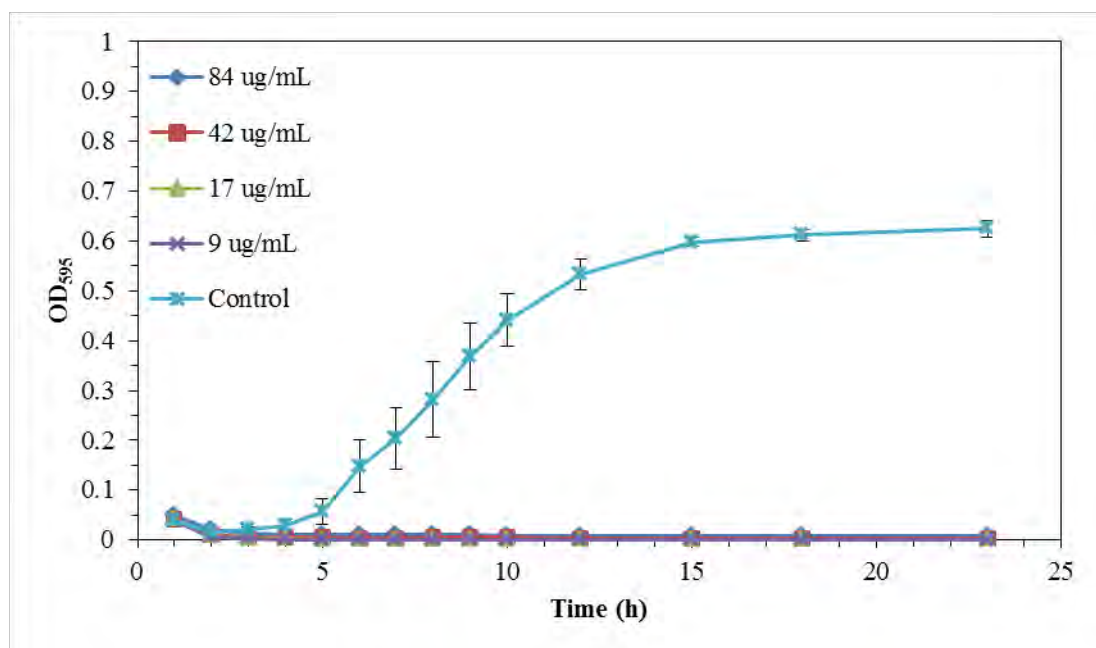


Figure 2-9. Antimicrobial activity of nisin against *Lb. bulgaricus* over 24 h at 37 °C (y-error bars present, indicating standard deviation among six replicates).

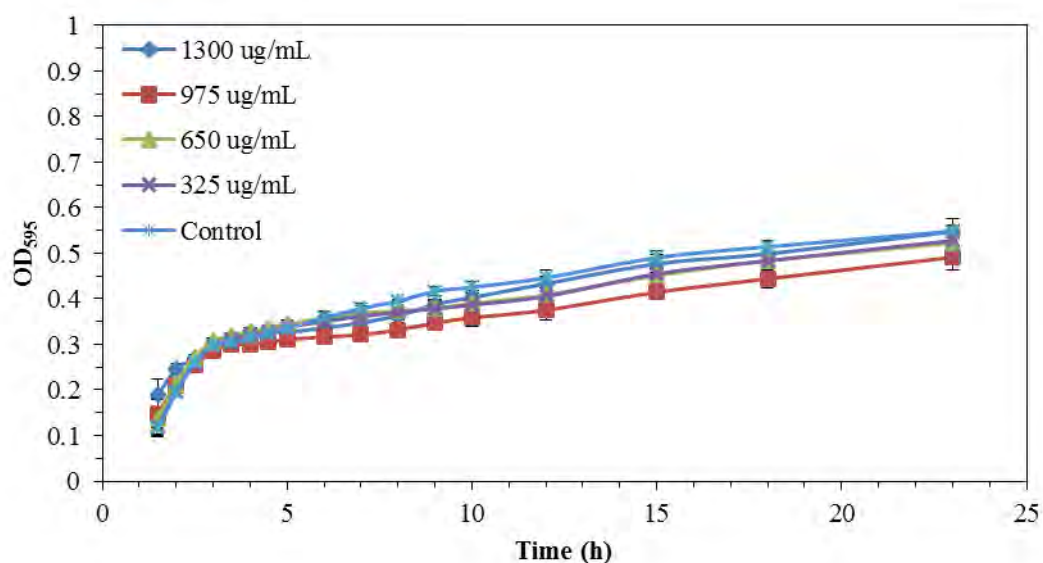


Figure 2-10. Antimicrobial activity of nisin against *E. coli* over 24 h at 37 °C (y-error bars present, indicating standard deviation among three replicates).

2.3.3 Stability testing

2.3.3.1 pH

As previously reported, nisin showed decreased stability at pH 7, with no significant change after 3 days incubation at pH 2 or 4.[213], [215] This was determined by monitoring Nisin AP solutions (0.5 mg/ml) in buffers at pH 2, 4 and 7 by RP-HPLC. The HPLC chromatograms of Nisin AP at pH 2 and pH 4 showed no

change. However, after 24 h at pH 7, a new peak ($t_r = 15$ min) had emerged accompanied by a reduction in peak areas of the three original Nisin AP components (Section 2.3.1). This new peak had the same molar mass as nisin A (3352 Da) and therefore could be an aggregated form of the peptide.

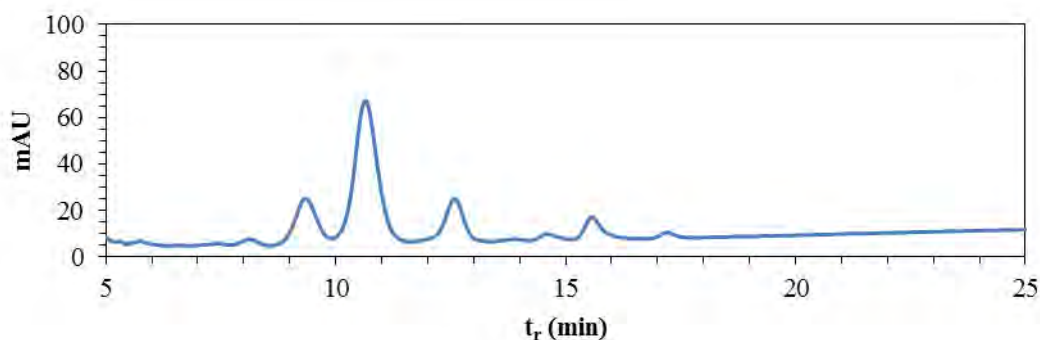


Figure 2-11. RP-HPLC chromatogram of Nisin AP after 24 h incubation at pH 7.

2.3.3.2 *Simulated gastrointestinal fluids*

To try and simulate the stability of nisin along the GI tract, Nisin AP solutions (0.5 mg/mL) were made up in SGF and SIF. After rocking at 37 °C for 4 days, Nisin AP remained stable in SGF. However, following 72 h rocking at 37 °C in SIF, Nisin AP was no longer detectable by RP-HPLC and appeared to have precipitated out of solution (Figure 2-12). This demonstrates the need for nisin formulation if it is to succeed as an orally deliverable antibiotic for infections of the GI tract.

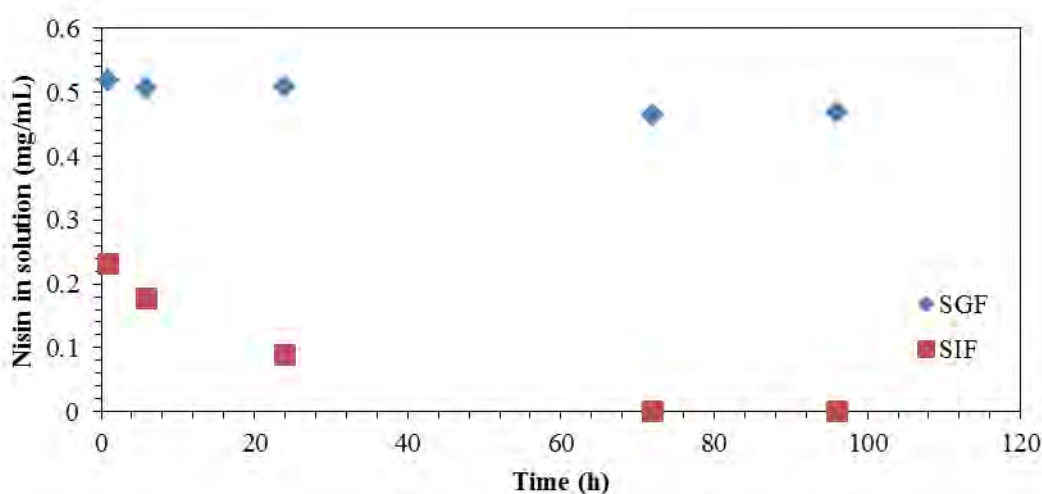


Figure 2-12. Stability of Nisin AP in SGF and SIF over time (initial nisin concentration = 0.5 mg/mL).

2.3.3.3 Trypsin activity assay

The activity of trypsin was tested at pH 4, 7 and 8, using L-BAPNA as a substrate, to investigate whether a lower pH could be used for the digestion study to prevent the stability issues observed for nisin at pH 7. Trypsin activity was found to be highest at pH 8, decreasing slightly at pH 7 and becoming negligible at pH 4 (Table 3). Since trypsin activity against L-BAPNA was negligible at pH 4, trypsin digestion studies were carried out at pH 7 in the knowledge that aggregates of nisin may form.

Table 3. Effect of pH on trypsin activity.

<i>pH</i>	<i>Trypsin activity (U.g⁻¹)</i>	<i>σ</i>
4	2.34	2.08
7	79.5	4.5
8	109.1	5.5

2.3.3.4 Protease digestion

Susceptibility to enzyme degradation is one of the major reasons that AMPs, such as nisin, have not yet been exploited for clinical use. In order to protect nisin from enzyme degradation, the effect of proteases on stability must first be understood. Previous studies have been carried out showing the susceptibility of nisin to trypsin, chymotrypsin and thermolysin and the stability of this peptide to the gastric protease pepsin.[214], [240] In this study, the effects of α -chymotrypsin and trypsin on the stability of Nisin AP were studied by RP-HPLC analysis following incubation with these proteases.

After 2 h incubation with α -chymotrypsin, the first component of Nisin AP to elute, Ser³³-nisin (t_r = 9 min), appeared to have completely degraded when analysed by RP-HPLC (Figure 2-13). However, the other two components of Nisin AP, nisin A (t_r = 10) and nisin¹⁻³² (t_r = 12 min), appeared to remain stable in the presence of this protease. Following 2 h incubation with trypsin, all three components of Nisin AP appeared to have degraded to some extent when analysed by RP-HPLC. Again, the Ser³³-nisin was fully degraded with only trace amounts of nisin A (t_r = 10) and nisin¹⁻³² detectable by RP-HPLC.

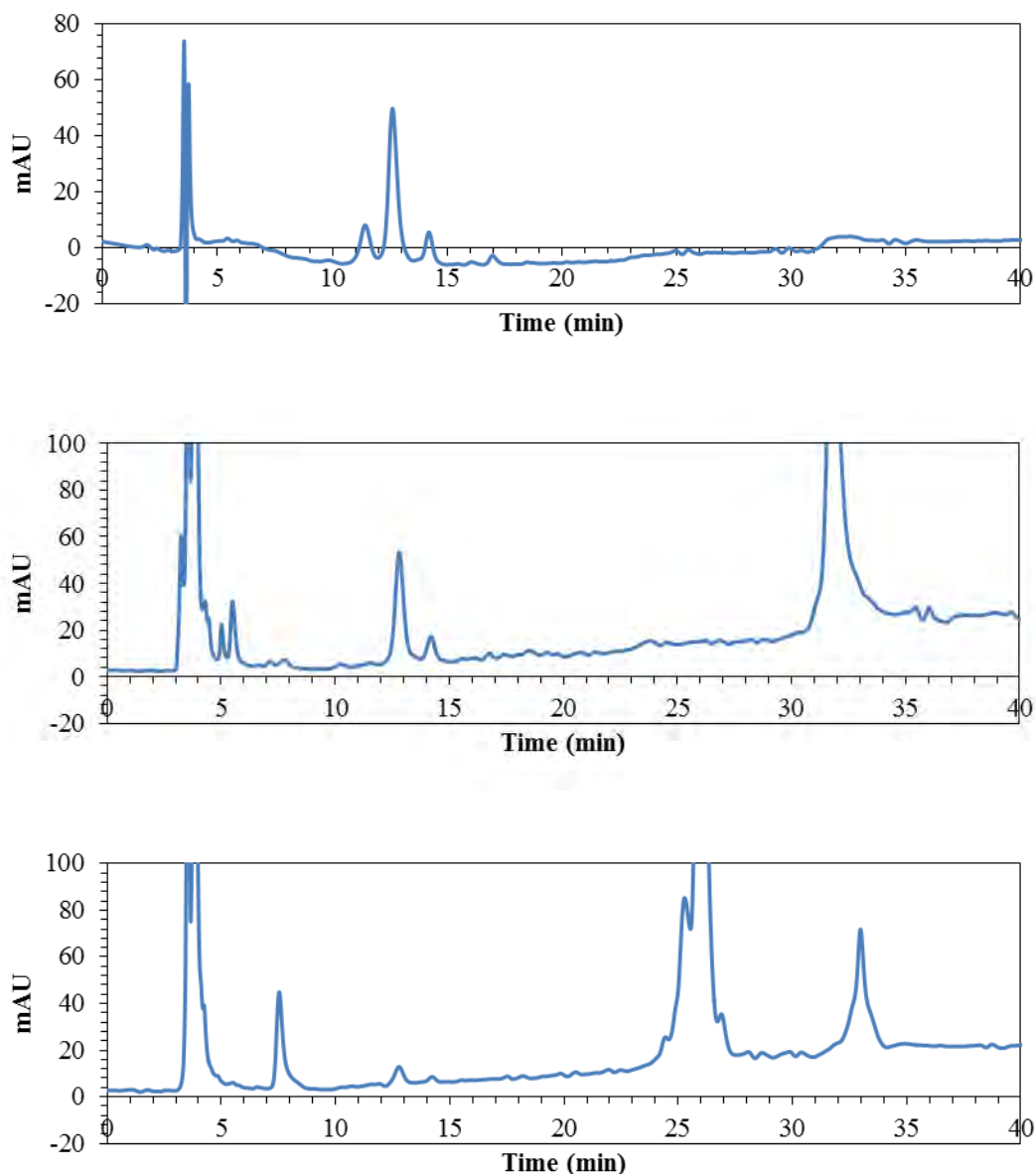


Figure 2-13. From top to bottom: (a) nisin (0.1 mg/mL), (b) nisin (0.1 mg/mL) after 2 h incubation with chymotrypsin (1.25 mg/mL) and (c) nisin (0.1 mg/mL) after 2 h incubation with trypsin (5 mg/mL) at pH 7.

2.3.3.5 Prolonged trypsin digestion

Due to the more obvious degradation profile observed for trypsin over α -chymotrypsin, the stability of nisin formulations towards trypsin was further investigated. The exposure of nisin to trypsin was repeated, extending the incubation period from 2 h to 24 h. RP-HPLC results were similar to those obtained after 2 h exposure of nisin to trypsin but after 24 h exposure to trypsin, all three Nisin AP components appeared to be completely degraded when analysed by RP-HPLC. The emergence of a new component ($t_r = 6$ min) was observed as before (Figure 2-14). The decrease in retention time indicated that this degradation product was more polar

than the original nisin components. The RP-HPLC chromatograms clearly show the digestion of all components of nisin by trypsin indicating that 24 h incubation with this protease would be suitable in determining whether the delivery matrices provide protection for nisin against trypsin degradation.

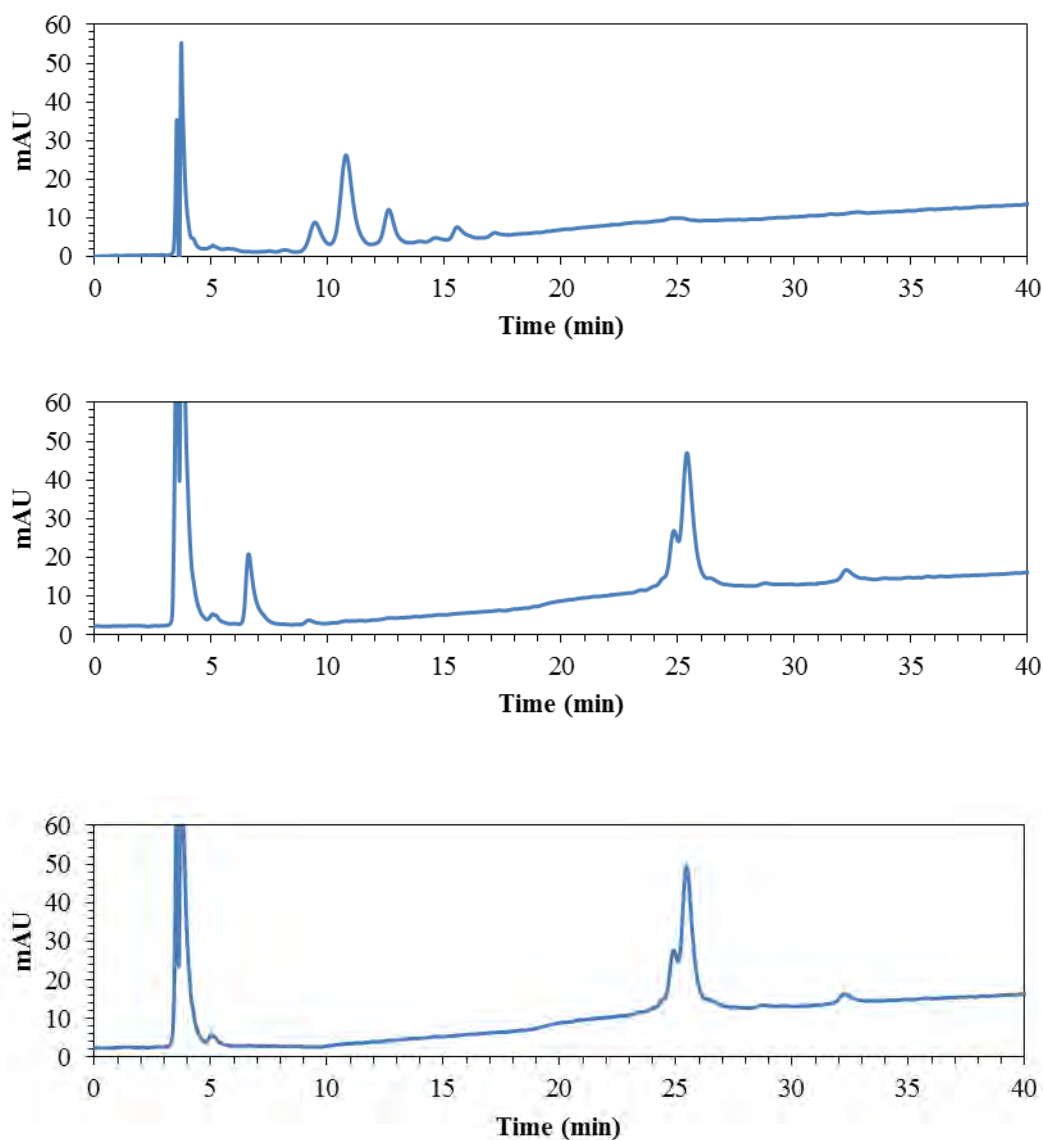


Figure 2-14. Trypsin digestion of nisin. From top to bottom: (a) nisin (0.5 mg/mL), (b) nisin (0.5 mg/mL) and trypsin (1 mg/mL) and (c) trypsin (1 mg/mL) after 24 h at pH 7.

When Nisin AP was tested for antimicrobial activity against *Lb. bulgaricus* following prolonged exposure to trypsin, only 87% of the *Lb. bulgaricus* growth was inhibited compared to the Nisin AP control (g/mL).

Table 4, Figure 2-15). While the antimicrobial activity of Nisin AP had decreased compared to the positive controls (i.e. Nisin AP without trypsin), the

trypsin-digested Nisin AP was still relatively active against *Lb. bulgaricus* compared to the negative controls (i.e. trypsin). Statistical analysis comparing the number of colonies that survived in the presence of trypsin-nisin and trypsin alone indicated that they were weakly significantly different (p-value < 0.0216). This implies that either the products of the trypsin digestion of Nisin AP are active against *Lb. bulgaricus* or that trace amounts of Nisin AP is still present at levels below the detection limit of RP-HPLC (10 µg/mL) but above the minimum inhibitory concentration against *Lb. bulgaricus* (<9 µg/mL).

Table 4. Effect of trypsin on the activity of nisin against *Lb. bulgaricus*.

<i>Sample</i>	<i>Lb. bulgaricus</i> growth (CFU.mL ⁻¹)	<i>Standard deviation</i>
Nisin	0	0
Nisin/trypsin	3.74 x 10 ⁷ *	±9.35 x 10 ⁶
Trypsin	2.34 x 10 ⁸ *	±9.26 x 10 ⁷

*p-value = 0.0216



Figure 2-15. MRS agar plates spread with *Lb. bulgaricus* after 2 h incubation with solutions of a) nisin, b) trypsin and c) nisin with trypsin; Nisin (0.5 mg/mL) had been exposed to trypsin (1 mg/mL) and pH 7 buffer for 24 h before incubation with bacteria.

2.3.3.6 Proteinase K digestion

Following 24 h incubation of Nisin AP with proteinase K (pH 7.5, 37 °C), RP-HPLC analysis showed 100% degradation of the Nisin AP component, nisin A (t_r = 10 min). RP-HPLC analysis also showed that the concentrations of the other components, Ser³³-nisin (t_r = 9 min) and nisin¹⁻³² (t_r = 12 min), had decreased by 78 and 96% respectively following digestion by proteinase K. New peaks emerged at lower retention times (t_r = 5 and 6.5 min) which most likely correspond to the degradation products produced by the digestion of Nisin AP. The component eluting at 15 min in the nisin control (previously seen for Nisin AP at pH 7, indicating

aggregation of the peptide as previously described, Section 2.3.3.1) was found to have a molar mass of 3352 Da when analysed by MALDI-TOF MS. The RP-HPLC chromatograms clearly show the digestion of nisin by proteinase K, indicating that this protease would also be suitable in determining whether the delivery matrices protect nisin against proteolytic degradation.

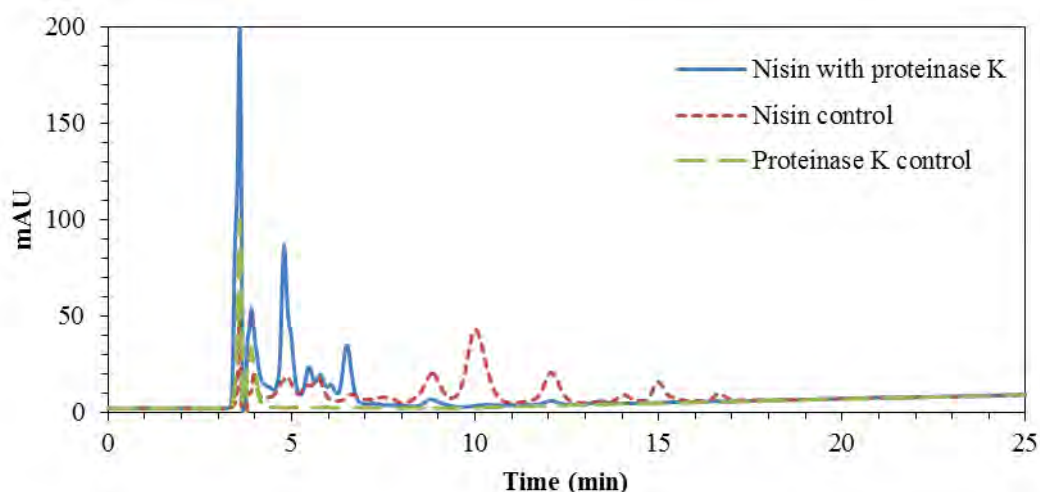


Figure 2-16. RP-HPLC chromatograms for nisin after 24 h incubation at 37 °C (red, dashed line) and in the presence of proteinase K with (blue, solid line).

Table 5. Effect of proteinase K on the activity of nisin against *Lb. bulgaricus*.

Sample	<i>Lb. bulgaricus</i> growth (CFU.mL ⁻¹)	Standard deviation
Nisin	0	0
Nisin/proteinase K	5.49 x 10 ³ *	±1.25 x 10 ³
Proteinase K	4.15 x 10 ⁸ *	±5.22 x 10 ⁷

*p-value = 1.6 x 10⁻⁴

Following exposure to proteinase K, Nisin AP still inhibited 99.9% of *Lb. bulgaricus* growth as compared to the Nisin AP control (Table 5). Statistical analysis comparing the number of colonies that survived in the presence of the nisin which had been exposed to proteinase K and proteinase K alone indicated that they were strongly significantly different (p-value < 1.6 x 10⁻⁴). This could imply that the products of the proteinase K digestion of Nisin AP are more active against *Lb. bulgaricus* than the products of the trypsin degradation. An alternative, which might also be the case for trypsin, is that trace amounts of Nisin AP are still present at levels below the detection limit of RP-HPLC (10 µg/mL) but above the minimum

inhibitory concentration against *Lb. bulgaricus* (<9 µg/mL). While trypsin appeared to be more effective in reducing the antimicrobial activity of nisin, the proportion of trypsin to Nisin AP was much higher (2:1) than proteinase K to Nisin AP (1:5). In the future, a 2:1 ratio of proteinase K to nisin could be tested to determine whether this decreases the antimicrobial activity of nisin to the same extent as trypsin. However, both show a suitable degradation profile for Nisin AP by RP-HPLC to allow delivery matrices to be tested for protecting the structural integrity of nisin.

2.3.4 Estimation of molecular dimensions

The estimated molecular dimensions for nisin (1WCO, modified to exclude lipid II) and the proteases, trypsin (5FXL), α-chymotrypsin (2CHA) and proteinase K (5B1D), are shown below in Table 6. The structure of nisin shown below in Figure 2-17 is the conformation when bound to the peptidoglycan precursor, lipid II, with the lipid II molecule edited out using PyMOL. In the absence of lipid II, nisin most likely adopts a different conformation. Therefore, the dimensions for nisin, measured using PyMOL, simply provide a rough estimate of the molecular size and shape of nisin. With these estimated dimensions for nisin and the estimated dimensions of the proteases, porous delivery matrices can be chosen for testing which are likely to accommodate nisin molecules but prevent the entry of the larger, globular protease molecules (Figure 2-17). The selective adsorption of nisin to such matrices would ideally protect nisin against degradation by these proteases.

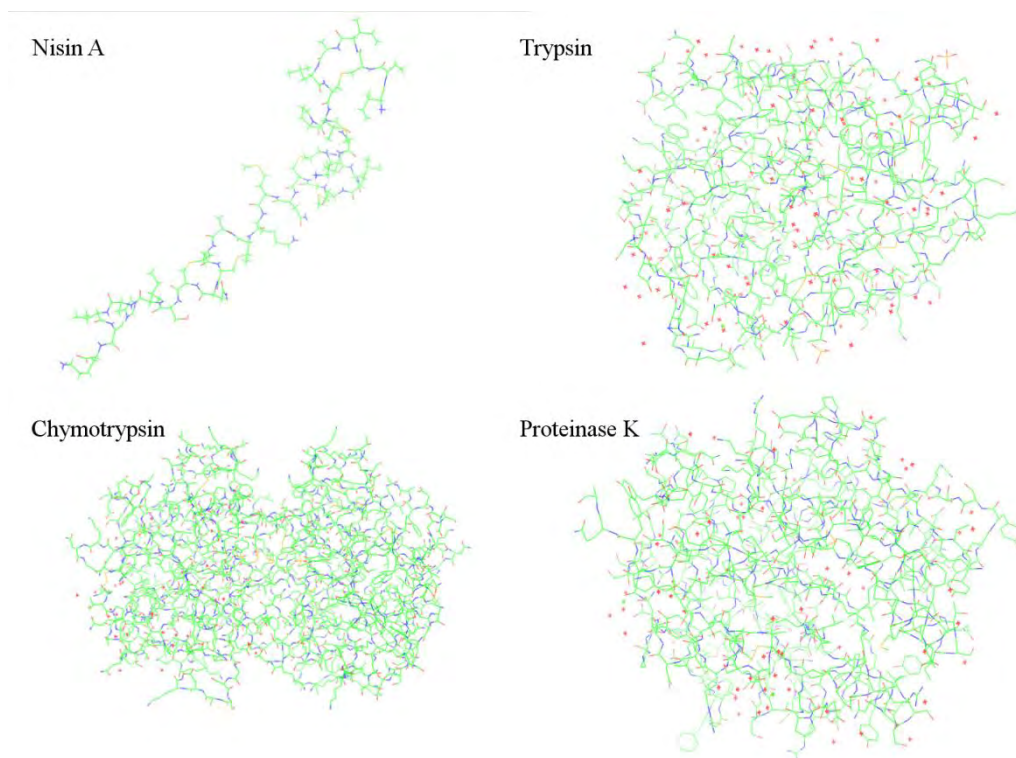


Figure 2-17. Top left to bottom right: Structures of nisin A (modified, 1WCO), trypsin (5FXL), α -chymotrypsin (2CHA) and proteinase K (5B1D) extracted from PDB viewed using PyMOL.

Table 6. Estimated molecular dimensions for nisin and the enzymes, trypsin, α -chymotrypsin and proteinase K.

<i>Molecule</i>	<i>Estimated molecular dimensions (\AA)</i>
Nisin component (from structure 1WCO)	57 x 19 x 15
Trypsin	51 x 41 x 41
α -Chymotrypsin	72 x 51 x 39
Proteinase K	51 x 45 x 39

2.4 Conclusions

Using RP-HPLC analysis, the composition of Nisin AP and the stability of its components, Ser³³-nisin, nisin A and nisin¹⁻³², in the presence of buffers and enzymes was determined. All components remained stable at low pH and in SGF. However, at pH 7, the stability of nisin A was compromised, resulting in a small degree of peptide aggregation. Stability was further compromised in SIF, with all three components precipitating out of solution over a prolonged time period. Exposure of Nisin AP to α -chymotrypsin resulted in the degradation of Ser³³-nisin,

with the other two components remaining relatively stable to this protease over 2 h. However, 24 h exposure to trypsin (2:1, trypsin/Nisin AP) and proteinase K (1:5, proteinase K/nisin) resulted in the degradation of all three components of Nisin AP by HPLC analysis. Therefore, either trypsin or proteinase K would be suitable in determining a suitable protective matrix for the oral delivery of nisin. While proteinase K is a protease synthesised by *Engyodontium album* rather than of human origin, it has broad substrate specificity.[267] This means that if a delivery matrix can be found which protects nisin against proteinase K, it will most likely protect nisin from degradation by most human GI proteases. As previously reported, Nisin AP was not active against the Gram-negative strain, *E. coli*. Nisin AP showed activity against all Gram-positive strains tested, *L. innocua*, *S. epidermidis* and *Lb. bulgaricus*. Due to the pronounced sensitivity of *Lb. bulgaricus* to Nisin AP, this Gram-positive bacterial strain was chosen to indicate the presence of Nisin AP. While the three components of Nisin AP were not tested individually for their antimicrobial activity, it has been reported in literature that Ser³³-nisin and nisin¹⁻³² exhibit similar antimicrobial activity to nisin A.[228]

3. Mesoporous silica

3.1 Introduction

While there have been some attempts made at improving the solubility and stability of nisin for pharmaceutical application, none as of yet have resulted in an FDA-approved dosage form of this AMP.[182], [258]–[263] In the last chapter, the behaviour of nisin in solution and its stability was studied to determine which factors need to be considered during formulation. The antimicrobial activity and susceptibility of nisin to protease degradation was also studied so that novel delivery systems for antimicrobial peptides could be tested for their preservation of antimicrobial activity and their protection against enzyme digestion following oral administration. This chapter will focus on the improvement of the bioavailability of nisin by using mesoporous silica (MPS) matrices as delivery systems.

MPS matrices have previously been used to deliver drug molecules and have also been applied in the areas of bone regeneration, tissue engineering and biocatalysis.[268]–[271] The advantages of using these matrices as drug delivery devices include the potential for homogenous distribution of the drug, due to the well-ordered porous structure, and the ability to control drug adsorption and release, through appropriate surface functionalisation and tuning of the pore size. In this chapter, a series of MPS matrices will be synthesised, followed by the adsorption of nisin onto these matrices. The antimicrobial activity of the MPS-nisin systems will be tested against the indicator strain, *Lb. bulgaricus*. Following adsorption, the release of nisin from the silica matrices and the ability of these matrices to protect nisin against protease degradation will be studied to determine if these materials are suitable matrices for the oral delivery of AMPs to treat local infections in the GI tract.

3.1.1 History

In 1992, the first synthesis of silica matrices with pore diameters in the mesoporous range (i.e. 20 to 500 Å) was reported by Kresge *et al* .[272] This research, conducted by the Mobil Oil Corporation, took advantage of the self-aggregation of surfactants as templates for well-ordered porous silica structures. This family of mesoporous silicates was labelled M41S and included the hexagonal MCM-41. Prior to this, single molecule templating had been the most common

method for forming inorganic porous structures, such as zeolites, cloverites, and cadoxenites. However, the pore size of inorganic structures synthesised by this method was limited to the microporous range, i.e. $\leq 20 \text{ \AA}$. [273] In 1990, Yanagisawa *et al* synthesised MPS with pore sizes of 20-40 \AA by intercalating surfactant into the layered polysilicate, kanemite. [274] However, the pores of the MPS produced by Kresge's method had the added advantage of being well-ordered with larger pore diameters, in the range of 15 to 100 \AA .

MCM-41 was initially studied for use as an adsorbent in the removal of volatile organic compounds (VOCs) in industry. However, it was found to be unsuitable for this application as high partial pressures were required for adsorption of the VOCs to occur. [273] In 1994, MCM-41 was used to adsorb metal catalysts. It was found that titanium-doped MCM-41 showed increased catalytic activity for the oxidation of alkanes compared to titanium-doped microporous silica. [275] The same year, MCM-41 was used in the area of nanoelectronics when copper-doped MCM-41 was loaded with polyaniline wires. [276] The copper in the pores of the MCM-41 oxidises aniline to form wires of the conductive polymer, polyaniline, confining the conductivity to within the pore channels of the MCM-41. In 1996, MCM-41 was used for the immobilisation of biocatalysts. This work was carried out by Diaz and Balkus who adsorbed the enzymes cytochrome c, papain and trypsin into MCM-41. [268] However, it was not until 2001 that the first report of MCM-41 as a drug delivery system was published. [269]

3.1.2 Synthesis and structure of MPS

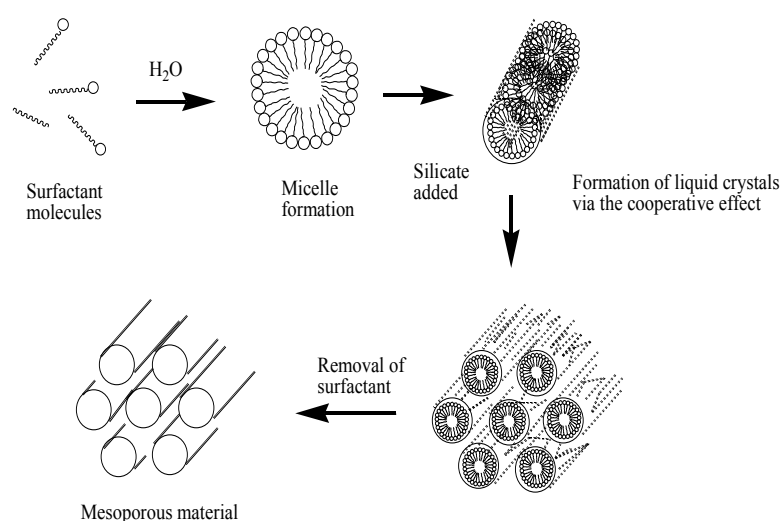


Figure 3-1. Synthesis of mesoporous silicates. [277]

Mesoporous silica matrices are generally synthesised by the surfactant template method, similar to the method used by Kresge *et al* in 1992 (Figure 3-1). This method involves the addition of a surfactant in concentrations above the critical micelle concentration (CMC). Above this concentration, the surfactant molecules aggregate to form micelles. A silica precursor added which condenses around the micelles. The surfactant template is then removed by Soxhlet extraction or calcination (under oxygen or air), leaving behind the mesoporous silica matrix. [278], [279] The resulting structure is a matrix of silica with pores of uniform size, presenting silanol groups at the surface (Figure 3-2).

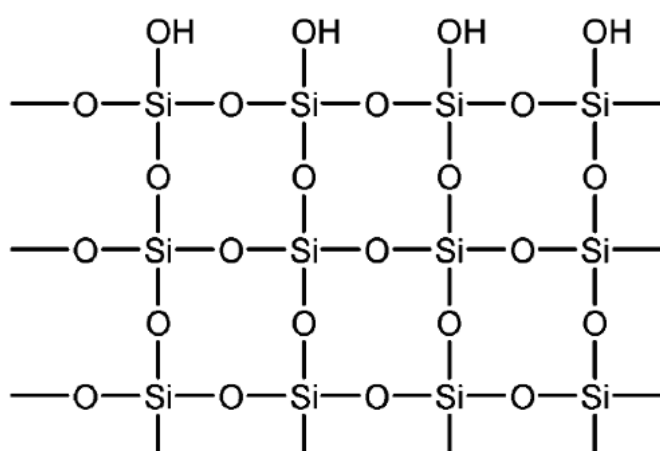


Figure 3-2. Structure of mesoporous silica.

The mesoporous silica structure can be altered by changing the type of surfactant or silica precursor used, altering the ionic strength, pH or temperature of the reaction mixture or by using additives. The reaction temperature must be above the critical micelle temperature (CMT) but, for non-ionic surfactants, below the “cloud point”, i.e. the point at which non-ionic surfactants precipitate out of solution.[280] The choice of surfactant influences the pore diameter, with surfactants of longer alkyl chain lengths creating MPS with larger pore diameters. The use of cetyltrimethylammonium bromide (CTAB) generates MPS with a smaller pore diameter than those synthesised using the Pluronic surfactants, P123 and F127.[281] Additives, such as 1,3,5-trimethylbenzene, can also be used to alter the pore size. 1,3,5-trimethylbenzene acts as a swelling agent to create an oil phase in the hydrophobic core of surfactant micelles, thereby increasing the size of the micelles and, in turn, the size of the pores in the resulting silica matrix.[282] The presence of Hofmeister anions can affect the formation of mesoporous silica materials by

changing the rate of silicate hydrolysis which has an impact on the morphology, order and porosity of the matrices.[283]

The surface chemistry of the mesoporous silica can be altered by changing the silica precursors used *in situ* or by the post-synthetic modification of surface silanol groups. The *in situ* method can be carried out either by the co-condensation of organosilanes, $(\text{RO})_3\text{Si-R}'$, with silanes or by using organosilanes alone as the silica precursor. For example, Bao *et al* used the organosilane, 1,2-bis(trimethoxysilyl)ethane (BTMSE), as a silica precursor to form the ethylene-bridged silica matrix, MSE.[284] Post-synthetic functionalisation of the mesoporous silica matrices is carried out by reacting organic molecules with the silanol groups of the formed mesoporous silica matrix. Yang *et al* used both co-condensation and post-synthetic modification to form carboxylic acid-functionalised MPS. In this work, 2-cyanopropyltrimethoxysilane (CPTES) was co-condensed with TEOS to form cyanopropyl-functionalised MPS which was then acid-hydrolysed post synthesis to form the carboxylic acid groups.[285] Co-condensation is a one-pot synthesis and is therefore preferred over post-synthetic functionalisation which is a multi-step process.[286] However, co-condensation requires both precursors to condense simultaneously to avoid the formation of two independent materials. Acid concentration is also an important factor for co-condensation. At higher acid concentrations, organosilane precursors condense at a faster rate than silane precursors due to hydrolysis. Therefore, the acid concentration must be monitored when functionalising by co-condensation to control the rate of condensation of organosilane in relation to silane molecules and produce a silica matrix with the desired degree of functionalisation.[284] Issues that may arise for post-synthetic functionalisation include low loading and uneven distribution of functional groups due to the aggregation of organic molecules at the pore surface. This aggregation can also block the pores of the MPS, reducing the available surface area for adsorption, e.g. of drug molecules. [281]

3.1.3 Application of MPS in drug delivery

In 2001, Vallet-Regi *et al* reported for the first time the use of MPS as a drug delivery system, by adsorbing ibuprofen onto MCM-41.[269] While Hata *et al* had previously shown the adsorption of taxol onto an FSM-type MPS, this was the first case where MPS was intended for use as a drug delivery system.[287] Since 2001,

MPS have been applied as delivery systems to a wide spectrum of drugs including small molecules, DNA, siRNA, proteins and peptides.[271], [288]–[290] The most commonly used MPS matrices in drug delivery to date are SBA-15 and MCM-41, both of which are unfunctionalised. MPS matrices are attractive drug delivery systems as they are inert, have a high surface area and tuneable pore size and can be functionalised to suit the drug cargo. Nanoparticles of MPS have also been shown to improve the dissolution properties of poorly water-soluble drugs by transforming the drug from a crystalline to an amorphous form and/or reducing the drug particle size.[291] The synthesis of MPS is simple, scalable, cost-effective and controllable – qualities which make it suitable for future commercialisation.[277]

The loading and release of drug molecules from MPS matrices is influenced by the pore diameter, surface area and surface functionalisation. The surface area and pore size correlate to the drug loading capacity of the MPS matrix as drug adsorption is a surface phenomenon.[292] Adsorption onto MPS matrices can occur through ionic interactions, hydrophobic interactions or hydrogen bonding. However, the interaction must be weak enough to allow the drug to be released from the delivery matrix. Functionalisation can be used to manipulate the interaction between the silica matrix and drug molecules. For example, the amine-functionalisation of SBA-15 and MCM-41 by Vallet-Regi *et al* resulted in an increased loading of alendronate.[293] These functionalised mesoporous materials also provided a slower release of alendronate compared to the unfunctionalised matrices. The method chosen for functionalisation, i.e. *in situ* or post-synthetic, can also alter the adsorption or release profile of the drug cargo. A study carried out by Zeng *et al*, demonstrated an improvement in the release profile of aspirin from aminopropyl-functionalised MCM-41 when the functionalisation was carried out by the *in situ* co-condensation method compared to post-synthetic functionalisation.[286]

MPS matrices which release drug cargo in response to stimuli such as pH, temperature, light, ultrasound, magnetic fields and chemicals have also been studied.[294]–[299] A pH-responsive MPS drug delivery system was synthesised by Yang *et al* for the delivery of the antibiotic, vancomycin. This delivery system used the functionalisation of SBA-15 with carboxylic acid groups to provide a pH-triggered release of vancomycin.[295] The delivery of vancomycin was also studied using a chemo-responsive MPS matrix. This chemo-responsive system was

synthesised by the functionalisation of MPS with thiol groups by co-condensation methods followed by capping with cadmium sulphide (CdS). Release of vancomycin was triggered by perfusion with disulphide bond reducing agents, such as mercaptoethanol, which removed the cadmium sulphide cap.[297] Light-responsive MCM-41 matrices were synthesised using β -cyclodextran and azobenzene derivatives. These matrices take advantage of the isomerisation of azobenzene from *trans* to *cis* configuration when irradiated with light of 351 nm. This isomerisation causes the dissociation of β -cyclodextran which releases the cargo. While this system was tested using dyes, it holds potential as a photo-responsive drug delivery system.[300]

MPS matrices have been studied for the delivery of many types of drugs, including analgesic, antibiotic, antihypertensive, anti-ulcer, anti-osteoporotic and anti-cancer drugs.[286], [292], [301]–[304] Some examples of these MPS delivery systems are shown below in Table 7. While the majority of adsorbed drugs to date have been small molecules, the adsorption of peptides has also been tested. Pentagastin, a pentapeptide which stimulates the production of gastric acid, was successfully adsorbed onto unfunctionalised mesoporous silica with an average pore size of 42 Å by Tourne-Petelil *et al.*[305] However, while it is suggested this adsorption may protect the peptide from enzyme degradation and/or provide controlled release, this study does not confirm these hypotheses. In 2013, Mendes *et al* adsorbed osteogenic growth peptide, a 14-residue peptide, onto unfunctionalised and calcium phosphate-functionalised mesoporous silica with an average pore size of 60 Å.[271] The release of this peptide into simulated body fluid (pH 7.4) was studied. It was found that the majority of the peptide was released over the first 9 h in a concentration-dependent manner. The antimicrobial peptide, LL-37, has also been incorporated into mesoporous silica by Izquiero-Barba *et al* and Braun *et al*. In the study conducted by Izquierdo-Barba *et al*, this 37-residue peptide was adsorbed onto both unfunctionalised and thiol-functionalised mesoporous silica matrices.[306] The thiol-functionalised matrices, with a higher degree of hydrophobicity, showed a slower release of the peptide compared with the unfunctionalised material. The adsorbed LL-37 showed very low cytotoxicity against HeLa cells and retained antimicrobial activity against *E. coli* and *S. aureus*, even after 10 months storage. In the study conducted by Braun *et al*, unfunctionalised and amino-functionalised MPS nanoparticles were used to adsorb LL-37.[307] LL-37 adsorbed to a much greater

extent onto the unfunctionalised silica compared to the amino-functionalised silica. This was likely due to the net positive charge on both the amino-functionalised silica and the LL-37 at this pH. A slow release was observed at pH 7.4 with approximately 17% release after 24 h. In this study, the proteolytic effect of both bacterial and human neutrophil elastase on the adsorbed peptide was also tested. It was found that the unfunctionalised silica nanoparticles offered partial protection against proteolysis by both elastases.

The biocompatibility of micron-sized MPS matrices was studied by Hudson *et al* in mice following intraperitoneal, intravenous and subcutaneous administration.[308] Administration via the intraperitoneal and intravenous route resulted in the death or euthanasia of the mice. This systemic toxicity was thought to be due to thrombosis. However, subcutaneous administration showed no signs of toxicity, indicating the potential for administration of MPS drug delivery systems via this route. Kupferschmidt *et al* tested the oral delivery of micron-sized MPS with good biocompatibility.[309] A study by Lopez *et al* demonstrated the biocompatibility of MPS as a drug delivery implant inserted into the brain tissue of rats. Necrosis and inflammation were not detected in response to MPS administered by this route.[310]

Table 7. Examples of MPS delivery systems reported in literature.

<i>Drug</i>	<i>MPS type</i>	<i>Intended application</i>	<i>Reference</i>
Alendronate	Amine-functionalised silica	Bone scaffolds	Nieto, 2008 [303]
Amikacin	Pure silica and aluminosilica	Drug delivery (unspecified route)	Nastase, 2013 [290]
Amoxicillin	Amine-, thiol- and methyl-functionalised silica	Drug delivery (unspecified route)	Sevimli, 2012 [301]

<i>Drug</i>	<i>MPS type</i>	<i>Intended application</i>	<i>Reference</i>
Aspirin	Amine-functionalised silica	Drug delivery (unspecified route)	Zeng, 2006 [286]
Camptothecin	Amine- and phosphate-functionalised silica	Drug delivery (unspecified route)	Lu, 2007 [311]
Captopril	Trimethyl silyl-functionalised silica	Drug delivery (unspecified route)	Qu, 2006 [292]
Ceftaroline	Pure silica	Antimicrobial coating for biomedical implants	Radulescu, 2016 [312]
Cephalexin	Amine-functionalised silica	Drug delivery (unspecified route)	Legnoverde, 2013 [313]
Cisplatin	Amine-functionalised silica conjugated to folic acid	Drug delivery (targeting cancer cells)	Pasqua, 2007 [304]
Cytochrome c	Citraconic amide-functionalised silica	Drug delivery (pH-dependent)	Park, 2010 [314]
Diflunisal	Pure silica	Drug delivery (unspecified route)	Heikkila, 2007 [315]

<i>Drug</i>	<i>MPS type</i>	<i>Intended application</i>	<i>Reference</i>
Doxorubicin	Poly(acrylic acid)-coated silica	Drug delivery (unspecified route)	Yuan, 2011 [316]
Famotidine	Carboxylate-functionalised silica	Drug delivery (unspecified route)	Tang, 2005 [302]
Gemcitabine	Carboxylate-functionalised and amine-functionalised silica	Drug delivery (unspecified route)	Bahrami, 2015 [317]
Gentamycin	Pure silica	Drug delivery (unspecified route)	Doadrio, 2004 [318]
Ibuprofen	Pure silica	Drug delivery (unspecified route)	Vallet-Regi, 2001 [269]
LL-37 (peptide), Chlorhexidine	Pure silica and thiol-functionalised silica	Antimicrobial coating for biomedical implants	Izquierdo-Barba, 2009 [306]
LL-37 (peptide)	Pure and amine-functionalised silica	Drug delivery (unspecified route)	Braun, 2016 [307]
Naproxen	Amine-functionalised silica	Drug delivery (unspecified route)	Halamova, 2012 [319]

<i>Drug</i>	<i>MPS type</i>	<i>Intended application</i>	<i>Reference</i>
Osteogenic growth peptide (peptide)	Pure silica and hydroxyapatite-functionalised silica	Bone regeneration	Mendes, 2013 [271]
Pentagastrin (peptide)	Pure silica	Drug delivery (unspecified route)	Tourne-Petelilh, 2003 [305]
Quercetin	Amine-functionalised silica conjugated to folic acid	Drug delivery (targeting cancer cells)	Sarkar, 2016 [320]
Telmisartan	Pure silica and amine-functionalised silica	Drug delivery (oral route)	Zhang, 2010 [321]
Tetracycline	Pure silica	Drug delivery (unspecified route)	Koneru, 2015 [322]
Valproic acid with sodic phenytoin	Pure silica	Drug delivery (brain implant)	Lopez, 2006 [310]
Vancomycin, Rifampicin, Linezolid	Pure silica	Antimicrobial coating for implants	Molina-Manso, 2012 [323]

3.1.4 Potential use of MPS as drug delivery system for nisin

In this chapter, adsorption of the antimicrobial peptide, nisin, onto MPS matrices was studied along with the effectiveness of these matrices in improving nisin stability under conditions simulating the gastrointestinal environment. The MPS matrices SBA-15 and MCM-41 (unfunctionalised), PMO-PA (amino-functionalised) and MSE (ethylene-functionalised) were investigated in this

respect.[281], [284], [288], [324] Due to its cationic nature, it was expected that nisin would show a higher adsorption onto the unfunctionalised silica compared to the amino-functionalised material, as seen for LL-37 by Braun *et al.*[307] Simulated gastric fluid (SGF) and simulated intestinal fluid (SIF) were used to study the release of the adsorbed nisin from the matrices. Proteinase K was used to determine if any of these MPS matrices would provide suitable protection of nisin against protease degradation.

3.2 Experimental

3.2.1 Materials

Nisin AP (95%) was purchased from Handary, Belgium. Tetraethoxysilane (TEOS), bis[3-(trimethoxysilyl)propyl]amine ($\geq 90\%$), 1,3,5-trimethylbenzene (98%), KCl, HCl (36.5-38%), sodium hydroxide (NaOH), citric acid monohydrate, sodium citrate dehydrate, $\text{CaCl}_2 \cdot 6\text{H}_2\text{O}$, acetic acid (99.8-100.5%) ethanol (EtOH), methanol (MeOH), MRS broth, MRS agar, Pluronic P123, Pluronic F127, bis(trimethoxysilyl)ethane (BTMSE), cetyltrimethylammonium bromide (CTAB), sodium taurocholate (NaTc, $\geq 95\%$), potassium phosphate monobasic (KH_2PO_4 , $\geq 99\%$), magnesium chloride (MgCl_2 , $\geq 98\%$) and L- α -lecithin ($\geq 99\%$, from egg yolk) were all purchased from Sigma Aldrich Ireland Ltd. Tris base was purchased from Fisher Scientific Ireland and sodium acetate was obtained from VWR International Ltd (Ireland).

3.2.2 MPS synthesis and characterisation

3.2.2.1 SBA-15

SBA-15 was synthesised by a method previously described by Sayari *et al.*[324] 4 g Pluronic P123 was weighed into a conical flask. 30 mL DI water and 120 mL 2 M HCl was added. The mixture was stirred vigorously at 35 °C until the P123 had dissolved. 9.11 mL TEOS was added under vigorous stirring and stirring continuously for 5 min. Following this, stirring was stopped and the mixture was maintained at 30 °C for 20 h.

After 20 h, the reaction mixture was autoclaved at 130 °C for 24 h. The crude product was vacuum-filtered and washed with DI water and EtOH. This was left to dry in a desiccator for 2-3 days. The P123 template was removed by 2 x 8 h Soxhlet extractions using EtOH. The resulting product was dried in a desiccator to yield a dry white powder (2.57 g).

3.2.2.2 MSE

MSE was synthesised using the method described by Bao *et al* ($\text{HCl}/\text{H}_2\text{O} = 2.54 \times 10^{-4}$).[284] 2 g Pluronic P123 was dissolved in 28 mL DI water, stirring at 40 °C. 2.62 mL BTMSE was added to 28 mL DI water and 4.8 mL 1M HCl, stirring at

40 °C. The P123 solution was added slowly to the BTMSE solution. The reaction mixture was covered with parafilm and stirred at 40 °C for 24 h.

After 24 h, the reaction mixture was autoclaved at 100 °C for 5 days. The product was then vacuum-filtered and washed with DI water and EtOH. This was left to dry overnight. The P123 template was removed by 2 x 5 h Soxhlet extractions using EtOH. The resulting product was dried in a desiccator and further dried in an oven at 60 °C to yield a white fluffy powder (1.42 g).

3.2.2.3 PMO-PA

PMO-PA was prepared by a method previously described by Hudson *et al.*[281] 2.5 g Pluronic F127, 12.5 g KCl, 3.47 mL trimethylbenzene and 150 mL 2M HCl were added to a conical flask and stirred at 15 °C for around 1 h. 11.15 mL TEOS and 5.45 mL bis[3-(trimethoxysilyl)propyl]amine were combined and added to the F127 solution. The reaction mixture was stirred at 15° for 24 h.

After 24 h, the reaction mixture was autoclaved at 100 °C for 24 h. The product was then filtered under vacuum and washed with DI water. The resulting white powder was added to 200 mL 2 M HCl and then autoclaved at 140 °C for 48 h. The F127 template was removed by 2 x 8 h Soxhlet extractions using EtOH. The product was stirred in DI water for 4 h to remove salts ions, followed by filtration and drying to yield a white powder (7.99 g).

3.2.2.4 MCM-41

MCM-41 was prepared by the method previously described by Radu *et al.*[288] 1 g CTAB was added to 3.5 mL 2 M NaOH in 480 mL DI water and stirred vigorously at 80 °C for 1 h. While stirring, 5 mL TEOS was added dropwise over approximately 5 min resulting in the formation of a white precipitate. This was stirred for a further 2 h at 80 °C. The precipitate was vacuum filtered and washed with DI water and MeOH. The precipitate was left to dry overnight before transferring to a clock glass and further dried in a desiccator.

The dried precipitate was added to 18 mL 12 M HCl in 380 mL MeOH and refluxed at 80 °C for 5 h twice. The powder was then vacuum filtered, washed with MeOH and dried. The dried powder was calcined under air at 500 °C for 6 h (ramping speed = 1 °C/min) to yield a fine white powder (0.87 g).

3.2.2.5 *Characterisation of silica matrices*

Characterisation of the silica matrices was carried out by attenuated total reflectance Fourier transform infrared spectroscopy (ATR-FTIR), scanning electron microscopy (SEM), transmission electron microscopy (TEM), N₂ adsorption analysis, powder X-ray diffraction (P-XRD), measuring the zeta potential and by solid state-nuclear magnetic resonance (SS-NMR),

ATR-FTIR analysis of the samples was carried out on an Agilent Cary 630 FTIR Spectrometer. Samples for SEM were prepared by mounting on double-sided carbon tape followed by gold coating (1.5 min, 20 mA, Emitech K55). SEM analysis was carried out on a Hitachi SU-70 system (accelerating voltage = 10 kV). For TEM analysis, samples were ground into a fine powder with a pestle and mortar, a few drops of IPA added and the suspension dropped onto carbon-coated copper TEM grids (200 mesh). TEM analysis was carried out on a JEOL JEM 2011 system at 200 kV. Nitrogen adsorption analysis was carried out on a Tristar II Plus surface area and porosity analyser at 77 K. P-XRD patterns were obtained on a PANalytical Empyrean diffractometer using Cu K α radiation at 2 θ angles of 0.5 to 8°. Prior to measuring the zeta-potential, the samples were sonicated for 10-15 min. The zeta potential was measured at pH 4 (citrate buffer; 0.1 M) and pH 7 (Tris acetate buffer; 25 mM NaOAc, 5 mM Tris, 5 mM CaCl₂; adjusted to pH 7 with 0.1 M acetic acid) on a Malvern Zetasizer Nano ZSP (Attn. 6 and 9).

¹³C CPMAS and ²⁹Si MAS SS-NMR analysis was carried out on a Bruker 400 MHz. A triple resonance probe (4 mm) in double resonance mode with a proton frequency of 400.14 MHz, a carbon frequency of 100.6MHz and a silicon frequency of 79.49 MHz was used for analysis. Optimisation of the magic angle was carried out by spinning a rotor packed with KBr at 5 kHz. Adamantane was spun at 10 kHz and the carbons were directly excited with decoupling from carbon. The chemical shift of the low field peak of adamantane was set to 38.48 ppm by adjusting the magnetic field. The magnet was shimmed so that this low field peak was symmetric with a peak width of less than 2 Hz. Glycine was then used to check that the signal to noise ratio was acceptable. ²⁹Si spectra were collected at a silicon frequency referenced to the low field peak of 3-(trimethylsilyl)propane-1-sulfonic acid (DSS) -23.1 ppm.

For SS-NMR analysis, MPS were packed into 4 mm zirconia rotors. Samples were spun at a rate of 10 kHz. Proton spin lattice relaxation times (T₁) were determined using a direct saturation recovery pulse sequence. To optimise contact time for cross polarisation conditions, ¹³C CPMAS experiments were run. Cross polarisation conditions were conducted at a ramp of 50 % - 100 % and spinal64 decoupling at 100 %. A recycle delay of 3 s, contact times between 1-1.5 ms were used and 1024 scans were collected for all samples. ²⁹Si MAS experiments were collected with proton decoupling and a recycle delay of 200 seconds. 300-500 scans were collected per sample. Line broadening of 50 Hz was applied to all spectra.

3.2.3 Nisin-loaded MPS

0.001, 0.005, 0.01, 0.05, 0.1, 0.2, 0.5 and 1.0 mg/mL solutions of Nisin AP were made up using buffer A (0.1% TFA). These solutions were analysed by RP-HPLC (as described in Chapter 2) and a calibration curve was constructed using the peak area which corresponded to nisin A (t_r = 10 min).

In preliminary studies, nisin solution (0.5 mg/mL) was made up in citrate buffer (100 mM, pH 4.01). 200 mg mesoporous silica (MPS) was transferred to a glass vial and 20 mL nisin solution was added. The remaining nisin solution was used as a control. Both the test and the control samples were sonicated until the MPS appeared evenly dispersed. While stirring, 15 x 1 mL aliquots of the test sample were transferred to Eppendorf tubes. This was repeated for the nisin control sample. All samples were placed on a rocker at 37 °C. After 0.5 h, three tubes of each sample were removed from the rocker, centrifuged (5000 RPM, 15 min) and the supernatants analysed for nisin content by RP-HPLC. The pellets were left to air dry at room temperature. This was repeated at 1, 3, 6 and 21 h.

To determine the effect of pH and ionic strength on adsorption, slurries containing 0.5 mg/mL nisin with 10 mg/mL SBA-15 (or MSE) were made up in a series of buffers to determine the effect of pH and ionic strength on adsorption. In this experiment, the nisin solution (0.5 mg/mL) was added to the dry silicate to ensure 10 mg/mL MPS was present in each sample. The slurries were sonicated for 20 min and placed rocking at 37 °C for 5 h. After 5 h, the slurries were centrifuged (5,000 RPM, 15 min) and the supernatants were analysed for nisin content by RP-HPLC. The nisin calibration curve was used to determine the amount of nisin in the

supernatant. The amount of nisin adsorbed to the mesoporous silica was then calculated by subtracting the nisin in the supernatant from the initial nisin in solution.

To determine the effect of pH, the slurries described above were made up in KCl/HCl buffer (105 mM, pH 2), citrate buffer (600 mM, pH 4) or Tris acetate buffer (5 mM Tris, 5 mM CaCl₂, 25 mM NaOAc). This was carried out in triplicate in each buffer. The effect of ionic strength on nisin adsorption was determined by making up buffers of different ionic strengths. KCl/HCl buffer was made up at 52, 105 and 677 mM. Citrate buffer was made up at 150, 300 and 600 mM. Tris acetate buffer was made up at 45, 90 and 500 mM. The ionic strength was calculated using Eq. (2). [325]

$$I = \frac{1}{2} \sum_{i=1}^n c_i z_i^2 \quad (2)$$

The optimal conditions for adsorption of nisin onto SBA-15 and MSE were applied to MCM-41. Adsorption was determined by analysing the nisin remaining in solution by RP-HPLC.

To construct an adsorption isotherm for nisin onto MSE, 0.2, 0.5, 1.0, 2.0 and 3.0 mg/mL solutions of Nisin AP were made up in KCl/HCl pH 2 buffer (677 mM) and used to make up slurries with MSE (10 mg/mL). The slurries for each nisin concentration were made up in triplicate, sonicated for 20 min and placed rocking at 37 °C for 5 h. After 5 h, the slurries were centrifuged (5,000 RPM, 15 min) and the supernatants were analysed for nisin content by RP-HPLC.

3.2.4 Release study

Simulated intestinal fluid (SIF, 6.8 mM KCl, 0.8 mM KH₂PO₄, 85 mM NaHCO₃, 38.4 mM MgCl₂, 0.6 mM CaCl₂(H₂O)₆, 15 mM HCl) was made up according to Minekus *et al.* [264]. Simulated gastric fluid (SGF, 80 µM NaTc, 20 µM L-α-lecithin, 34.2 mM NaCl, 25 mM HCl) was made up based on the fasted state simulated gastric fluid reported by Vertzoni *et al.* [154].

20 mg of SBA-15-nisin (adsorbed at pH 2, $I = 677$ mM; 31.0 µg/mg loading) was weighed into 6 tubes. 1 mL SGF was added to three of the tubes. 1 mL SIF was added to the other three tubes. Controls were made up by adding SGF or SIF to 20

mg SBA-15. The tubes were placed on a rocker at 37 °C. After 1h, the samples were centrifuged (5000 RPM, 15 min). 0.5 mL supernatant was removed to analyse the nisin that had released by RP-HPLC. 0.5 mL fresh media (SGF/SIF) was added to the samples and the tubes were returned to the rocker at 37 °C. This was repeated after 5, 24, 48 and 72 h. For release into SGF, samples were also taken at 96 and 120 h.

When no further nisin release was detected by RP-HPLC, the antimicrobial activity of the supernatant and the remaining pellet suspension was tested against *Lb. bulgaricus*. 100 µL of the supernatant or pellet suspension to be tested was added to 3 mL of an overnight culture of *Lb. bulgaricus* (pre-diluted to OD₅₉₅ = 0.4 using MRS broth). The samples were incubated with the cells for 3 h at 37 °C (5% CO₂). After this incubation period, the samples were diluted and plated. After 24 h, the activity of the sample against *Lb. bulgaricus* was determined by counting the colony-forming units (CFU).

This release study was repeated for MCM-41-nisin (32.6 µg/mg loading) and MSE-nisin (31.4 µg/mg loading).

3.2.5 Proteinase K digestion study

15 mg SBA-15-nisin (31.0 µg/mg loading) was added to six tubes. 90 µL proteinase K solution (1 mg/mL) was added to three of these and the total volume in all six tubes was made up to 1 mL with Tris.HCl buffer (200 mM, pH 7.5). The samples were placed on a rocker at 37 °C. After 24 h, the supernatants were removed for RP-HPLC analysis and the pellets were washed twice with DI water. 10 µL 1M HCl was added to the washed pellets to stop proteinase K activity. To release the adsorbed nisin, 1 mL SGF was added to each tube and the samples were placed on a rocker at 37 °C for 5 days. After 5 days, the samples were centrifuged and the supernatants analysed by RP-HPLC to determine the effectiveness of the silica matrices in protecting nisin against proteolysis.

This was repeated for MCM-41-nisin (32.6 µg/mg loading) and MSE-nisin (31.4 µg/mg loading).

3.3 Results

3.3.1 MPS synthesis and characterisation

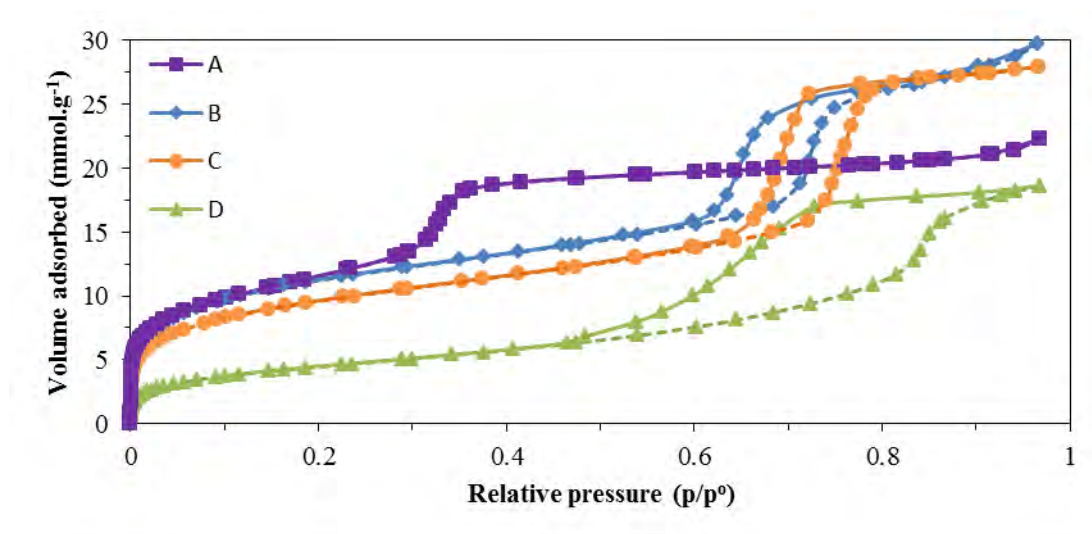


Figure 3-3. Nitrogen adsorption (dashed line) and desorption (solid line) isotherms for (A) MCM-41 (B), MSE (C), SBA-15 (D) and PMO-PA.

The mesoporous silica matrices SBA-15, MCM-41 MSE and PMO-PA were synthesised successfully by the surfactant templating methods reported in literature.[281], [284], [288], [324] The adsorption/desorption isotherms for all four MPS matrices synthesised (MCM-41, MSE, SBA-15 and PMO-PA) were Type IV isotherms with a H1 hysteresis loop, characteristic of mesoporous materials (Figure 3-3). The capillary condensation pressure, i.e. the relative pressure at which the steep increase in nitrogen adsorption occurs, of each porous matrix is correlated to the pore diameter. The hysteresis loop for MCM-41 was undetectable due to the narrow pore size (<4 nm).[280] The average pore diameters for the MPS matrices were calculated from the desorption curves, which slightly underestimate the values, using the Barrett-Joyner-Halenda (BJH) theory.[326] SBA-15, PMO-PA and MSE all showed similar average pore diameters (68, 59 and 60Å respectively) with MCM-41 presenting a smaller value (28 Å) (Figure 3-4). The smaller pore size of MCM-41, may allow for the adsorption of long, narrow molecules of nisin (57 x 19 x 15 Å), but prevent the entry of the globular enzyme, proteinase K (51 x 45 x 39 Å) (Figure 2-17). The narrow hysteresis loop for MCM-41 indicates that the pores of this silica matrix have a narrow size distribution. From this, it can be determined that the pore size distribution of SBA-15 and MSE is slightly broader with PMO-PA having quite

a broad pore size distribution. The broad pore size distribution for PMO-PA can be attributed to the disordered porous array of this material.

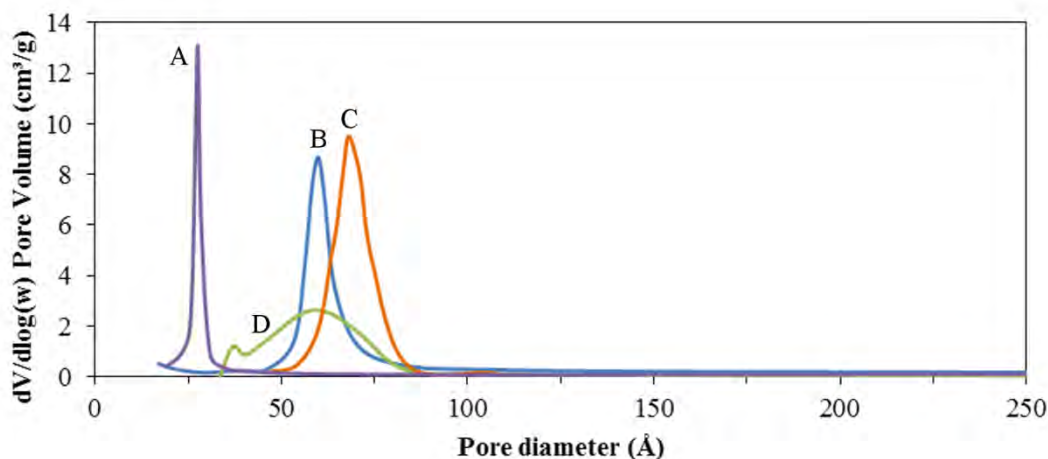


Figure 3-4. Average pore diameters (calculated from the desorption curve using BJH theory) for MCM-41 (A), MSE (B), SBA-15 (C) and PMO-PA (D).

The disordered pores of PMO-PA were further confirmed by TEM (Figure 3-6) and by the absence of peaks when this material was analysed by P-XRD (Figure 3-5). This is most likely due to the co-condensation method used to synthesise this material. While this method of functionalisation provides better functional group uniformity and more stable products than post-synthetic grafting, the order of the mesopores is compromised.[327] P-XRD patterns of the other materials, MCM-41, MSE and SBA-15, contained peaks corresponding to the (100, 110 and 200) planes which confirmed their ordered hexagonal porous structure. The ordered porous structures were also seen using TEM analysis (Figure 3-6). The $d_{(100)}$ spacing was used to calculate the distance between two adjacent pore centres, i.e. the unit cell parameters (a), for the ordered mesoporous silica matrices using Eqn. (3).[328]

$$a = 2d_{(100)}/\sqrt{3} \quad (3)$$

This value was calculated as 50, 122 and 125 Å for MCM-41, MSE and SBA-15 respectively. By subtracting the average pore diameter (determined by N₂ adsorption analysis) from a , the pore wall thickness was determined as 22, 62 and 57 Å for MCM-41, MSE and SBA-15 respectively.

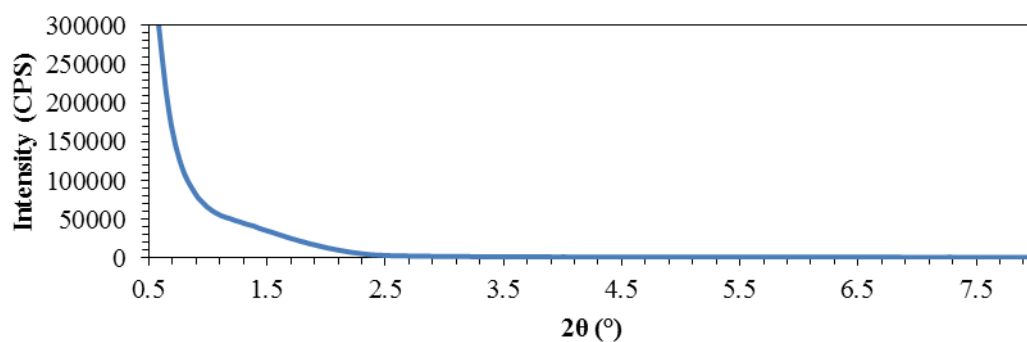
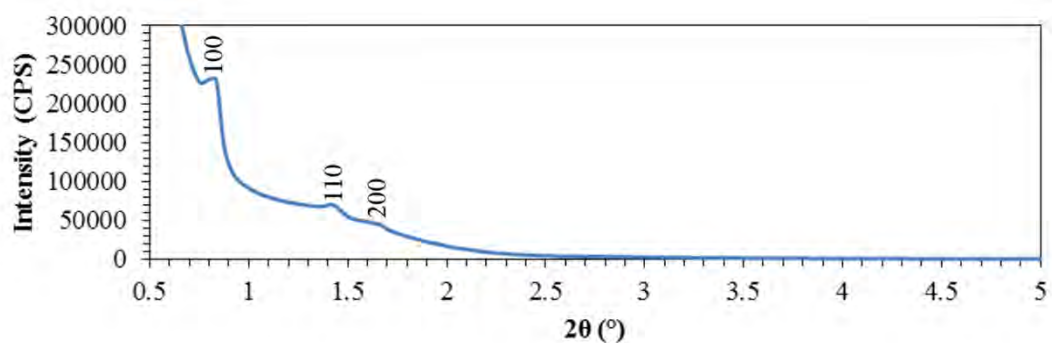
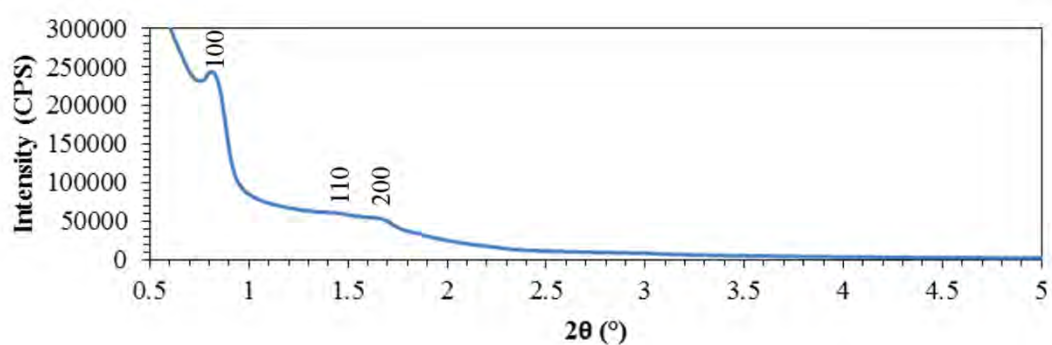
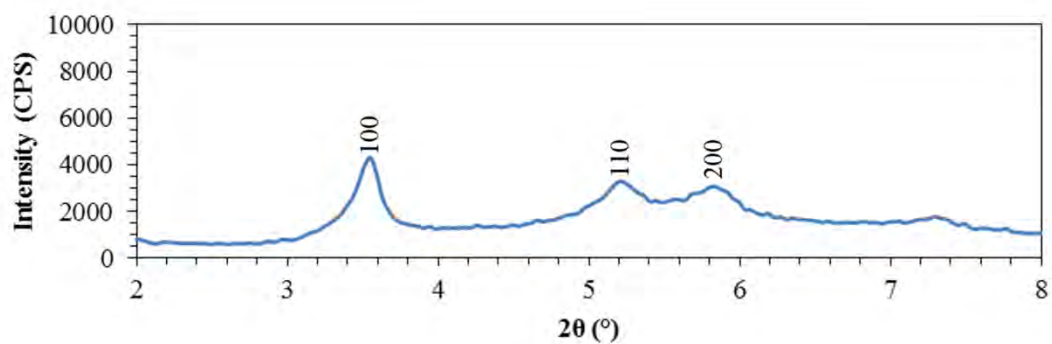


Figure 3-5. From top to bottom: P-XRD patterns for MCM-41, MSE, SBA-15 and PMO-PA.

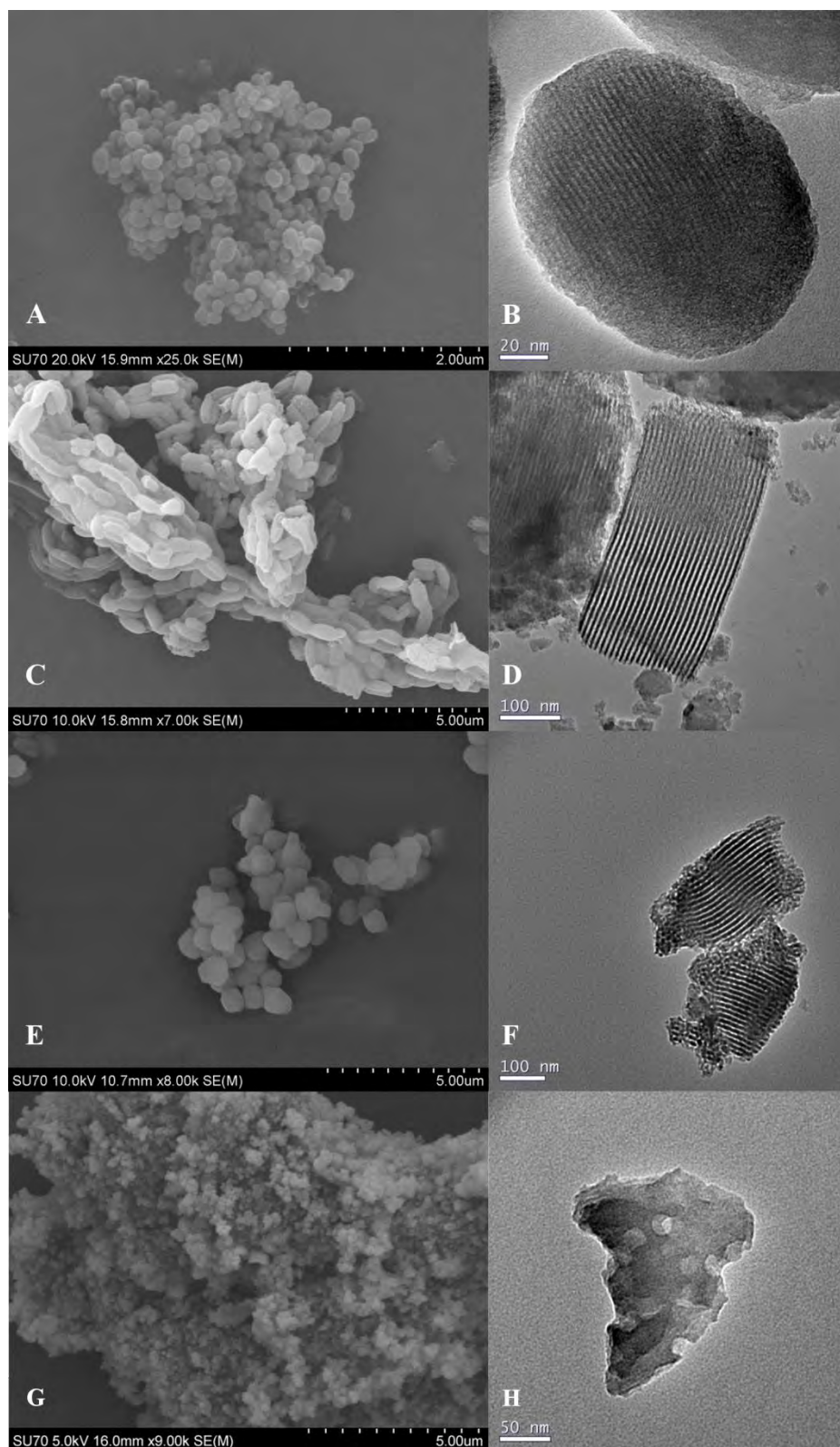


Figure 3-6. SEM images of (A) MCM-41, (C) MSE, (E) SBA-15 and (G) PMO-PA; TEM images of (B) MCM-41, (D) MSE, (F) SBA-15 and (H) PMO-PA.

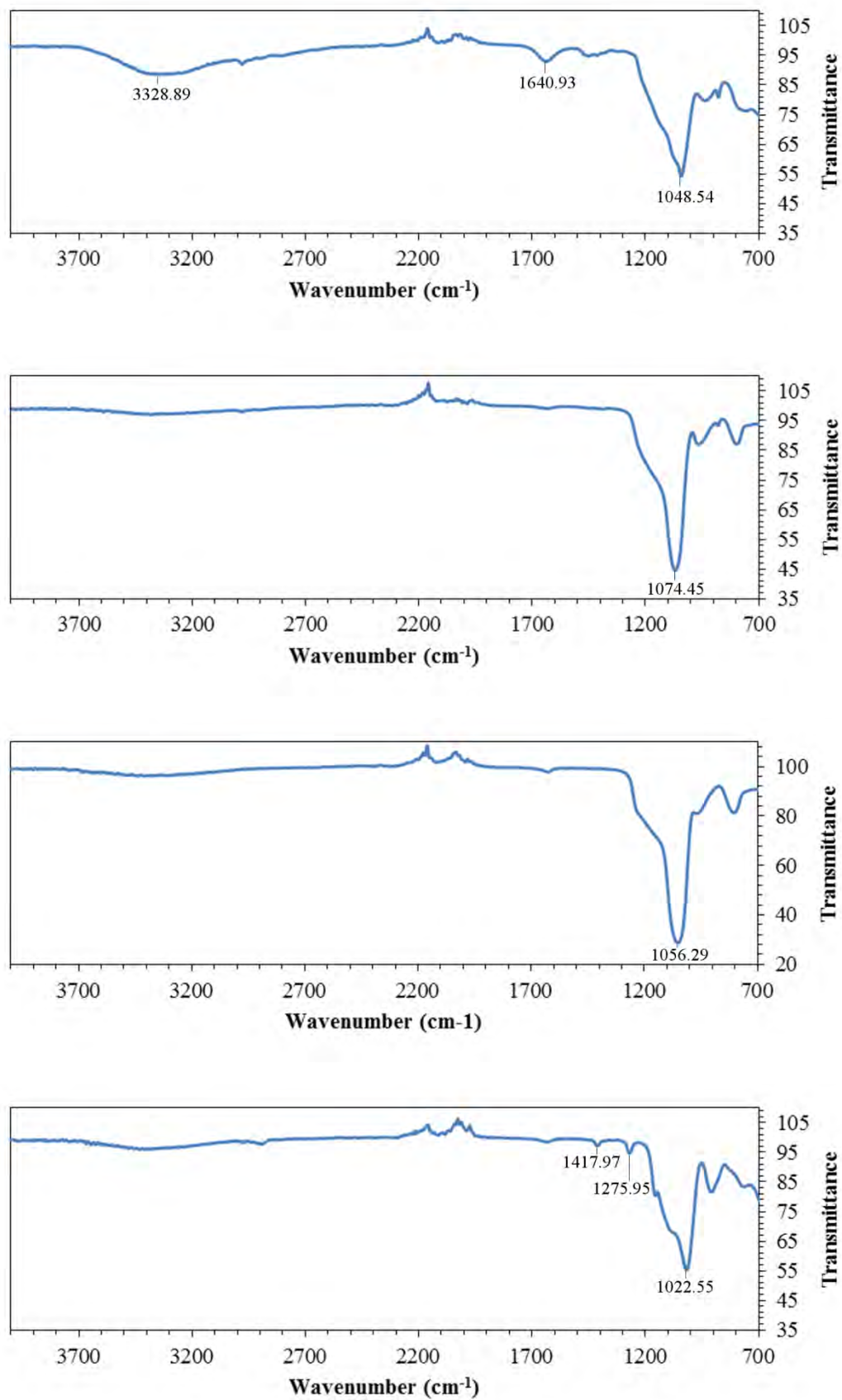


Figure 3-7. From top to bottom: ATR-FTIR spectra for PMO-PA, SBA-15, MCM-41 and MSE.

SEM analysis was carried out to determine the surface morphology and average particle size of the silica matrices (Figure 3-6). MCM-41 particles were spherical with an average particle size of $0.173 (\pm 0.017) \mu\text{m}$. MSE particles were rod-shaped and had an average particle size of $0.917 (\pm 0.177) \mu\text{m}$. SBA-15 particles were oval-shaped with an average particle size of $0.938 (\pm 0.127) \mu\text{m}$. The irregular shape of the PMO-PA particles made it impossible to determine the average particle size using SEM.

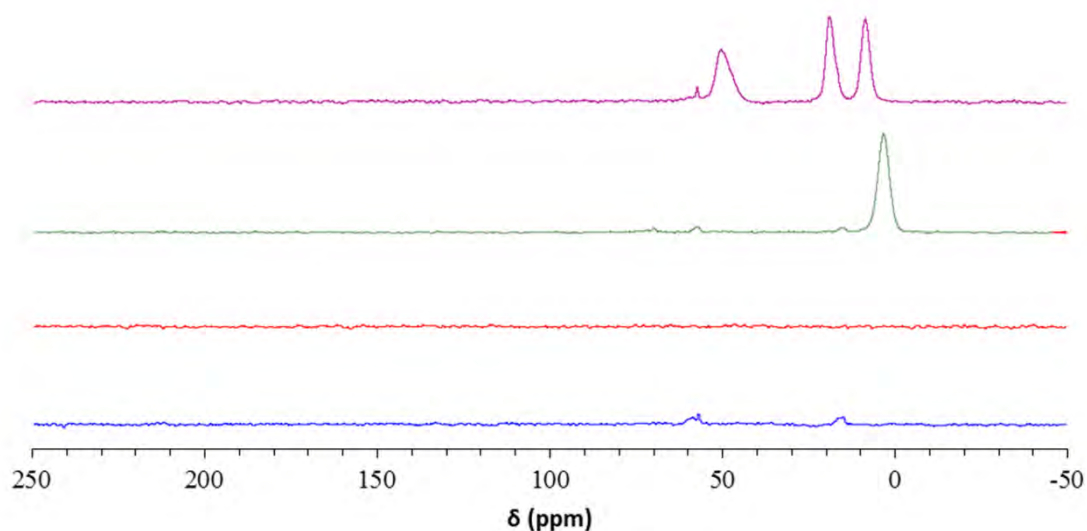


Figure 3-8. From top to bottom: ^{13}C CPMAS spectra for PMO-PA, MSE, MCM-41 and SBA-15. Contact times = 2.5 ms, recycle delay = 10 seconds, number of scans = 1024.

SS-NMR and ATR-FTIR were used to confirm the functionalisation of MSE (ethyl-bridged) and PMO-PA (aminopropyl-functionalised). The amine-functionalisation of PMO-PA was confirmed by the presence of the N-H stretch at 3328 cm^{-1} and the N-H bending at 1641 cm^{-1} in the FTIR spectrum (Figure 3-7).[301], [329] The ethylene-functionalisation can be seen from the ATR-FTIR spectrum of MSE in the C-H deformation vibrations at 1276 and 1418 cm^{-1} . [330]

The ^{13}C CPMAS SS-NMR spectrum of MSE showed one peak at $\delta = 5.3$ ppm, corresponding to the ethyl carbons (Figure 3-8). For the PMO-PA material, three peaks were present at 10.2, 20.1 and 50.8 ppm were present, corresponding to the three environments for the aminopropyl bridging carbons. The peak furthest upfield most likely corresponds to the carbons bound to silicon, with the peak at 20.1 ppm corresponding to the carbons bound to other carbons and the peak downfield

from that at 50.8 ppm corresponding to the carbons bound to the amino groups, due to deshielding of carbon by the more electronegative nitrogen atoms. Similar signals observed by Hartono *et al* for their aminopropyltriethoxysilane functionalised silicates.[331] These initial experiments indicated the organic functional groups, -CH₂-CH₂- and -CH₂-CH₂-NH-CH₂-CH₂- had successfully been incorporated into the silica matrices for MSE and PMO-PA respectively. The ¹³C CPMAS spectrum of SBA-15 and MSE showed small signals ($\delta \approx 59$ ppm, 15 ppm) indicating the presence of a small amount of residual surfactant (P123), undetected by ATR-FTIR.

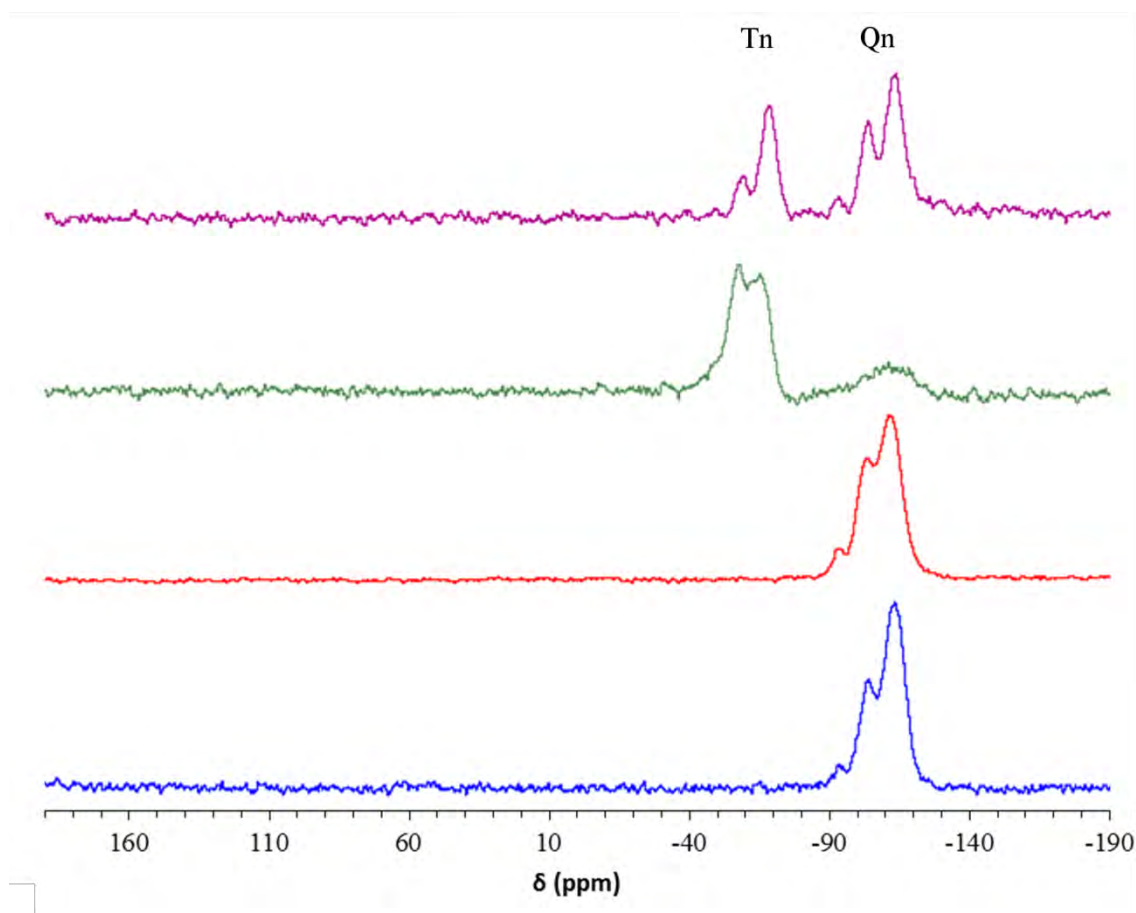


Figure 3-9. From top to bottom: ²⁹Si MAS spectra of PMO-PA, MSE, MCM-41 and SBA-15.

²⁹Si SS-NMR was also used to confirm functionalisation of MSE and PMO-PA. ²⁹Si-NMR can distinguish between siloxane species (Si(OSi)_n(OH)_{4-n}, Q_n) and organosiloxane species (T_n). The peaks at $\delta = -90$ to -120 ppm in the SBA-15 and PMO-PA-40 ²⁹Si-NMR spectra correspond to the Q_n species (Figure 3-9). It appears that three peaks are present in this range which are most likely the three types of Q_n species reported in literature - siloxane bridges (SiO₄, Q₄), silanol ((SiO)₃SiOH, Q₃) and geminal silanol ((SiO)₂Si(OH)₂, Q₂).[332] However, these peaks could not be

deconvoluted to determine the exact ratio of each of the three species present in each material. The MSE spectrum also featured a broad peak within this chemical shift range. For MSE and PMO-PA, there was an additional set of peaks at $\delta = -50$ to -70 ppm in the ^{29}Si -NMR spectra. This region corresponds to the T_n species present in the MSE and PMO-PA matrices due to organic functionalisation.[333]

The peaks in the Q_n region and the T_n region could be integrated to approximate the relative quantities of Q_n to T_n species (Table 8). From these approximations, it appears that 94 % of the silicon atoms in MSE are bound to ethane groups with a small percentage (6 %) bound to other silicon atoms via a siloxane bridge which may arise from Si-C bond cleavage during synthesis. The PMO-PA material was found to contain 30 % of silicon atoms bound to the propyl amine functional groups with 70 % of the silicon atoms existing as Q_n species. Both MCM-41 and SBA-15 contained only Q_n type silicon species, as expected. The structures for all the materials synthesised are shown above in Figure 3-10.

Table 8. Relative abundance of Q_n and T_n Si species in MPS, approximated by integration on single pulse ^{29}Si MAS spectra.

<i>MPS</i>	<i>Q_n Species (%)</i>	<i>T_n Species (%)</i>
SBA-15	100	0
MCM-41	100	0
MSE	6	94
PMO-PA	70	30

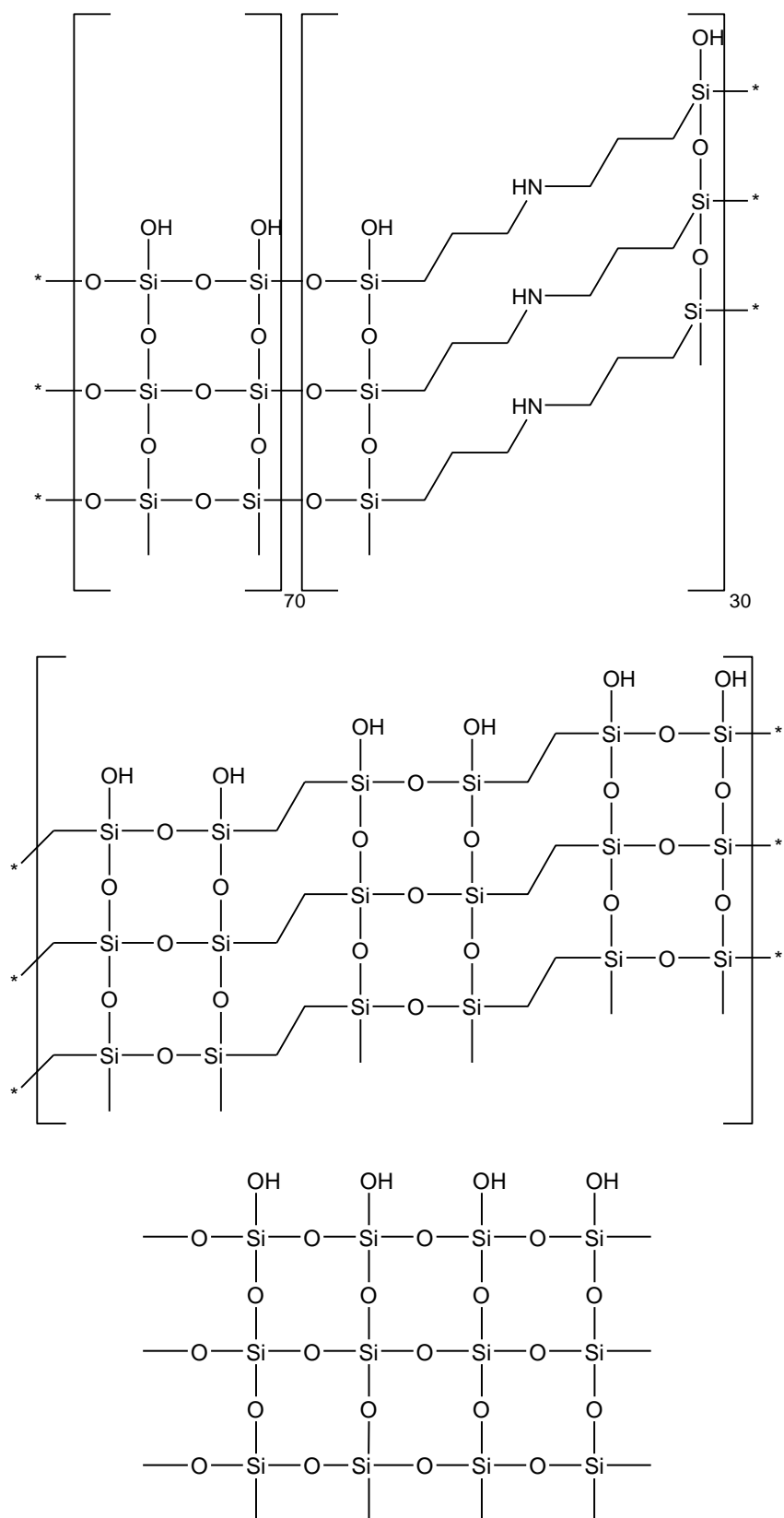


Figure 3-10. From top to bottom: structures of a) PMO-PA (70:30 m/n), b) MSE and c) SBA-15 or MCM-41.

The surface charge on the MPS particles was estimated by zeta potential measurements at pH 4 and pH 7. At pH 4, MCM-41, SBA-15 and MSE are close to their isoelectric points and therefore had very little overall surface charge (**Error! Not a valid bookmark self-reference.**).[334] However, at pH 7, the matrices were negatively charged. The amine-functionalised silica, PMO-PA, was positively charged at both pH 4 and pH 7 due to the presence of the aminopropyl bridging groups and the higher isoelectric point of this MPS (pI 9.1).[281] Based on these values, it was assumed that the cationic AMP, nisin (pI 8.8), was likely to adsorb onto SBA-15, MCM-41 and MSE and less likely to adsorb onto the positively charged PMO-PA. The physical properties determined for the mesoporous silica matrices synthesised are summarised below in **Error! Not a valid bookmark self-reference.**.

Table 9. Summary of the properties of the as-synthesised mesoporous silica matrices.

<i>Material</i>	<i>Avg pore size (Å)</i>	<i>d₁₀₀ (Å)</i>	<i>a (Å)</i>	<i>Wall thickness (Å)</i>	<i>BET surface area (m²/g)</i>	<i>Primary particle size (µm)</i>	<i>Zeta-potential (mV)</i>	
							<i>pH 4</i>	<i>pH 7</i>
MCM-41	28	43	50	22	702.20	0.173	-0.52	-10.52
					(± 1.48)	(± 0.017)	(± 0.13)	(± 2.42)
SBA-15	68	108	125	62	748.29	0.938	0.668	-10.06
					(± 7.94)	(± 0.127)	(± 0.074)	(± 0.075)
MSE	60	106	122	63	816.00	0.917	-0.26	-8.08
					(± 13.66)	(± 0.177)	(± 0.87)	(± 0.25)
PMO-PA	59	n/a	n/a	n/a	336.15		8.53	7.74
					(± 4.06)		(± 0.40)	(± 1.76)

3.3.2 Nisin-loaded MPS

A calibration curve (Figure 3-11) was constructed using RP-HPLC analysis of nisin solutions at concentrations of 0.05, 0.1, 0.2, 0.5 and 1.0 mg/mL (in 0.1% TFA). Nisin concentrations of 0.01 mg/mL and lower could not be detected using

RP-HPLC ($\lambda = 214$ nm). The calibration curve was constructed using the peak area of the component which eluted at 10 min corresponded to nisin A (see Chapter 2). This calibration curve was used to determine the amount of nisin which had adsorbed to the mesoporous silica matrices following 5 h incubation with rocking at 37 °C. This was calculated by subtracting the amount of nisin remaining in solution from the initial nisin concentration in solution.

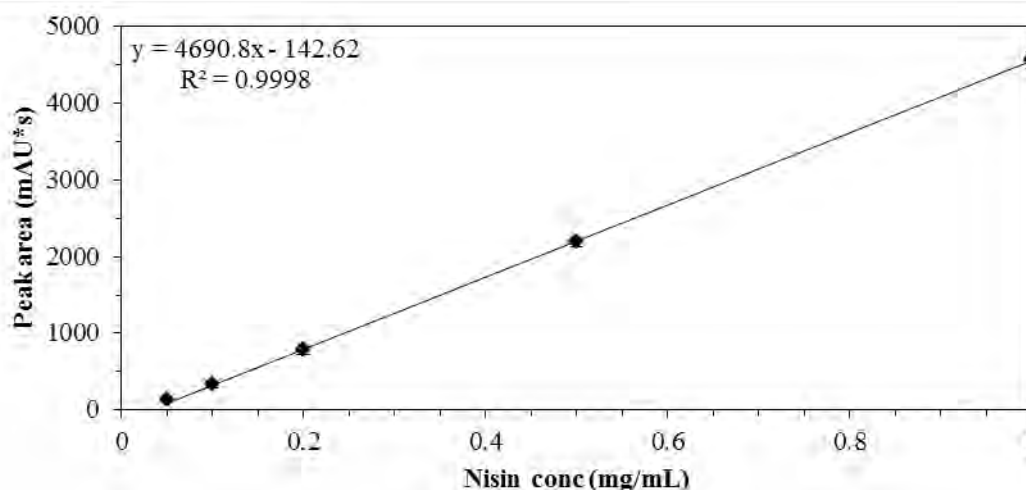


Figure 3-11. Calibration curve for nisin A ($t_r = 10$ min) in 0.1% TFA using the peak area, as determined by RP-HPLC analysis.

Preliminary adsorption studies were carried out at pH 4 over 21 h. As predicted by the zeta potential measurements for the MPS, nisin (pI 8.8) did not adsorb onto PMO-PA (8.53 ± 0.4 mV at pH 4) but adsorbed successfully onto SBA-15 (0.668 ± 0.074 mV at pH 4) and MSE (-0.26 ± 0.87 mV at pH 4) with loadings of 40 $\mu\text{g}/\text{mg}$ and 98 $\mu\text{g}/\text{mg}$ respectively (Figure 3-12).

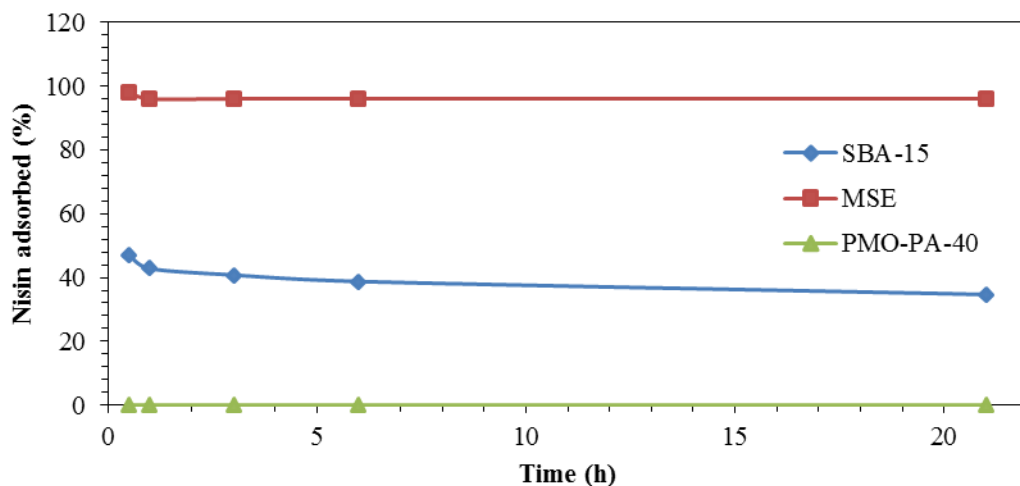


Figure 3-12 Nisin adsorbed onto SBA-15 (blue), MSE (red) and PMO-PA-40 (green) at pH 4, shown as a percentage of the concentration of nisin in solution at $t = 0$ h (initial nisin concentration = 0.5 mg/mL).

When studying the effect of pH and ionic strength on nisin adsorption to the MPS matrices, neither parameter seemed to greatly affect the adsorption of nisin onto SBA-15. At least 60% nisin adsorption onto SBA-15 was observed under all conditions tested (Figure 3-13). Nisin appeared to adsorb to SBA-15 best in the Tris acetate buffer (pH 7) of lower ionic strength. However, in the nisin stability studies (Section 2.3.3.1) the RP-HPLC profile of nisin exhibited an extra peak at pH 7 ($t_r = 15$ min), indicating that nisin did not remain stable at this pH. At pH 2, the nisin was shown to remain stable. Nisin showed good adsorption onto SBA-15 in KCl/HCl buffer (pH 2) at higher ionic strength and therefore this pH and ionic strength was chosen when scaling up the adsorption process.

pH and ionic strength appeared to have less of an effect on the adsorption of nisin onto MSE (Figure 3-14). The nisin appeared to absorb completely (i.e. no nisin detected in the supernatant by RP-HPLC) in KCl/HCl (pH 2) and Tris acetate (pH 7) buffers of higher ionic strengths. Again, KCl/HCl buffer was chosen for the scaling up of this adsorption due to the stability issues presented at pH 7.

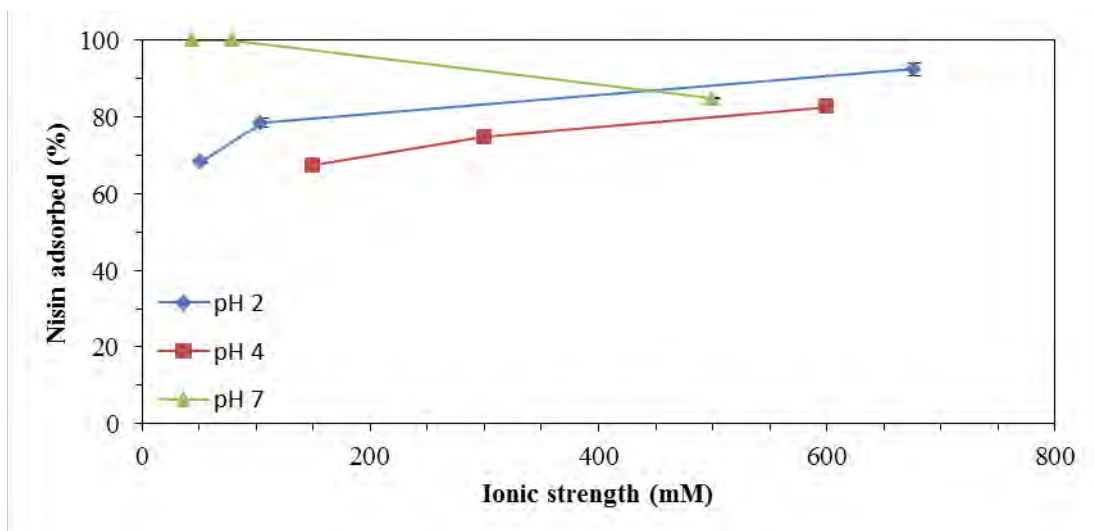


Figure 3-13. Effect of pH and ionic strength on nisin adsorption to SBA-15 (y-error bars present, indicating standard deviation among three replicates).

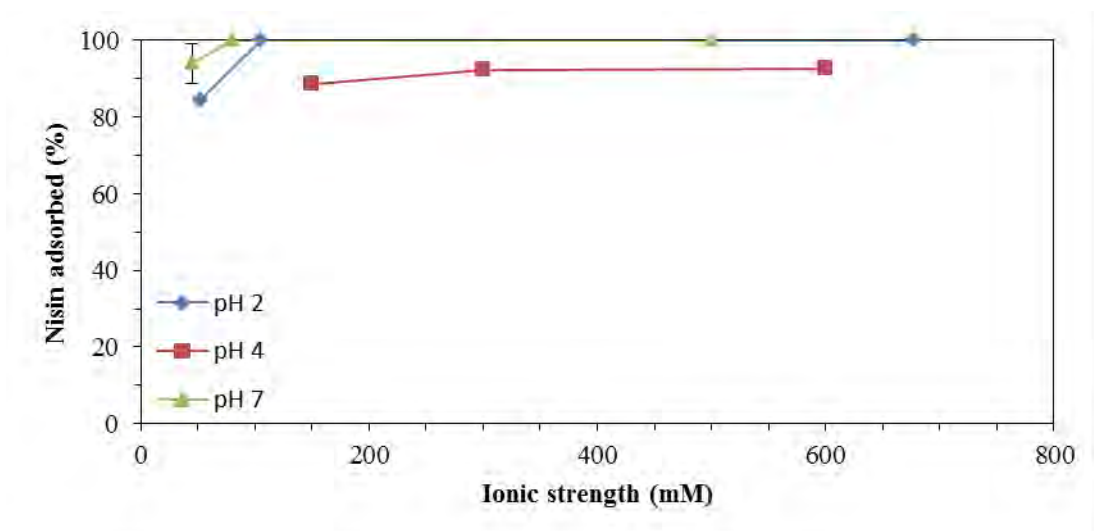


Figure 3-14. Effect of pH and ionic strength on the adsorption of nisin to MSE (y-error bars present, indicating standard deviation among three replicates).

The adsorption of nisin onto SBA-15 and MSE was scaled up from 1 mL to 35 mL using KCl/HCl pH 2 buffer ($I = 677$ mM). Nisin was also adsorbed to MCM-41, a mesoporous silica matrix with comparable surface chemistry to SBA-15 but smaller pore size, using these conditions. After washing twice with 25 mL DI water, the supernatants were analysed by RP-HPLC for nisin content (Figure 3-15). The pellets were dried and the moisture content was determined by thermogravimetric analysis (TGA). TGA of the samples confirmed the presence of $23.3\% \pm 0.2\%$, $15.6\% \pm 0.8\%$ and $15.8 \pm 0.3\%$ moisture respectively. Therefore, allowing for the moisture content, the nisin loading onto the mesoporous matrices was found to be

31.4, 31.0 and 32.6 $\mu\text{g}/\text{mg}$ for MSE, SBA-15 and MCM-41 respectively after washings.

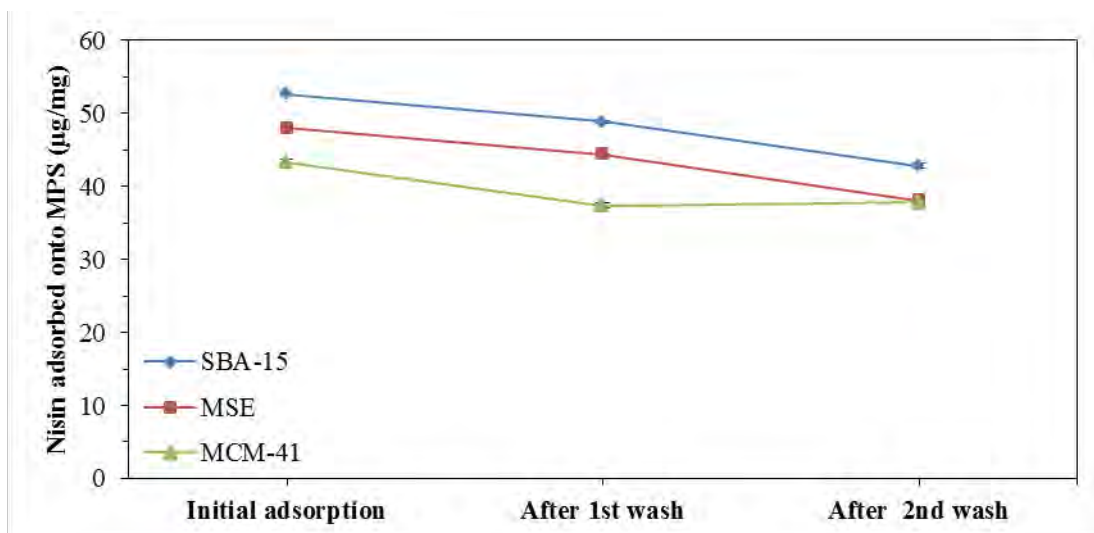


Figure 3-15. Scaled up adsorption of nisin onto the mesoporous silica matrices (SBA-15, MSE and MCM-41) at pH 2 (677 mM KCl), showing loss of nisin after washes with 25 mL DI water (y-error bars present, indicating standard deviation among three replicates).

When MSE was incubated with solutions increasing in nisin concentration, up to 3 mg/mL, the MSE appeared to approach a maximum nisin loading at 163 $\mu\text{g}/\text{mg}$. Therefore, the proportion of nisin to silicate used for studying the effects of pH and ionic strength on nisin adsorption to MSE (i.e. 50 $\mu\text{g}/\text{mg}$) was well below the maximum loading. This means that the abundance of adsorption sites, both hydrophilic (-OH) and hydrophobic (-CH₂CH₂-) available for nisin to adsorb may have prevented ionic strength and pH having an influence on the final loading. Testing the effects of pH and ionic strength using higher loadings would give a better indication of the influence of these parameters on loading and may give an insight into the preferred adsorption mechanism, i.e. ionic, hydrophobic, etc.

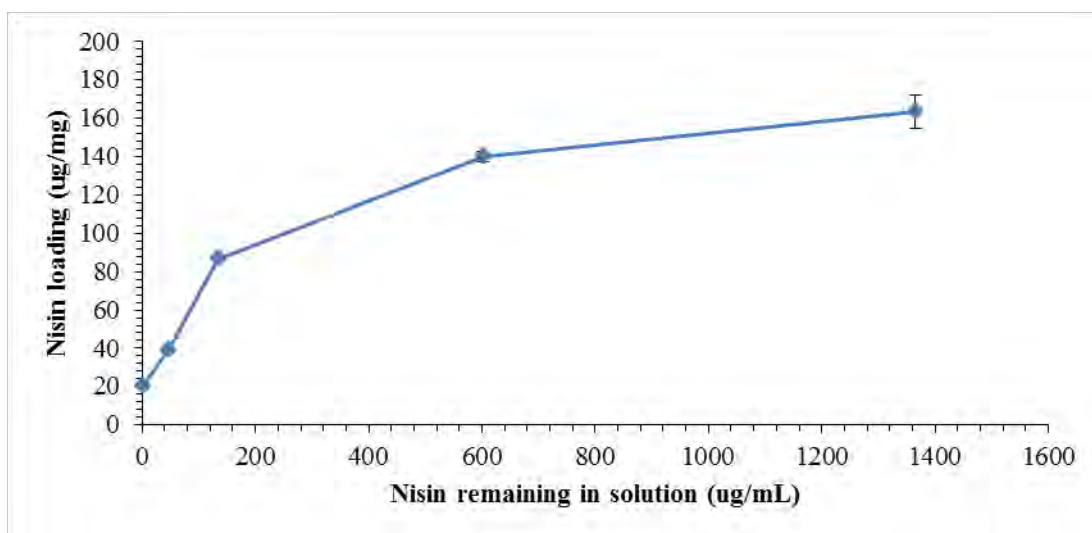


Figure 3-16. Adsorption isotherm of nisin onto MSE at pH 2 (677 mM KCl) (y-error bars present, indicating standard deviation among three replicates).

At the lower initial concentration of nisin (0.5 mg/ml) for the adsorption studies onto the MPS silicates, the high adsorption levels of all three variants meant that preferential adsorption could not be detected. However, when the adsorption isotherm of nisin onto MSE was generated using higher initial nisin concentrations (1.0 mg/mL, 2.0 mg/mL), nisin¹⁻³² showed preferential adsorption onto MSE over Ser³³-nisin. This was determined by the higher proportion of Ser³³-nisin and lower proportion of nisin¹⁻³² remaining in solution following adsorption, compared to the ratios of the three components observed in the Nisin AP control, (Table 10). This preferential adsorption of nisin¹⁻³² is most likely due to the increased hydrophobicity of this fragment, as confirmed by the increased retention time on the RP-HPLC column (Figure 2-5), which likely forms hydrophobic interactions with the ethylene-bridged surface of the MSE matrix.

Table 10. Ratios of Nisin AP components in pH 2 buffer and in pH 2 buffer in the presence of MSE, incubated at 37°C for 5 h

<i>Sample</i>	<i>Ratio of component in supernatant</i>		
	<i>Ser33-nisin</i>	<i>Nisin A</i>	<i>Nisin1-32</i>
Nisin AP control	0.30 ± 0.00	1.00 ± 0.00	0.36 ± 0.01
MSE-Nisin AP	0.46 ± 0.04	1.00 ± 0.00	0.23 ± 0.00

3.3.3 Release study

Release of nisin from the MPS matrices into SGF and SIF was carried out in an attempt to predict the pharmacokinetics of the nisin formulations *in vivo*.

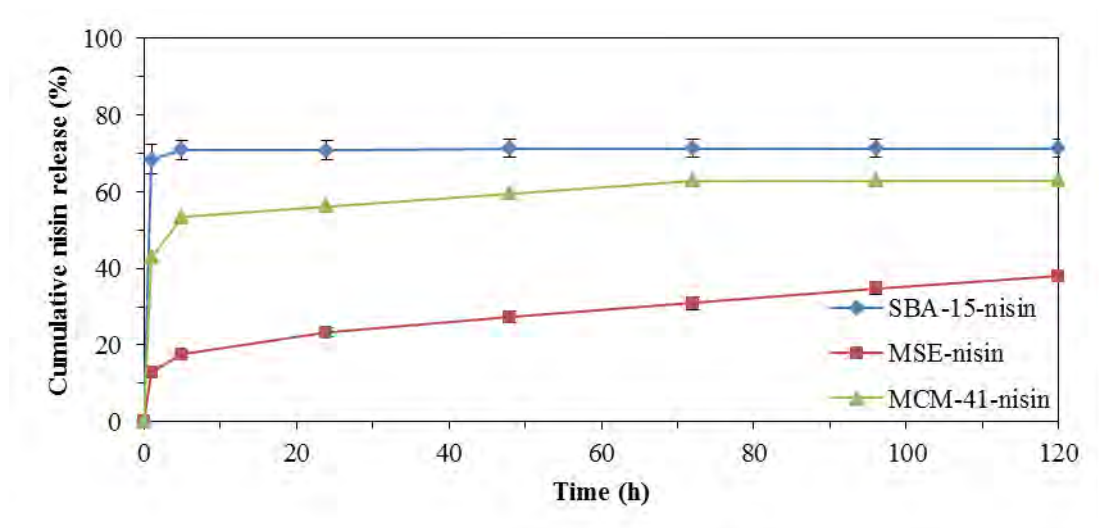


Figure 3-17. Release of nisin from MPS matrices into SGF (y-error bars present, indicating standard deviation among three replicates).

The release of nisin into simulated gastric fluid (SGF; pH 1.6) and simulated intestinal fluid (SIF; pH 7.0) from SBA-15-nisin (31.0 $\mu\text{g}/\text{mg}$), MCM-41-nisin (32.6 $\mu\text{g}/\text{mg}$) and MSE-nisin (31.4 $\mu\text{g}/\text{mg}$) was studied over 120 h and 72 h respectively. After 5 h in SGF, a burst release of 53.3 and 70.9% nisin was observed from MCM-41 and SBA-15 respectively, with a total release of 62.9 and 71.3% respectively after 72 h and no further release up to 120 h. Nisin release from MSE into SGF was more gradual, with a total release of 37.9% following 120 h.

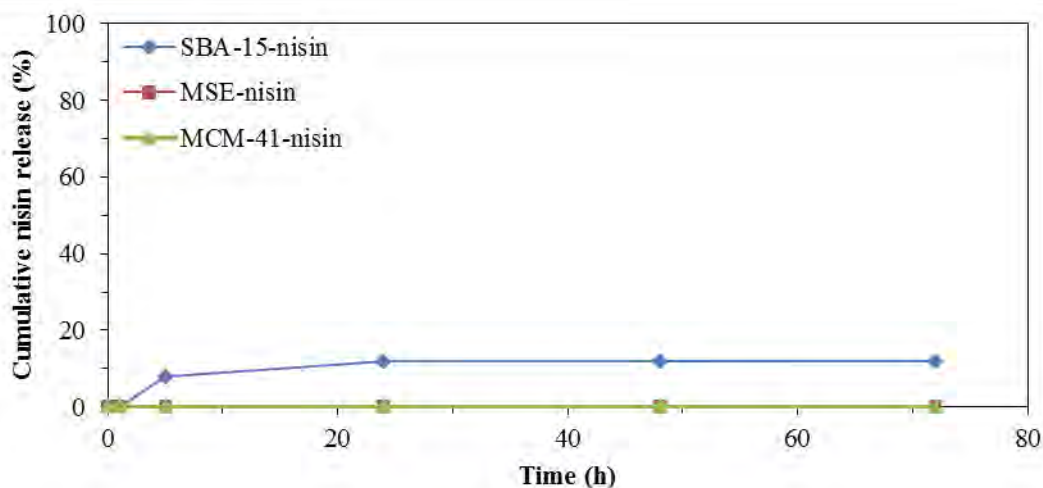


Figure 3-18. Release of nisin from MPS matrices into SIF (y-error bars present, indicating standard deviation among three replicates).

8% of the nisin adsorbed to SBA-15 released into SIF following 5 h, with a total release of 12% following 24 h and no further release up to 72 h (Figure 3-18). No release of nisin was detected by RP-HPLC analysis from MSE or MCM-41 into SIF over this time period. These results agreed with preliminary release studies carried out at pH 7.

Since the rate of dissolution of mesoporous silica matrices in aqueous solution is relatively low, release of nisin from these matrices is primarily due to dissociation from the surface of the matrix or diffusion from the pores.[293] The burst release of nisin observed from SBA-15 and MCM-41 into SGF could be due to the nisin at the surface dissociating from these matrices, followed by the slow diffusion from inside the porous matrix as seen previously for the release of drugs from unfunctionalised silica matrices.[269], [318] Another proposed explanation for this 2-step release is the release of electrostatically bound nisin followed by the release of the hydrogen bonded nisin.[335] The higher cumulative release from SBA-15 versus MCM-41 in this media may be due to the increased pore size. Although MSE is similar in pore size to SBA-15, the release of nisin from this matrix into SGF is more controlled and gradual. This may be due to the nisin adsorbing to MSE through hydrophobic interactions. No release of nisin was detected from MSE or MCM-41 into SIF over 72 h, with 12% cumulative release observed from SBA-15 into this media. This is similar to the release of LL-37,

another cationic AMP, at pH 7.4 from calcined silica matrices with pore sizes of 31-35 Å observed by Braun *et al.*[307]

Table 11. Activity of release media and pellets against *Lb. bulgaricus* (N = nisin, S = supernatant, P = pellet).

<i>Sample</i>	<i>Lb. bulgaricus</i> growth (CFU.mL ⁻¹)	σ
MSE (control)	2.34×10^7	5.57×10^5
MSE-N (S)	6.80×10^4	3.56×10^4
MSE-N (P)	0	0
SBA-15 (control)	3.78×10^8	3.12×10^7
SBA-15 -N (S)	1.24×10^4	1.79×10^4
SBA-15 -N (P)	0	0
MCM-41 (control)	2.31×10^8	5.82×10^7
MCM-41 -N (S)	4.07×10^2	1.76×10^2
MCM-41 -N (P)	0	0

The pellets and supernatants of the SIF release samples at 72 h were tested for activity against *Lb. bulgaricus*. Although nisin release was not detected by RP-HPLC in the supernatants after 72 h, they still inhibited more than 99% of *Lb. bulgaricus* growth compared to the controls (Table 11). This activity may be due to the presence of nisin at concentrations below the limit of detection on HPLC (10 µg/mL), which are sufficient to effectively inhibit *Lb. bulgaricus* growth (Section 2.3.2). The pellets, which still contained between 88 to 100% of the adsorbed nisin, showed complete inhibition of *Lb. bulgaricus*, indicating that further nisin release from the matrices occurred during the activity assay.

3.3.4 Proteinase K digestion study

The effect of proteinase K on the stability of the adsorbed nisin was studied to determine if any of the MPS matrices provided protection for nisin against this enzyme. RP-HPLC analysis was carried out following 24 h incubation of MPS-nisin with proteinase K and subsequent release of the adsorbed nisin into SGF over 5 days. During the 24 h exposure to proteinase K at pH 7.5, no release of nisin was detected by HPLC for the test samples or the controls (MPS-nisin at pH 7.5). Following this 24 h period, the samples were acidified to stop further proteolytic degradation and then released into SGF over a 5 day period to determine the effects of proteinase K

on the released nisin A. The MPS-nisin controls, with no proteinase K present, were used to determine the quantity of undigested nisin released from the matrices. After 5 days in SGF, 20, 14 and 19% of the nisin A adsorbed to SBA-15, MSE and MCM-41 respectively had been released from the controls. For the test samples pre-exposed to proteinase K before release into SGF, a reduction in the concentration of nisin A released of 82.6, 61.8 and 40.4% was observed for SBA-15, MSE and MCM-41 respectively. This implies that at least 17.4, 38.2 and 59.6% of the nisin A was protected by SBA-15, MSE and MCM-41 respectively (Table 12). These results infer that MCM-41 provides the best protection for nisin A against digestion by proteinase K. Since both MCM-41 and SBA-15 have similar surface functionality, the improved protection provided by MCM-41 is most likely due to the smaller pore size of this MPS matrix (28 Å vs. 68 Å). MSE has a slightly smaller pore size than SBA-15 (60 Å) which may contribute to the improved protection of nisin A. However, MSE also presents ethylene-bridged surface functionality which may also contribute to the increased protection of nisin A provided by this matrix compared to SBA-15.

Table 12. Protection of nisin A from proteinase K digestion (%) following adsorption onto MPS matrices.

<i>MPS matrix</i>	<i>Nisin A protected (%)</i>
SBA-15	17.4
MSE	38.2
MCM-41	59.6
No matrix	0

3.4 Conclusions

Nisin was successfully adsorbed onto unfunctionalised silica (MCM-41 and SBA-15) and ethylene-functionalised silica (MSE) but did not adsorb to the amine-functionalised silica (PMO-PA). When adsorption onto MSE was carried out using higher initial nisin concentrations, a preferential adsorption of nisin¹⁻³² was observed, which is thought to be related to the hydrophobic nature of both the matrix and the peptide. Although the nature and degree of this preference need to be studied further, this opens the possibility for MSE to be used in the preferential adsorption of hydrophobic peptides. A burst release of nisin was observed from MCM-41 and

SBA-15 into SGF. This may prove useful for immediate-release, high dosing of cationic peptide therapeutics into the gastric environment to treat acute bacterial infections or inflammation in the stomach. Release of nisin from MSE was more controlled and therefore this system may be applicable for prolonging the release of cationic peptides past the stomach and targeting delivery to the small intestine. Little to no release of nisin was observed from the matrices into SIF but the particles themselves were active against *Lb. bulgaricus*. However, this antimicrobial activity may be due to small amounts of nisin being released from the matrices and needs to be confirmed using a less sensitive bacterial strain, e.g. *L. innocua* or *S. epidermidis*. While none of the matrices provided 100% protection of nisin against digestion by proteinase K, all three matrices provided partial protection of nisin up to 60% at pH 7.5 (MCM-41 > MSE > SBA-15). The increased protection of nisin provided by MCM-41 compared with MSE or SBA-15 may be due to the smaller size of the pores which may hinder the entry of proteinase K (18 kDa).[267] This partial protection provided by MCM-41 may prove useful in reducing the proteolytic degradation of peptide drug molecules similar in size to nisin (i.e. around 3.4 kDa) in the protease-rich environment of the small intestine. To prevent release of nisin from MCM-41 in the acidic gastric environment, an enteric coating, such as HPMC, could be utilised.[182] MSE provided better protection of nisin against proteinase K compared to SBA-15. Since these matrices have very similar pore sizes, the improved protection provided for nisin by MSE is most likely due to the hydrophobic adsorption sites on this matrix.

4. Overall conclusions and future work

In this work, mesoporous silica matrices were synthesised which allowed the effects of pore size and surface functionality to be studied for the adsorption and release of the bacteriocin, nisin. It was found that nisin successfully adsorbed onto unfunctionalised (SBA-15 and MCM-41) and ethylene-functionalised (MSE) silica, with no adsorption observed onto silica with amine functionality (PMO PA). Pore size along with the pH and ionic strength of the environment appeared to have little effect on nisin adsorption. However, this may be due to the nisin loading being well below the maximum adsorption achievable, as seen in adsorption studies for MSE-nisin. The higher nisin concentrations in solution for the MSE adsorption isotherm also allowed the preferential adsorption of nisin¹⁻³² onto MSE to be observed.

A burst release of nisin into SGF was observed from the unfunctionalised MPS matrices, with an increase in pore size resulting in a higher cumulative nisin release from SBA-15 over MCM-41. This may prove useful in a targeted immediate-release formulation of cationic peptides similar in size to nisin (3.4 kDa). A more controlled release into SGF was observed from MSE, which is possibly due to the hydrophobic, ethylene functionality interacting with the hydrophobic N-terminal with nisin. However, this theory needs to be further investigated. The controlled release of nisin from MSE into SGF may be useful in preventing release of cationic peptides in the stomach and delivering them to the small intestine or colon. No release of nisin from MSE or MCM-41 into SIF was observed over 72 h, with only low amounts of nisin releasing from SBA-15. Although the release media following this study did not present peaks for nisin when analysed by RP-HPLC, activity against *Lb. bulgaricus* was still observed from both the supernatant and the suspension of all three MPS-nisin materials. This may be due to nisin being released at concentrations below the limit of detection for RP-HPLC but above the MIC against *Lb. bulgaricus* and due to further release of nisin from the MPS during the activity assay. In the future this could be further probed by using a less sensitive indicator strain than *Lb. bulgaricus*, e.g. *L. innocua* or *S. epidermidis*, which has an MIC of nisin above the RP-HPLC detection limit. If the particles themselves prove to be active, MSE could prove to be a successful delivery system for delivering cationic peptides to the intestine.

While all MPS matrices appeared to provide some degree of protection for nisin against digestion by proteinase K, MCM-41 shows the highest degree of protection. This is most likely due to the smaller size of the MCM-41 mesopores (28 Å) which it is thought may allow for the entry of narrow nisin molecules (57 x 19 x 15 Å) but prevent the entry of larger, globular molecules of proteinase K (51 x 45 x 39 Å). The higher protection of nisin provided by MSE over SBA-15 is possibly due to a small amount of nisin being released from the SBA-15 during the 24 h exposure to proteinase K (pH 7.5). Although MCM-41 shows the highest degree of protection for nisin against proteinase K, a burst release of nisin from this matrix is observed in SGF. However, enteric coatings, e.g. HPMC, could potentially be used to deliver antimicrobial peptides to the intestine using this protective matrix – provided the adsorbed peptide is active. Proteinase K is not present in the GI tract of humans, it is synthesised by the fungus *Tritirachium album*. [267] However, it has broad substrate specificity and so it is likely that protection of nisin from degradation by this enzyme will, in turn, protect against proteases of the GI tract. However, the protection provided for nisin by these MPS matrices against enzymes present *in vivo* such as trypsin and α -chymotrypsin will need to be investigated.

While *in vitro* and *in vivo* biocompatibility studies need to be carried out to confirm the safety of these MPS matrices, there is a great potential for their application in the controlled release of peptide drugs and the protection of these peptides from proteolytic degradation following oral delivery to treat local infections or diseases in the GI tract. In these studies, nisin was used as a model peptide. While nisin itself has interesting antimicrobial, anticancer and immunomodulatory properties, the properties of other novel bacteriocins, such as thuricin CD, which shows narrow-spectrum activity against *Clostridium difficile*, and bactofencin A, which shows potency against *S. aureus*, may also be exploited in the future using the proposed formulation techniques. [205], [206]

5. References

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6. Appendix

Table 13. Naturally occurring amino acids

Property	Amino acid
	Cysteine (Cys, C)
	Serine (Ser, S)
Polar, hydrophilic	Threonine (Thr, T)
	Glutamine (Gln, Q)
	Asparagine (Asn, N)
	Leucine (Leu, L)
	Valine (Val, V)
Non-polar, hydrophobic	Isoleucine (Ile, I)
	Alanine (Ala, A)
	Methionine (Met, M)
	Tyrosine (Tyr, Y)
Aromatic	Tryptophan (Trp, W)
	Phenylalanine (Phe, F)
Positively charged (at pH 7)	Arginine (Arg, R)
	Lysine (Lys, K)
	Histidine (His, H)
Negatively charged (at pH 7)	Aspartic acid (Asp, D)
	Glutamic acid (Glu, E)
Other	Glycine (Gly, G)
	Proline (Pro, P)