Material Characterisation and \textit{in vitro} Biological Investigation into Acrylic Bone Cements Incorporating Multi-Walled Carbon Nanotubes (MWCNTs)

By

Ahmad H. al-Sharif

A thesis submitted to Faculty of Science and Engineering, Department of Civil Engineering and Materials Science, University of Limerick, Ireland.

In fulfilment for the award of Doctor of Philosophy

Under the supervision of: Prof. Martin Buggy

October 2011
Declarations

I, Ahmad H. al-Sharif, declare that this is my own work and it has not previously been submitted to this or any other university or higher education institution support of any degree or other academic award.

Where use has been made of the work of other people, it has been acknowledged and fully referenced.

The library of University of Limerick may lend or copy this thesis on request.

---------------------------------
Ahmad al-Sharif

October 2011

Chairman: Prof. Stuart Hampshire (University of Limerick)
External Examiner: Prof. Brian Meenan (University of Ulster)
Internal Examiner: Dr. Eamonn deBarra (University of Limerick)
Supervisor: Prof. Martin Buggy (University of Limerick)
Acknowledgments

I am ever grateful to the almighty God for his blessings and for having made this work possible. I would like to acknowledge many people and collaborators who helped me during the course of this work.

First and foremost, I am heartily thankful to my supervisor, Prof. Martin Buggy, whose guidance and support from the initial to the final stage ensured the success of this work. I sincerely thank him for giving me the opportunity to work for him.

Further, I owe my deepest gratitude to Prof. Conleth Hussey for his unwavering support throughout the duration of my time at the University of Limerick. Along with Prof. Hussey, I must recognize the people at the Civil Engineering and Material Science Department of University of Limerick for their patience and assistance.

Additionally, I thank the Centre for Applied Biomedical Engineering Research (CABER) at University of Limerick for their help with training, processing, testing, instrumentation, analysis, and, in general, for their advice. Specifically, I thank Dr. Grāinne Carroll for her invaluable assistance throughout this project.

Last, but certainly not the least, I would like to acknowledge the commitment, sacrifice and support of my family and friends, who have always motivated me. In reality this thesis is partly theirs too.
Abstract

Acrylic bone cements, based on poly(methylmethacrylate) (PMMA), have been widely used in orthopaedic surgery for the fixation and force distribution of joint prostheses in cemented arthroplasties. However, PMMA continues to have less than ideal in vivo performance. A variety of materials have been added to bone cement to enhance its performance. Multi-Walled Carbon Nanotubes (MWCNTs) have emerged as a viable augmentation candidate because of their superior properties. This study investigated if the incorporation of small amounts of MWCNTs to a commercially available Simplex-P® bone cement matrix can improve its properties and in vivo performance. The MWCNTs examined were unfunctionalised, hydroxyl and carboxyl functionalised MWCNTs with weight loading varied from 0 to 0.5 wt.%. In most Total Joint Arthroplasty (TJA) there exists a state of biaxial loading: a combination of hoop, axial, and flexural loading, thus evaluating the stress within the cement mantle by traditional uniaxial tests may not provide accurate material characterisation. To address this issue, biaxial flexural test was used to determine the strength and stiffness of the resultant bone cement nanocomposites. Furthermore, finite element analysis (FEA) was performed to verify the analytical biaxial theory. Along with the mechanical properties of the reinforced nanocomposites, the rate of reaction exotherm generated, and the degree of polymerisation attained, thus, the residual monomer content were investigated by differential scanning calorimetry (DSC) using isothermal and dynamic modes. The maximum polymerisation temperature ($T_{\text{max}}$) and the thermal necrosis index (TNI) values were also measured using a k-type thermocouple wire according to (ISO 5833:02) specifications. In order to investigate the in vivo biomedical potential of these bone cement nanocomposites, the influence of MWCNTs chemical functionality and loadings in bone cement composites on the adhesion and viability of an in vitro model of human osteoblast cells was studied. On the basis of the determined mechanical properties, this work demonstrated that the biaxial flexural test can be used for testing orthopaedic bone cement nanocomposites, and that specific MWCNTs loading ($\leq 0.1$ wt.%) can favourably improve the performance of acrylic bone cement. The thermal analysis demonstrated that the chemically functionalised MWCNTs altered the bone cement polymerisation kinetics, reducing the exothermic polymerisation reaction for acrylic bone cement, which could potentially reduce the hyperthermia experienced in vivo. The in vitro biocompatibility investigation showed that the biological response of osteoblasts onto the surface of MWCNTs reinforced Simplex-P® bone cement nanocomposites was not cytotoxic and exhibited good cell functionality related to cellular adhesion, growth, and viability.
Contents

Declarations ............................................................................................................................................. i
Acknowledgments................................................................................................................................... ii
Abstract .................................................................................................................................................. iii
Contents ................................................................................................................................................. iv
List of Figures ....................................................................................................................................... vii
List of Tables .......................................................................................................................................... x
1 Introduction ...................................................................................................................................... 1
2 Literature Review ............................................................................................................................. 3
   2.1 Polymer Composites Biomaterials ............................................................................................. 4
      2.1.1 Introduction ....................................................................................................................... 4
      2.1.2 Classification ....................................................................................................................... 6
      2.1.3 Biocompatibility .............................................................................................................. 7
      2.1.4 Mechanical Properties ......................................................................................................... 8
      2.1.5 Applications ...................................................................................................................... 11
   2.2 Carbon Nanotubes .................................................................................................................... 13
      2.2.1 Introduction ....................................................................................................................... 13
      2.2.2 Structure and Defects ........................................................................................................ 14
      2.2.3 Synthesis of CNTs ............................................................................................................ 16
         2.2.3.1 Arc-Discharge ....................................................................................................... 17
         2.2.3.2 Laser Ablation ....................................................................................................... 17
         2.2.3.3 Chemical Vapour Deposition ................................................................................ 18
      2.2.4 Properties of Carbon Nanotubes ....................................................................................... 18
      2.2.5 Purification ........................................................................................................................ 20
      2.2.6 Functionalisation ................................................................................................................. 21
      2.2.7 Nanotoxicology .................................................................................................................. 23
      2.2.8 Toxicity of Carbon Nanotubes .......................................................................................... 24
      2.2.9 CNTs for Tissue Engineering Applications ...................................................................... 26
   2.3 Bone Cement ............................................................................................................................. 30
      2.3.1 Introduction ....................................................................................................................... 30
      2.3.2 Applications ...................................................................................................................... 32
4.1 Mechanical Studies ................................................................. 90
  4.1.1 Flexural Properties .......................................................... 90
  4.1.2 Finite Element Analysis ..................................................... 101
  4.1.3 Single Lap Shear Test ......................................................... 108
4.2 Thermal Studies ...................................................................... 111
  4.2.1 DSC Measurement ............................................................ 111
  4.2.2 Thermal Necrosis Evaluation ............................................. 117
4.3 Biocompatibility Studies .......................................................... 120
  4.3.1 Cytotoxicity Assessments ............................................... 120
  4.3.2 Cellular Adhesion and Morphology .................................... 124
  4.3.3 Cell Viability ................................................................. 130
5 Discussion ............................................................................... 133
  5.1 Mechanical Analysis ......................................................... 134
  5.2 Thermal Analysis ............................................................... 148
  5.3 Biocompatibility Analysis .................................................... 157
6 Conclusions ........................................................................... 165
  6.1 Conclusions ......................................................................... 166
  6.2 Recommended Future Work ................................................ 168
References ................................................................................. 169
Appendix I .................................................................................. 184
Appendix II ................................................................................ 188
List of Figures

Figure 1-1: Schematic of a total hip replacement. ................................................................. 2
Figure 2-1: Different structures of fullerenes. .......................................................................... 14
Figure 2-2: Structure of single wall carbon nanotubes. .......................................................... 15
Figure 2-3: Structure of multi-wall carbon nanotubes ............................................................ 15
Figure 2-4: Some defects structures of CNTs ........................................................................... 16
Figure 2-5: Examples of covalent side-wall, non-covalent side-wall,
and covalent tip functionalisation of CNTs ......................................................................... 22
Figure 2-6: FE-model of the (B3B) test assembly. ................................................................. 44
Figure 3-1: Raman spectra of MWCNTs with 20-30 nm OD .................................................. 58
Figure 3-2: TEM image of MWCNTs, (20-30 nm OD). ........................................................... 59
Figure 3-3: Biaxial flexural test setup ..................................................................................... 62
Figure 3-4: Schematic of the test setup for (B3B) biaxial flexural test .................................... 63
Figure 3-5: Relationship of the theoretical deflection to number of supports ................. 64
Figure 3-6: Schematic of the test setup for ring-on-ring equibiaxial flexural test .................... 67
Figure 3-7: Four-point bend test rig ....................................................................................... 68
Figure 3-8: schematic of single lap shear joint ....................................................................... 71
Figure 3-9: Differential scanning calorimeter ......................................................................... 75
Figure 3-10: Laser Scanning Confocal Microscope ............................................................... 83
Figure 3-11: The xCELLigence RTCA DP Instrument components ....................................... 85
Figure 3-12: Electronic sensor technology applied to cell biology .................................... 85
Figure 3-13: Determination of the cells biology status .......................................................... 86
Figure 3-14: Electrode layer on the bottom of one well ......................................................... 87
Figure 4-1: Load-displacement curves of various bone cements when tested under: 4-point
bend, ring-on-ring, and B3B biaxial test methods ............................................................... 90
Figure 4-2: Biaxial and Bending modulus of bone cement composites ............................... 91
Figure 4-3: The mean flexural strength obtained with different equivalent contact radii ...... 94
Figure 4-4: Weibull plot of biaxial strength of bone cements obtained with (3rd r0 •)
approximations .................................................................................................................. 95
Figure 4-5: Weibull moduli of various bone cement composites with different contact radii .... 95
Figure 4-6: Characteristic flexural strength acquired with different equivalent contact radii. ..........96

Figure 4-7: The variation and central tendency of biaxial flexural strength distributions in terms of filler loadings and chemical functionality for all cement composites. ..........97

Figure 4-8: Effect of MWCNTs loadings and chemical functionality on the survival rates...........97

Figure 4-9: Survival distributions from the ranked biaxial flexural strength data........................99

Figure 4-10: Bending, equibiaxial and biaxial flexural strengths.............................................100

Figure 4-11: Fracture patterns of bone cement discs tested in the (B3B) test assembly..............101

Figure 4-12: FE-model of a disc deforming under flexural load in a (B3B) test assembly..........101

Figure 4-13: The displacement magnitude and distribution in a disc for a typical loading conditions.........................................................................................................................102

Figure 4-14: Stress field in a disc for a typical loading condition in a (B3B) test..........................104

Figure 4-15: Effects of loading force, disc thickness, and loading ball radius on the biaxial strength, contact radius, and displacement of a disc loaded in (B3B) test..........................107

Figure 4-16: Top surface of fractured biaxial specimens...............................................................108

Figure 4-17: Single lap shear strength of various joint systems. .................................................109

Figure 4-18: Failure surfaces of Aluminium-bone cement joints.............................................110

Figure 4-19: Failure surface of the rigid polyurethane foam-bone cement joints......................110

Figure 4-20: DSC thermograms for the control samples subjected to isothermal polymerisation at 37°C, first scan at 10°C/min, and second scan at 10°C/min. .........................111

Figure 4-21: Example of typical DSC thermograms for fully polymerised control bone cement and SUNF5 samples obtained at 10°C/min.........................................................112

Figure 4-22: The effect of MWCNTs loadings and chemical functionality on DSC thermograms for cement obtained during isothermal polymerisation at 37°C. .............113

Figure 4-23: The conversion of variety of acrylic bone cement composites with different MWCNTs contents polymerised at 37°C (isothermal conditions). .........................115

Figure 4-24: Experimental curves for the determination of rate constants $k$ and $k^*$ for SUNF5 bone cement composite polymerised isothermally at 37°C.................................116

Figure 4-25: The effect of MWCNTs loading on the released temperature for variety of acrylic bone cement composites.................................................................118

Figure 4-26: The effect of MWCNTs chemical functionality and loading on the TNI for variety of acrylic bone cement composites.........................................................119
Figure 4-27: Percentage of HOB-c cell population after 24 h exposure to extracts of the main constituents of bone cement composites ................................................................. 120

Figure 4-28: Percentage of HOB-c cell population after 24 h exposure to extracts of varies bone cement composites .................................................................................................. 121

Figure 4-29: Dynamic monitoring of cell line at different densities observed during 100 h .......... 122

Figure 4-30: Dynamic monitoring of cell adhesion & viability using xCELLigence system ......... 124

Figure 4-31: Cellular population of osteoblasts cultured onto the surfaces of bone cement- MWCNTs composite samples for 24 h ............................................................................. 125

Figure 4-32: SEM micrographs for ostoblasts cultured on cement samples for 3 h &24 h .......... 126

Figure 4-33: Live-dead LSCM images for bone cement samples .................................................... 128

Figure 4-34: Live-dead LSCM images for bone cement samples .................................................... 129

Figure 4-35: Effects of MWCNTs loading and chemical functionality on cell viability of osteoblasts cultured for 1, 3, and 7 days on various bone cement composites ................. 130
List of Tables

Table 2-1: Mechanical properties of hard and soft tissues. ................................................................. 9
Table 2-2: Mechanical properties of typical biomaterials. ................................................................. 9
Table 3-1: Composition of surgical Simplex-P®. ............................................................................... 57
Table 3-2: Properties of MWCNTs used. ......................................................................................... 58
Table 3-3: Average Material Properties of solid rigid polyurethane foam 20 PCF. ......................... 59
Table 3-4: Notation and compositions of bone cement nanocomposites. ....................................... 61
Table 4-1: Biaxial and Bending modulus of all bone cement composites. ................................. 91
Table 4-2: Mean biaxial flexural strength & Weibull analytical results for all bone cements. ....... 93
Table 4-3: Means bending and biaxial strengths results. ................................................................. 99
Table 4-4: Relationship between FE load-displacement gradient, deflection function, 
loading force, loading ball diameter, & thickness for a FE modulus of 2.5 GPa. ........... 103
Table 4-5: Tensile lap-shear strength. ............................................................................................. 109
Table 4-6: Heat evolved for fully polymerised cement composites (dynamic conditions). ............ 113
Table 4-7: The effect of different MWCNTs chemical functionality and loadings on $\Delta H$, $t_p$, $t_f$, and $\Delta t$ at 37°C (isothermal conditions). ................................................................. 114
Table 4-8: The degree of MAA monomer conversion of bone cement composites polymerised isothermally at 37°C. ................................................................. 115
Table 4-9: The effect of MWCNTs chemical functionality and loadings on polymerisation reaction rate constants......................................................................................... 117
Table 4-10: Exothermic polymerisation characteristics for variety of acrylic bone cements. .... 118
1 Introduction
A wide diversity of sophisticated materials are used in biomedical applications [1]. Polymers and their composites present the largest class of biomaterials and are widely used in biomedical devices that include orthopaedic, dental, soft tissue, and cardiovascular implants. Acrylic bone cements, namely poly(methylmethacrylate) (PMMA) have been widely used in orthopaedic surgery for the fixation for joint prostheses in cemented arthroplasties [2]. Cemented total joint replacements are cost-effective interventions for reducing pain, improving function, and enhancing the quality of life in patients with arthritis of the hip and knee. The most common example is total hip replacement, figure (1-1).

Although the in vivo longevity of cemented total joint replacements is very good [2] (10-years survival rate between 94 and 96%), the drawbacks of commercially available acrylic bone cement brands used in cemented arthroplasties are well recognised. Therefore, many long-standing problems such as strength, adhesion, and bone processing have been discussed widely to determine the efficiency of current acrylic bone cements.

One of the main efforts in the improvement of bone cements is to reinforce the existing cements by incorporating various additive materials into the cement matrix. Nanotechnology provides a new avenue to develop composites with superior properties. Current studies have reported enhancements of the mechanical properties of acrylic bone cements by incorporating small amounts of multi-walled carbon nanotubes (MWCNTs) into a cement matrix [4-8], but to date none of these augmented PMMA/CNTs nanocomposites has proven successful. Furthermore, there is a sustained debate on the biocompatibility of MWCNTs and it has been suggested that the reinforced bone cement may have adverse effects on the living cells surrounding the prosthesis [6, 7]. Thus, the aim of this study is to highlight and overcome some limitations of previous studies, which used MWCNTs to reinforce acrylic bone cements, and to conduct a comprehensive investigation into the in vitro biocompatibility of the aforementioned acrylic bone cement nanocomposites using human osteoblasts.
2 Literature Review
2.1 Polymer Composites Biomaterials

2.1.1 Introduction

Biomaterials are materials of natural or man-made origin in the form of implants and medical devices that are widely used to replace and/or restore the function of traumatised or degenerated tissues or organs, to assist in healing, to improve function, to correct abnormalities of living tissues of the human body, and thus improve the quality of life of the patients [9, 10]. The use of biomaterials dates far back into ancient civilisations where artificial eyes, ears, teeth, and noses were found on Egyptian mummies. Chinese and Indians used waxes, glues, and tissues in reconstructing missing or defective parts of the body [11]. Over the centuries, advancements in synthetic materials, surgical techniques, and sterilisation methods have permitted the use of biomaterials in many ways [12]. Medical practice today utilises a large number of medical devices (pacemakers, biosensors, artificial hearts, blood tubes, etc.) and implants (sutures, bone plates, joint replacements, ligaments, vascular grafts, heart valves, intraocular lenses, dental implants, etc.). According to a report published in 2007 by the BCC Research, Wellesley, USA [13], the advanced orthopaedic technology and product market is likely to reach $31.9 billion by 2012, with average growth estimated at 10.3%, and the global catheter market is forecast to grow at a compound annual growth rate of 8.3% to reach $19.4 billion. Within this industry, the global medical market for nanotechnology applications is expected to reach $3.8 billion by 2013, at a compound annual growth rate of 14.9%.

Biomaterials perform in the aggressive internal biological environment of the human body where the pH of body fluids in various tissues varies in the range from 1 to 9, depends on the body's conditions and activities. During daily activities bones are subjected to a stress of approximately 4MPa whereas the tendons and ligaments experience peak stresses in the range 40–80MPa. The average load on a hip joint is up to 3 times body weight and peak load during jumping can be as high as 10 times body weight. More importantly, these stresses are repetitive and fluctuating depending on the activities such as standing, sitting, jogging, stretching, and climbing [9]. In a year, the stress cycles of finger joint motion or hip joint motion estimated to be as high as $1 \times 10^6$ cycles and for a typical heart $0.5 \times 10^7 – 4 \times 10^7$ cycles.
This information roughly indicates the acute and instantaneous biological environment in which the biomaterials need to survive\textsuperscript{[10]}.

Composites are considered to be engineered materials formed two or more constituent materials with significantly different physical or chemical properties which remain separate (do not dissolve or merge completely into each other) and distinct (exhibit an interface between one another) on a macroscopic level within the finished structure, and having bulk properties significantly different from those of any of the constituents. Many tissues of our body, bone, skin, dentine, cartilage, etc. are the best example of composites materials. The fact that the properties of composite material can be tailored by changing the amount, type, and geometry of constituent materials, has lead researchers to design composite materials for various applications in human tissue engineering. Essentially, these composites consist of two constituents: matrix and reinforcement. As the name suggests, the matrix material surrounds and supports the reinforcement materials by maintaining their relative positions, while the reinforcement phase is incorporated into the matrix phase to carry the bulk of the structural loads imposed on a composite and improve its mechanical properties\textsuperscript{[14]}.

Bone is a composite material composed of bone cells (osteocytes), suspended in a matrix consisting of collagen fibres, blood vessels and minerals (hydroxyapatite). The high elastic modulus mineral portion imparts great strength and rigidity to bone. The low elastic modulus collagen fibrils are aligned in bone so that their strong primary bonds are parallel to applied stress. Thus, bone is an anisotropic material with superior properties in the longitudinal direction compare to the radial or circumferencial direction. Two types of bone tissue are observed in the mature human skeleton: Cortical (dense) and Trabecular (cancellous) bones. Although macro- and microscopically different, all bones are identical in their chemical composition and the difference only is in the pattern of arrangement. Cortical bone (also called compact bone) which comprises about 80\% of the skeleton, is dense, has low turn-over rate, a high resistance to bending and torsion. The major part of the cortical bone is calcified and localised in the outer part of all skeletal structure. Cortical bone function is to provide mechanical strength and protection. Trabecular bone (also called spongy bone) is less dense, more elastic, and has a higher turn-over rate than cortical bone. Trabecular bone represents 20\% of the skeletal mass, and 80\% of trabecular bone is found inside the long bones through the skeleton of vertebra, and in the inner portions of the pelvis and other large flat bones\textsuperscript{[15]}.
2.1.2 Classification

The various materials available for use in biomedical applications may be grouped based on the atomic bonding forces of a particular materials into metals, ceramics, polymers, and additionally, different materials can be combined to create a composite material. These classes can be further broken into various sub-groups, each with different applications such as bioinert and bioactive, biostable and biodegradable, etc. or combinations of these classes\[^{16}\].

Gold, tantalum, stainless steel, and cobalt chrome, and nickel titanium alloys are widely considered as biocompatible metals and alloys. Ceramics which are successful as biomaterials include: alumina, titania, zirconia, glass, carbon, and hydroxyapatite (HA). A large number of polymers such as polyethylene (PE), polyurethane (PU), polytetrafluoroethylene (PTFE), polyacetal (PA), poly(methylmethacrylate) (PMMA), polyethylene terephthalate (PET), silicone rubber (SR), polyetheretherketone (PEEK), polysulfone (PSU), poly(lactic acid) (PLA), and poly(glycolic acid) (PGA) are also used in various biomedical applications. Few examples of polymer composite biomaterials are: HA/PE, silica/SR, carbon fibre/ultra high molecular weight polyethylene (CF/UHMWPE), carbon fibre/epoxy (CF/epoxy), and CF/PEEK. Each type of material has its own positive aspects that are particularly suitable for specific application\[^{10}\].

Traditionally, man-made composite materials fall into one of these classes that are based on the matrix material type or the nature of the reinforcement phase. Based on the characteristic of the matrix material, composites are categorised into metal matrix composites, ceramic matrix composites, and polymer matrix composites. The composite properties are influenced by the amounts (volume fractions) and kind of the reinforcement and matrix materials, as well as by the geometry of the reinforcement (particulates, whiskers, fibres, and fabrics)\[^{14}\].

Polymers can be easily fabricated into complex shapes and structures and are available in a wide variety of forms, compositions, and properties, therefore, polymer composite materials are more successful than the other two types of composites, and were developed and investigated for possible biomedical applications. Based on the characteristic of the polymer matrix type, they are further classified into thermoset and thermoplastic polymer composites.

A current trend in biomaterials development is to grow tissues in the laboratory using cells of the target tissue (replaced or augmented) and porous scaffolds. The combination of polymers
(avital or non-living) in the form of foams or fabrics and cells (vital or living) results in special type of composite materials, namely “vital/avital composites” \[17\]. Alternatively, composite material made of (avital or non-living) matrix and reinforcement phases, is called “avital/avital composite”.

The avital/avital composites are further divided into non-resorbable, partially resorbable, and fully resorbable composite biomaterials. The non-resorbable composites are designed not to degrade in the \textit{in vivo} (inside the body) environment. They are mainly proposed for long-term implants such as total joint replacements (TJRs), but they are also promising for short-term applications such as bone plates, and screws. On the other hand the resorbable composites are particularly promising short-term or transient implants (screws, vascular grafts, and artificial skin) as they are intended to lose their mechanical integrity in \textit{in vivo} conditions \[10\].

\subsection*{2.1.3 Biocompatibility}

Clinical experience clearly indicates that not all commonly used engineering materials are suitable for biomedical applications. In the early days, natural materials such as wood, gum and rubber, and tissues from living forms, and manufactured materials such as iron, gold, zinc and glass were used as biomaterials based on the trial and error method. Some materials were tolerated by the body whereas others were rejected. It has been recognised that there are deep differences between non-living (avital) and living (vital) materials. Also, depending on the characteristics of the host tissues and surgical procedure, the host responses to the same material were extremely varied from tolerated by the body in one situation and rejected in another.

To understand the interactions between the tissues and the biomaterials, considerable progress has been made over the last four decades and the words ‘biomaterial’ and ‘biocompatibility’ have been invented to indicate the biological performance of materials \[18\]. Materials that are biologically compatible are called biomaterials, and the biocompatibility is a descriptive term which indicates the ability of a material to perform with an appropriate host response, in a specific application. In simple terms it implies compatibility or harmony of the biomaterial with the living systems \[19\].
This definition was extended by Wintemantel et al.\cite{20} who distinguished between surface and structural compatibility of an implant. They defined the chemical, biological, and physical (including surface morphology) suitability of an implant surface to the host tissues as surface compatibility, whereas structural compatibility refers to the mechanical properties of the implant material at the implant-tissue interface, such as elastic modulus and strength, implant design, and optimal load transmission (minimum interfacial strain mismatch). Optimal interaction between biomaterial and host is reached when both the surface and structural compatibilities are met. Furthermore, the success of a biomaterial in the body also relies on the activities and health condition of the patient, as well as the surgical technique such as the degree of trauma imposed during implantation, sterilisation methods, etc. \cite{10}.

2.1.4 Mechanical Properties

Despite the high strength, ductility, and resistance to wear of metals, of many have shortcomings including low biocompatibility, corrosion, too high stiffness compared to tissues, high density, and release of metal ions which may cause allergic tissue reactions \cite{21}. With ceramics positive characteristics are good biocompatibility, high compression resistance, and corrosion resistance. Disadvantages of ceramics include: brittleness, low mechanical reliability, low fracture strength, difficult to fabricate, lack of resilience, and high density. As implants in orthopaedic surgery, polymers tend to be too weak and flexible to meet the mechanical demands of these applications \cite{10}. Also, depending on the application and usage, polymers may leach undesirable products (e.g. monomers, fillers, plasticisers, antioxidants), absorb liquids and swell. Moreover, the sterilisation processes may affect the polymer properties.

In order to overcome many shortcomings of the homogenous materials mentioned above, polymer composite materials provide an alternative choice. The primary development of polymer composites for biomedical applications are derived from the orthopaedic implant applications, although, traditionally metal alloys are used in implants and prostheses applications such as (TJR$s$).

Generally, tissues are classified into hard and soft tissues. As the names suggest, in general the hard tissues are stiffer (elastic modulus) and stronger (tensile strength) than the soft tissues. Bone and tooth are examples of hard tissues, and skin, blood vessels, cartilage and
ligaments are a few examples of soft tissues. Tables (2-1) & (2-2) show that the metal alloys and ceramics are about 10-20 times stiffer than the hard tissues, therefore, metals or ceramics are chosen for hard tissue applications, and polymers for the soft tissue applications.

Table 2-1: Mechanical properties of hard and soft tissues.[10]

<table>
<thead>
<tr>
<th>Material</th>
<th>Modulus (MPa)</th>
<th>Tensile Strength (MPa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hard tissue</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cortical bone (longitudinal direction)</td>
<td>17,700</td>
<td>133</td>
</tr>
<tr>
<td>Cortical bone (transverse direction)</td>
<td>12,800</td>
<td>52</td>
</tr>
<tr>
<td>Cancellous bone</td>
<td>400</td>
<td>7.4</td>
</tr>
<tr>
<td>Enamel</td>
<td>84,300</td>
<td>10</td>
</tr>
<tr>
<td>Dentine</td>
<td>11,000</td>
<td>39.3</td>
</tr>
<tr>
<td>Soft tissue</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Articular cartilage</td>
<td>10.5</td>
<td>27.5</td>
</tr>
<tr>
<td>Fibrocartilage</td>
<td>159.1</td>
<td>10.4</td>
</tr>
<tr>
<td>Ligament</td>
<td>303.0</td>
<td>29.5</td>
</tr>
<tr>
<td>Tendon</td>
<td>401.5</td>
<td>46.5</td>
</tr>
<tr>
<td>Skin</td>
<td>0.1-0.2</td>
<td>7.6</td>
</tr>
<tr>
<td>Arterial tissue (longitudinal direction)</td>
<td>—</td>
<td>0.1</td>
</tr>
<tr>
<td>Arterial tissue (transverse direction)</td>
<td>—</td>
<td>1.1</td>
</tr>
<tr>
<td>Intraocular lens</td>
<td>5.6</td>
<td>2.3</td>
</tr>
</tbody>
</table>

Table 2-2: Mechanical properties of typical biomaterials.[10]

<table>
<thead>
<tr>
<th>Material</th>
<th>Modulus (MPa)</th>
<th>Tensile Strength (MPa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Metal alloys</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stainless steel</td>
<td>190</td>
<td>586</td>
</tr>
<tr>
<td>Co-Cr alloy</td>
<td>210</td>
<td>1085</td>
</tr>
<tr>
<td>Ti-alloy</td>
<td>1116</td>
<td>965</td>
</tr>
<tr>
<td>Amalgam</td>
<td>30</td>
<td>58</td>
</tr>
<tr>
<td>Ceramics</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Alumina</td>
<td>380</td>
<td>300</td>
</tr>
<tr>
<td>Zirconia</td>
<td>220</td>
<td>820</td>
</tr>
<tr>
<td>Bioglass</td>
<td>35</td>
<td>42</td>
</tr>
<tr>
<td>Hydroxyapatite</td>
<td>95</td>
<td>50</td>
</tr>
<tr>
<td>Polymer</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Polyethylene (PE)</td>
<td>0.88</td>
<td>35</td>
</tr>
<tr>
<td>Polyurethane (PU)</td>
<td>0.02</td>
<td>vary</td>
</tr>
<tr>
<td>Polytetrafluoroethylene (PTFE)</td>
<td>0.5</td>
<td>27.5</td>
</tr>
<tr>
<td>Polycetel (PA)</td>
<td>2.1</td>
<td>67</td>
</tr>
<tr>
<td>Poly(methylmethacrylate) (PMMA)</td>
<td>2.55</td>
<td>59</td>
</tr>
<tr>
<td>Polyethylene terephthalate (PET)</td>
<td>2.85</td>
<td>61</td>
</tr>
<tr>
<td>Polyetheretherketone (PEEK)</td>
<td>8.3</td>
<td>139</td>
</tr>
<tr>
<td>Silicone rubber (SR)</td>
<td>0.008</td>
<td>7.6</td>
</tr>
<tr>
<td>Polysulfone (PS)</td>
<td>2.65</td>
<td>75</td>
</tr>
</tbody>
</table>
Stiffness mismatch between bone and metallic or ceramic implants is one of the major problems in orthopaedic surgery. In the load sharing between the bone and implant, the amount of stress carried by each of them is directly related to their stiffness. Thus, bone is insufficiently loaded compared to the implant, and this phenomenon is called ‘stress-shielding’ or stress protection, and the degree of stress protection is proportional to the degree of stiffness mismatch \[22\]. The stress-shielding affects the bone remodelling and healing process leading to increased bone porosity (also known as bone atrophy).

Considering the structural or mechanical compatibility with tissues, matching the stiffness of host tissues with the implant’s stiffness decreases the stress-shielding effect and produces desired tissue remodelling. Therefore, the use of low-modulus materials such as polymers becomes viable; however, low strength usually associated with low modulus limits their potential use. For this reason, polymer composites which exhibit concurrently low elastic modulus and high strength have been proposed for several orthopaedic applications \[23\].

Additional advantage of composite materials is that the properties and design of an implant can be varied and tailored by controlling the volume fractions and the arrangement of the reinforcement phase to suit the mechanical and physiological conditions of the host tissues. Thus, composite materials with modified properties offer a greater potential of structural biocompatibility than the homogenous monolithic materials \[24\]. Moreover the human tissues are essentially composite materials with anisotropic properties, which depend on the roles and structural arrangements of various components of the tissues (e.g. the longitudinal mechanical properties of cortical bone are higher than the transverse direction properties). These similarities have led to the development of composite biomaterials.

Other reasons for the development of polymer composite biomaterials include: absence of corrosion and fatigue failure of metal alloys and release of metal ions such as Nickel or Chromium which may cause loosening of the implant, patient discomfort, and allergic skin reactions, as well as low fracture toughness of ceramic materials which make them a difficult choice for load bearing applications. Polymer composite materials offer several significant advantages over metal alloys and ceramics in correcting the above mentioned or perceived deficiencies \[25\].
Metals alloys and ceramics are radio opaque and in some cases they result in undesirable artefacts in x-ray radiography \cite{26}. In the case of polymer composite materials the radio transparency can be adjusted by adding contrast medium to the polymer. Moreover the polymer composite materials are fully compatible with the modern diagnostic methods such as computed tomography (CT) and magnetic resonance imaging (MRI) as they are non-magnetic with lightweight and superior mechanical properties \cite{10, 14}.

### 2.1.5 Applications

Over the years, several studies have developed and investigated a wide variety of polymer composite materials for various biomedical applications in terms of soft and hard tissue applications,

Depending on the intended application, the soft tissue implants perform various functions. Bulk space fillers are used to restore cosmetic defects, atrophy, or hypoplassty to an aesthetically satisfactory condition. Composites comprising PET or PTFE fabrics and PU are used to fill the defects in the articular surfaces or to replace meniscus or fibrous tissues following the condylar shave or high condylectomy in the treatment of painful arthritis and to restore normal joint function \cite{27}. Woven carbon fibre fabrics and their composites are used for the treatment of cartilage defects \cite{28}. Burn victims are often treated with hybrid skin dressings that are a combination of synthetic polymers and cultured cells to form vital/avital composites. Silicone rubber reinforced with silica particles is widely used as a material for making catheters. Newer designs, with complete \textit{in vivo} recovery from kinking situations, consist of polymers (PU, LDPE, and PVC) reinforced with braided Nitinol (Ni–Ti alloy) ribbons were also reported \cite{29}. Ligament prosthesis were reproduced to mimic the natural ligaments by reinforcing a hydrogel matrix (PHEMA) with helically wound rigid PET fibres \cite{30}. Composites made of polyurethane fibres in a matrix of polyurethane and PELA (block copolymer of lactic acid and polyethylene glycol) mixture were developed for vascular graft materials \cite{31}. Otology prostheses made of CF/PTFE composites have been tried to replace defective ossicles \cite{32}. PET fabrics coated with collagen and PU materials are used for repairing hernia and abdominal wall \cite{33}.

For hard tissue applications, casting materials used in the external bone fraction fixation essentially are composite materials made of woven cotton fabrics and Plaster of Paris matrix
(calcium sulphate), and other reinforcements include fabrics of glass and polyester fibres. Also, casts made of glass or polyester fibre fabrics, and water-activated polyurethanes have been reported \cite{10}. External fixators constructed from CF/epoxy composite materials are also used \cite{34}. For internal bone fixation, a variety of polymer composite materials, including fully and partially resorbable composites, were proposed for bone plate applications \cite{35, 36}. A large amount of polymer composites were developed for vertebral body replacement (Bioglass/PU) \cite{37}, grafting purposes (Bioglass/PS) \cite{38}, and cages for lumbar interbody fusion (CF/PEEK and CF/PS) \cite{39}. Carbon fibres and biocompatible epoxy resin is also used to correct structural abnormalities or curvatures of spine \cite{40} and used for intramedullary application.

Numbers of artificial joints have been designed to replace or augment many joints in the body. Researchers proposed reinforcing UHMWPE with carbon fibres or UHMWPE fibres for acetabular cups \cite{41}. A prosthesis made of polymer composite with spatially or locally varying mechanical properties along the boundary of the prosthesis, results in a more uniform and efficient transfer of stress from the stem to the bone. This may lead to better bone remodeling and longer implant service life. CF/epoxy and CF/PEEK composite stems with behaviour similar to that of the femur were developed \cite{42, 43}.

One of the earliest methods for fixing the artificial joints to the bones is to press-fit the joint prosthesis into the bone using grouting material called bone cement. The most widely used bone cement is based on PMMA, also called acrylic bone cement. Researchers have tried to improve bone cement mechanical and thermal properties by reinforcing with variety of fillers to improve its mechanical and biological properties \cite{44}. Reinforced PE with bioactive HA particles and Bioglasses have been investigated as synthetic bone grafts to fill or replace fractured bones \cite{45}.

Dental composite resins, comprised of bisphenol-alpha-glycidyl methacrylate (Bis-GMA) as the matrix polymer and quartz, barium glass, and colloidal silica as fillers are very commonly used to restore posterior teeth as well as anterior teeth. Dental posts made of zirconia, short glass fibre reinforced polyester, and carbon fibre reinforced epoxy composite posts are also introduced. Inexpensive and easy to use CF/PMMA, GF/PMMA, and UHMWPE/PMMA composite bridges and dentures have been replaced the high cost and time consuming preparation of current gold bridges \cite{46}.
A typical artificial leg system consists of socket, shaft, and foot. Sockets are made using a combination of knitted or braided carbon or glass fibre fabrics and water-curable (water-activated) resins. The shaft or stem is often made of filament wound or laminated woven/braided fabric carbon fibre reinforced epoxy composites. The foot unit consists of heel and forefoot components, which are made of laminated CF/epoxy composites [47].

2.2 Carbon Nanotubes

2.2.1 Introduction

Carbon nanotubes (CNTs) that possess exceptional mechanical, thermal, and electrical properties are promising to revolutionise several fields in material science and are a major component of nanotechnology. Further market development will depend on material availability at reasonable prices. Nanotubes have a wide range of unexplored potential applications in various technological areas such as aerospace, energy, automobile, medicine, or chemical industry [48]. In the medical field, biomaterials are expected to be developed using CNTs for clinical use, in which they can be used as local drug delivery systems (DDS) and/or scaffolds to promote and guide bone tissue regeneration [49].

Carbon nanotubes are molecular-scale tubes of graphitic carbon with remarkable unique characteristics. They are among the stiffest and strongest fibres known. The current enormous academic and industrial growing interest in carbon nanotubes is a direct consequence of the synthesis and detection of large spherical unstable molecules of fullerenes C$_{60}$ and C$_{70}$. Fullerenes are large, closed-cage, carbon clusters and have several special properties that were not found in any other compound before [50]. Since the discovery in 1991 by Iijima et al. [51] that carbon could form stable, ordered structures other than graphite and diamond, carbon nanotubes have been investigated and inspired many researchers all over the world. Their relatively large length (up to several microns) and small diameter (a few nanometres) result in a large aspect ratio. They can be seen as the nearly one-dimensional form of fullerenes. Therefore, these materials are expected to possess additional interesting properties.
According to a report published in 2010 by the BCC Research, Wellesley, USA \[52\], the Global market for CNTs grades based on committed production reached $103 million in 2009. This market is projected to reach $167.2 million in 2010 and $1 billion in 2014 at a compound annual growth rate of 58.9%. Different types of carbon nanotubes can be produced in various ways. Economically feasible large-scale production and purification techniques still have to be developed \[48\].

2.2.2 Structure and Defects

Many interesting structures of fullerenes exist: regular spheres, cones, tubes and also more complicated and strange shapes; figure (2-1).

Carbon nanotubes can be considered as nearly one-dimensional structures due to a length to diameter ratio. The bonding in carbon nanotubes is sp², with each atom joined to three neighbours, as in graphite \[53\].

Single-Walled Nanotubes (SWNTs) are long wrapped graphene sheets consist of two separate regions with different physical and chemical properties. The first is the sidewall of the tube (cylinder), and the second is the end cap of the tube. Carbon atoms placed in hexagons and pentagons form the end cap structures with structure similar to fullerene C_{60}. 
The graphene sheet of a certain size can be rolled into a tube in three distinct ways to generate the cylinder structure, as shown in figure (2-2).

![Figure 2-2: Structure of single wall carbon nanotubes.](image)

Based on the arrangement of hexagons around the circumference, the first two are known as “armchair” and “zig-zag” with high degree of symmetry. The third class is the most common in practice known as “chiral”, meaning that it can exist in two mirror-related forms. Multi-Walled Nanotubes (MWNTs) can be considered as a collection of concentric SWNTs with different diameters, figure (2-3).

![Figure 2-3: Structure of multi-wall carbon nanotubes.](image)
The length and diameter of these structures differ a lot from those of SWNTs and, of course, their properties are also very different \cite{54}. As engineering materials, the existence of a crystallographic defect critically changes the carbon nanotube properties.

Defects can occur in the form of atomic vacancies, thus, desirable or undesirable defects are possible. Deformations, such as bends and nanotube junctions, are introduced by replacing a hexagon with a heptagon or pentagon, and it can be inwards or outwards.

Another class of defects is caused by impurities that are built in during or after the nanotube growth process. Under certain conditions, defects can be introduced in a controlled way to design various new unique structures such as Y-branches, T-branches, or SWNT junctions, that will have more interesting properties than their original forms \cite{55}; figure (2-4).

![Some defects structures of CNTs]{48}

2.2.3 Synthesis of CNTs

Even though scientists are intensively researching more economic ways to produce these structures, CNTs are generally produced by three main techniques; arc discharge, laser ablation and chemical vapour deposition.
2.2.3.1 **Arc-Discharge**

The carbon arc discharge method, initially used for producing $C_{60}$ fullerenes, is the most common and perhaps easiest way to produce CNTs as it is rather simple to undertake. This method creates nanotubes through arc-vaporisation of two carbon rods placed end to end, separated by approximately 1 mm, in an enclosure usually filled with inert gas (helium, argon) at low pressure (50-700 mbar).\(^{[55]}\) Recent investigations have shown that it is also possible to create nanotubes with the arc method in liquid nitrogen \(^{[56]}\). Producing nanotubes in high yield depends on the uniformity of the plasma arc and the temperature of the deposit formed on the carbon electrode \(^{[57]}\). Different diameter distributions have been found depending on the mixture of helium and argon, temperature and carbon and metal catalyst densities. Depending on the exact technique, it is possible to selectively grow SWNTs or MWNTs. Two distinct methods of synthesis can be performed with the arc discharge apparatus. If SWNTs are preferable, inert gas, optical plasma control, catalyst, improvement of oxidation resistance, and open air synthesis with welding arc torch, are among many ways to improve the process of arc discharge method \(^{[58-60]}\). However, synthesis in liquid nitrogen \(^{[56]}\), Magnetic field synthesis \(^{[61]}\), and Plasma rotating arc discharge \(^{[62]}\) are the most possibly economical route to mass production of MWNTs.

2.2.3.2 **Laser Ablation**

In 1996, Smalley et al.\(^{[63]}\) reported the synthesis of high yields CNTs by laser vaporisation of graphite rods with small amounts of Ni and Co at 1200°C. The tube grows until too many catalyst atoms aggregate on the end of the nanotube. The large particles either detach or become over-coated with sufficient carbon to poison the catalysis \(^{[64]}\). This allows the tube to terminate with a fullerene-like tip or with a catalyst particle. Both arc-discharge and laser-ablation techniques have the advantage of high (>70%) yields of SWNT but the drawback is that they rely on evaporation of carbon atoms from solid targets at temperatures $>3000$°C, and the nanotubes are tangled which makes the purification difficult and limits application of the samples \(^{[48]}\). MWCNTs are synthesised if graphite is mixed with Co, Ni, Fe, or Y catalysts instead of pure graphite for SWCNTs. The Ni/Y mixture catalyst gave the best yield \(^{[55]}\). Laser vaporisation results in a high yield for purer SWCNTs with better properties, and narrower size distribution than SWCNTs produced by arc-discharge.
2.2.3.3 Chemical Vapour Deposition

In 1996 a chemical vapour deposition CVD method emerged as a new candidate for nanotube synthesis and is capable of controlling growth direction on a substrate and synthesising a large quantity of nanotubes \[65\]. It is achieved by using a carbon source in the gas phase and applying an energy source, such as plasma or a resistively heated coil, to transfer energy to gaseous carbon molecules. Commonly used gaseous carbon sources include methane, carbon monoxide and acetylene. The energy source is used to “crack” the molecule into reactive atomic carbon. Then, the carbon diffuses towards the substrate, which is heated and coated with a catalyst where it will bind. Carbon nanotubes will be formed if the proper parameters are maintained \[55\]. Excellent alignment, as well as positional control on nanometre scale can be achieved by using CVD \[66\]. Control over the diameter, as well as the growth rate of the nanotubes can also be maintained. The appropriate metal catalyst can preferentially grow single rather than multi-walled nanotubes \[67\]. CVD is essentially a two-step process consisting of a catalyst preparation step followed by the actual synthesis of the nanotube. The catalyst is generally prepared by sputtering a transition metal onto a substrate and then using either chemical etching or thermal annealing to induce catalyst particle nucleation. Ammonia may be used as the etchant and the temperatures for the synthesis of nanotubes by CVD are generally within the 650–900°C range. Typical yields for CVD are approximately 30%. Different techniques for the carbon nanotubes synthesis with CVD have been developed, such as plasma enhanced, thermal chemical, alcohol catalytic, vapour phase growth, aero gel-supported and laser-assisted CVD \[68\].

2.2.4 Properties of Carbon Nanotubes

The unique electrical properties and conductivity of carbon nanotubes are derived to a large extent from their nearly one dimensional character and the peculiar structure of graphite. Depending on their diameter, carbon nanotubes are either semi-conducting or metallic. The differences in conducting properties are caused by the molecular structure that results in a different band structure and thus a different band gap \[69\]. Nanotubes have been shown to be superconducting at low temperatures. The resistance to conduction is determined by quantum mechanical aspects and was proved to be independent of the nanotube length \[70\]. In addition, they can carry the highest current density of any known material, measured as high as
10^9 A/cm^2 [71]. A nanotube formed by joining nanotubes of two different diameters end to end can act as a diode, suggesting the possibility of constructing electronic computer circuits entirely out of nanotubes [48].

Prior to CNTs, diamond was the best thermal conductor. CNTs have now been shown to have a thermal conductivity at least twice that of diamond. CNTs are very good thermal conductors along the tube, but good insulators lateral to the tube axis. That is, CNTs have the unique character of feeling cold to the touch, like metal, on the sides with the tube ends exposed, but similar to wood on the other side’s [48]. The thermal conductivity of SWCNTs across its axis at room temperature is about 1.52 W/m.K, which is about as thermally conductive as soil [72]. The measurements of the thermoelectric power of nanotube systems give direct information for the type of carriers and conductivity mechanisms.

SWNT has a room-temperature thermal conductivity along its axis of about 3500 W/m.K, compare this to copper, a metal well-known for its good thermal conductivity, which transmits 385 W/m.K [73]. The temperature stability of carbon nanotubes is estimated to be up to 2800°C in vacuum and about 750°C in air [74].

Compared with a graphene sheet, the chemical reactivity of CNTs is enhanced as a direct result of the curvature of the CNT surface. Carbon nanotube reactivity is directly related to the pi-orbital mismatch caused by an increased curvature. Therefore, a distinction must be made between the sidewall and the end caps of a nanotube. For the same reason, a smaller nanotube diameter results in increased reactivity. The solubility of CNTs in different solvents can be controlled by covalent chemical modification of either sidewalls or end caps of the CNT [75].

The carbon nanotubes are expected to have high stiffness and axial strength as a result of the carbon–carbon sp² bonding [53]. The small diameter of the tubes leads to macroscopic quantization of the electronic and vibrational states in the transversal direction and to a reduced dimensionality along the tube axis. However, these properties vary widely depending on the production method used to grow the nanotubes, the number of defects, and whether the nanotubes are SWNTs and MWNTs. It is also very hard to accurately measure the mechanical properties of CNTs due to their small size [76].
The nanotube as a whole is very flexible because of the length to diameter ratio. Therefore, these compounds are potentially suitable for applications in composite materials that need anisotropic properties. The tensile strength of MWNTs has been measured experimentally to be 11–63 GPa, with no dependence on outer wall diameter \(^{[77]}\). Nanotubes are the stiffest known fibre; the elastic modulus of CNTs is estimated at greater than 1TPa, based on the in-plane elastic modulus of graphite, with an expected elongation to failure of 20-30\% \(^{[78]}\). For comparison, the Young’s modulus of high-strength steel is around 200 GPa, and its tensile strength is 1-2 GPa \(^{[79]}\). However, CNTs produced by chemical vapour deposition (CVD) are known to contain many more defects than arc-grown CNTs, drastically affecting their mechanical properties. The mechanical behaviour of CNTs in composites is more complicated. Whether or not the strength and stiffness of CNTs can be transferred to the matrix depends on the amount of interfacial bonding between the two phases, which is affected by the CNTs’ wettability and interfacial area. Additionally, CNTs can buckle and remain twisted and curved in the matrix, meaning full advantage of the interfacial area may not be taken advantage of\(^{[76]}\).

It is also suggested that SWNTs might serve as better fillers than MWNTs. Firstly; SWNTs have a higher aspect ratio and greater surface area than MWNTs, allowing for more interfacial bonding. Secondly, while the outer shells of MWNTs can bond with the matrix material, the inner shells can rotate and slide freely, being only held by weak Van der Waals forces \(^{[80]}\). The drawback is that SWNTs are much harder to synthesize in large quantities and to process, compared with MWNTs. Ruoff and Lorents \(^{[81]}\) reported another advantage of using CNTs as a filler when postulate that fracture can occur via collapse of the inside hollow of the CNT, providing extra absorption energy and increased toughness when used in composites.

### 2.2.5 Purification

Purification of CNTs is a major challenge with nanotube application next to large scale synthesis. It generally refers to the separation of as-produced CNTs in soot from other impurities, such as graphite (wrapped up) sheets, carbon nanoparticles, amorphous carbon, residual catalyst, smaller fullerenes, and other unwanted species. These impurities will interfere with most of the desired properties of the CNTs. Also in the fundamental research,
in order to understand the measurements better, the CNTs samples have to be as pure and homogeneous as possible \cite{48}. Several purification techniques are used. Basically, these techniques can be divided into two mainstreams, structure selective (separate the CNTs from the impurities) and size selective (give more homogeneous diameter or size distribution) separations. The commonly used techniques are: oxidation, acid treatment, annealing, ultrasonication, micro-filtration, ferromagnetic separation, cutting, functionalisation and chromatography techniques. Most of the techniques used, are combined with other techniques and the use of strong oxidation, cutting, and acid refluxing techniques may have an effect on the structure of the tubes. Desired techniques are tearing down the carbon impurities and the metals, without changing the nanotubes. When a technique is chosen, care should be taken in adjusting the process variables such as temperature, scale, and time as well as the composition and the amount of the sample \cite{55}.

2.2.6 Functionalisation

All these potential applications in compound materials require new characteristics prepared by adding various chemical functional groups to CNTs to make the nanotubes processable and tune their properties to match a desired purpose by improving both their solubility and wettability. The main disadvantage of all carbon nanotubes is their insolubility in any simple solvents. Solubility is a vital property for processability, when only suspensions of the tubes can be produced. High molecular weights and strong intertube forces keep CNTs together in bundles, making their manipulation, characterisation and analytical investigation very difficult. Functionalisation by chemical reactions with extended molecular chains offers the advantage of creating a new soluble and easy-to-handle class of materials with new properties. As a consequence, properly functionalised CNTs become soluble in many solvents, so that their solution properties can be investigated, and the compatibility of CNTs with other materials, such as polymers, is expected to improve, thus, the new CNTs may find useful applications in the field of materials science and technology.

Functionalisation may help to separate semiconducting tubes from metallic ones, to purify nanotubes from carbonaceous impurities or to reduce the width of diameter dispersion. Alternative ways to functionalise carbon nanotubes are by substitution reactions such as replacement of carbon atoms from the tube wall by boron or nitrogen. In principle
functionalization should also be possible from the inside of the tubes. Graphite, and similarly graphene are well known to be chemically highly inert structures. Since CNTs reactivity is activated by curvature effects, curvature in nanotubes is much smaller than in conventional fullerenes because the tube diameters are generally larger and they are curved in one direction only \cite{82}. The areas of the CNTs to which the functional groups are added are the side-walls, figures (2-5A) & (2-5B) and on the end tips, figure (2-5C).

![Figure 2-5: Examples of (A) covalent side-wall, (B) non-covalent side-wall, and (C) covalent tip functionalisation of CNTs.\cite{49}]

In addition, several available strategies have been devised to solubilise nanotubes. Among these, the most successful are: the covalent functionalisation of sp$^2$ carbons at the sidewalls with organic pendant groups, figures (2-5A) & (2-5C) and the non-covalent functionalisation through supramolecular interactions, figure (2-5B), which allows the formation of stable suspensions \cite{49}.

Chemical oxidation is by far the most common method of functionalising CNTs, both for enhancing their reactivity and enabling them to disperse better in water and organic solvents. Typical oxidisers include HNO$_3$, H$_2$SO$_4$, KMnO$_4$, OsO$_4$, and RuO$_4$. This method covalently attaches a wide range of functional groups, including carboxyl (−COOH), carbonyl (−C=O), and hydroxyl (−OH) groups, to the CNT ends and sidewall defects, while also purifying the nanotubes, ridding them of any amorphous carbon and nanoparticles. CNT tips and defects are particularly reactive; thus, oxidation tends to attack and remove nanotube ends first, and then attacks any defects from synthesis, attaching functional groups at those locations. Sonicating the acid/CNTs mixture during functionalisation increases the reaction and also creates more defects in the CNTs, offering more sites for functional group attachment.
However, this can also cleave the CNTs, cutting them into shorter segments, and weaken them mechanically [76].

2.2.7 Nanotoxicology

Particle toxicology is the study of the adverse effects of tissue exposure to particulate matter. The emergence of nanotechnology and nanoparticulate pollution has raised questions as to the effect of these nanomaterials on human health. The term nanotoxicology, currently defined as ‘‘science of engineered nanodevices and nanostructures that deals with their effects in living organisms’’, was coined for a toxicological evaluation of nanosized particles and fibres [83].

The potential of a nanoparticle to cause harm is determined by the following factors [84]: (1) The reactivity or inherent toxicity of the chemical(s) contained within the particle; (2) The large surface area of the particle(s) providing a larger area of contact with the cellular membrane and capacity for absorption, hence greater transport of toxic substances; (3) The longer the retention time with the cellular membrane is the greater the chance for damage. Thus, the concept of particle mobility through clearance or migration to surrounding tissue is also integrated with retention time. Therefore, as particles become smaller, their high surface area enhances the inherent toxic effects, and the potential to cause harm becomes greater [83].

Fibrous materials present a different pathology to particulates. In particular, exposure via respiration is far more pathogenic than other methods of entry. The pathogenicity of respirable fibres is determined by the following characteristics [84]: (1) As with particulates, the toxicity of a fibrous material will also depend largely on the reactivity or inherent toxicity of its chemical components; (2) The dimensions of the fibre determine its respirability (the ability to penetrate into the lungs). Long fibres become difficult for macrophages to phagocytose, which promotes chronic release of inflammatory mediators and contributes to fibrosis. Short fibres, however, are usually easily phagocytosed and hence, cleared by the macrophages; (3) High biopersistence (the ability of a fibre to persist in the lung) increases the toxicity of all fibres while low biopersistence decreases the toxicity of even very long fibres. Fibres that are biosoluble are capable of having their structural components leached out over time until they eventually break into smaller fragments which then phagocytosed
and cleared. These characteristics apply to conventional diameter fibres and it is not yet known whether they will also be applicable to nano-fibres. Similarly, it is not clear how the inherent chemical stability of CNTs will impact on their biopersistence \[85\].

### 2.2.8 Toxicity of Carbon Nanotubes

One of the major concerns surrounding the use of CNTs based materials is the unknown impact on workers involved in their manufacture and handling. If CNTs are to be incorporated into composite materials for medical applications, evidence of their toxicity is essential. It is also imperative that the occupational health and safety of CNTs exposure be investigated before the use of CNTs-based materials becomes widespread.

Some *in vivo* studies have assessed the potential impact of CNTs on the lungs. Despite no initial indication of lung toxicity, other studies have found histological evidence of lung inflammation and granuloma formation. The presence of CNTs resulted in granuloma formation and all CNTs products induced dose-dependent lung lesions, regardless of the levels of impurities. Abnormal lung resistance was observed in animals exposed to CNT samples and the exposure time was critical for induction of lung pathology \[86\]. Histopathological examinations show large CNTs agglomerations in the lungs of treated animals \[87\]. Current handling procedures employed by nanotube manufacturers do not produce significant quantities of airborne CNTs \[88\]. However, the possibility of cumulative effects, especially if increased quantities are handled, justifies the introduction of safety measures.

Dermatological tests into skin irritation by CNTs showed that MWCNTs do not cause strong inflammatory reactions and had no visible effect on tissue healing, suggesting good tissue compatibility \[49\]. However, more *in vitro* studies involving human epidermal keratinocytes have raised concerns over this assessment \[89\].

The cytotoxicity and long-term impact of CNTs on human health need to be characterised very carefully before applying them *in vivo*. A cytotoxicity test is a screening tool determines whether a product or compound will have any toxic effect directly or due to leachables on living cells before they are put into the design of a medical device. It has been well known that nanoparticles can create reactive forms of oxygen that damages the cells. Cells can defend themselves by producing anti-oxidants when they encounter low concentrations of
nanoparticles. It has been shown that cells can become inflamed or die when the concentrations of nanoparticles increase \[^{[90]}\]. Just like most other nanoparticles, CNTs also possess a small size and large surface area. Therefore, it is likely that they will evoke the generation of reactive oxygen species (ROS) when used \textit{in vivo}. Recently, a number of groups have come out with contradictory reports on the nature of CNT’s toxicity, with some indicating CNTs are highly toxic and others showing lack of any toxic effects \[^{[84]}\].

It has been reported that treatment of human myeloid leukemia cells with SWCNTs can lead to time and dose dependent reduction in cell viability and has no effect on cell proliferation and viability if the cells are exposed to MWCNTs for a short time period \[^{[91, 92]}\]. Cui et al.\[^{[92]}\] investigated SWCNTs cytotoxicity and reported that SWCNT inhibited human embryonic kidney cells by inducing apoptosis and decreasing cellular adhesion ability. Both cell proliferation and adhesion ability decreased in a dose- and time-dependent manner.

The discrepancy in the cytotoxicity and biocompatibility studies of CNTs can be due in part to various factors such as the size, the nature (soluble or insoluble), and the method of functionalisation, the concentration and exposure time of CNTs used \textit{in vitro} or \textit{in vivo}. Compared with the solubilised CNTs, the exposure to the hydrophobic CNTs are particularly toxic to the cells and tissues \[^{[84]}\]. Although the CNTs are now being used even along with various stem cells. Though successful stem cell differentiations were reported, it is also interesting to note that severe DNA damages were observed in certain stem cells \[^{[93, 94]}\].

Drawing a general conclusion about the safe use of CNTs \textit{in vivo} is difficult because most studies are conducted based on varying concentrations and different conditions leading to contradictory results \[^{[95]}\]. Obviously, the functionalisation or solubilisation of CNTs cannot be a single and affirmative solution for preventing the CNTs cytotoxicity. The presence of modified CNTs can be hazardous in various routes. For example, a study conducted by Roberts et al.\[^{[96]}\] demonstrated that soluble CNTs can be modified during the digestion process by the organism and rendered insoluble back into the environment. This means that the nanotubes can be soluble and stable before they are taken up by organisms, but their interaction with biological systems can quickly convert the nanotubes into an insoluble form. Leaking of the nanotubes from implanted CNTs-based sensors and scaffolds and its accumulation in the body may then have serious and wide spread adverse effects.
The effect of CNTs on the formation of extracellular matrix (ECM) has also been characterised. One of the major components of extracellular matrix is collagen. Hence, collagen has been widely used in the regenerative medicine, it can be assumed that the incorporation of CNTs with collagen has the potential of improving the mechanical and electrical properties of these biomaterials [97]. Mac-Donald et al. [98] examined the cytotoxicity of a carboxyl functionalised SWCNTs collagen composite and found that the cell viability is uniformly high throughout the collagen matrix and the cell morphology and the collagen gel formation are unaffected by the presence of CNTs in the matrix.

Another aspect of the CNTs cytotoxicity studies involves examining the clearance of CNTs from the body after their introduction. Singh et al. [99] found that both SWCNTs and MWCNTs follow a rapid first order clearance from the blood compartment through the renal excretion route without any toxic side effects or mortality. This study suggested that soluble CNTs are safe for use in vivo. The modification of the CNTs surface coating reinstates the need for developing better methods for functionalisation and solubilisation of the CNTs. Otherwise the use of CNTs may result in unanticipated effects on the tissue or organism exposed [97].

2.2.9 CNTs for Tissue Engineering Applications

In the relatively new arena of nanobiotechnology, a vast majority of applications are based on CNTs, ranging from miniaturized biosensors to organ regeneration. Nevertheless, the complexity of biological systems poses a significant challenge in developing CNTs-based tissue engineering applications. The interaction between living cells/tissues and the nanotubes have been transformed by a variety of approaches and novel techniques to medical science and disease treatment. This integration has already resulted in a revaluation of tissue engineering and organ regeneration techniques. Some of the new treatments that were not possible previously become reachable now [100]. As CNTs are highly hydrophobic and the lack of solubility in physiological solutions is one of the main difficulties in integrating them into biological systems. Hence, a number of methods have been developed to solubilise CNTs through surface modification. Due to its surface chemistry, the biocompatibility of CNTs has been significantly improved, making it possible to serve as tissue scaffolding materials to enhance the organ regeneration [101, 102]. Despite the evidence of CNTs
cytotoxicity, there have been a number of published studies into interactions between CNTs-based biomaterials, neural cells, osteoblasts, fibroblasts, antibodies and the immune system, ion channels and cellular membranes, which support the biocompatibility of CNTs and its composites \[84\]. The biocompatibility of CNTs-based materials will be discussed and explained in terms of its applications.

Currently, the majority of studies on biological applications of CNTs revolve around the biosensor development in which the CNTs are integrated with biomolecules such as proteins, nucleic acids or carbohydrates and used as a sensor for ultrasensitive disease diagnosis \[103\].

As it is chemically inert and because of its incredible mechanical strength, CNTs can be ideally, a potential material for blood compatible biomaterials \[98\]. It has been reported that incorporating MWCNTs into the polyurethane matrix leads to relatively lower level of platelet adhesion owing to the presence of CNTs, thus, a reduction in thrombosis and also the hemolysis index, (a measure of disruption of red blood cells). Hence, such a composite may be able to find its application in cardiovascular surgery and in other blood contact environments \[104\].

It has been confirmed that the improved mechanical strength of the CNTs-nylon catheter also has a lower reactivity with blood and in vivo thrombus formation in an artery \[105\], thus, these catheters will be valuable for use in cardiopulmonary bypass, vascular dilating devices, intravascular catheters, and during hemodialysis.

In bone regeneration applications, developing biocompatible scaffold materials that can support the growth and proliferation of osteoblasts and thereby increment to replace the bone tissues still remains as a major challenge for biomedical engineers. Because of its high mechanical strength, flexibility, elasticity, and low density, CNTs can be a promising material for casting bone scaffolds \[106\]. Giannona et al.\[107\] demonstrated that the presence of vertically aligned CNTs in the scaffold significantly influences osteoblast-like cell growth, morphology, and orientation. CNTs-reinforced porous polyurethane nanocomposite scaffold showed osteoblast growth and mineralisation due to the changes in surface chemistries and nanoscale architectures in the scaffold \[108\].

In a scaffold used for bone graft, the growth of hydroxyapatite crystals depends on the ability of the scaffold to attract calcium ions and initiate the crystallisation process. Zanello et al.\[109\]
reported that calcium ions were attracted to nanotubes injected into a bone fracture for supporting the growth of new tissues to heal the fracture due to the negatively charged functional groups introduced on the CNTs surface. In addition, the presence of CNTs in the scaffold improved the cell adhesion, which is crucial for cell growth, proliferation, differentiation, and migration within the scaffold.

Another application of CNTs in bone engineering is to enhance the mechanical and thermal properties of poly(methylmethacrylate) (PMMA), a common polymer material for bone cement and dental prostheses. It has been found that the static and fatigue mechanical properties of bone cement can be improved remarkably by incorporating CNTs into the PMMA polymer, and improve the longevity of the implants\(^4\),\(^8\). Due to the high thermal conductivity of the carbon nanotubes, the presence of CNTs in the bone cement can significantly reduce the high temperatures at cement-bone interfaces during \textit{in vivo} polymerisation, thus the hyperthermia based destruction of bone adjacent to cement mantle\(^7\). It has also been found that the elevated surface roughness due to the presence of CNTs in the bone cement lead to a higher adsorption of the proteins on the composite to enhance the adhesion of cells onto the surface of the matrices as well as increases the osteoblastic cell proliferation\(^110\).

Studies of biocompatibility of CNTs for bone regeneration have demonstrated that the MWCNTs did not inhibit the bone repair and the nanotubes become integrated into the bone tissue\(^111\), also, the newly formed bone was remodeled around the nanotubes\(^112\). The cultivation of bone-forming cells on these CNTs revealed that surface charged nanotubes inhibited the growth and the proliferation of osteoblasts, whereas surface-neutralised nanotubes promotes the cell growth and the formation of the mineralised bone\(^109\).

CNTs are electrically conductive and possesses diameters less than 100 nm and aspect ratio close to that of nerve fibres, therefore, they are potential candidates for neuron regeneration applications\(^98\). Mattson et al\(^113\) reported that neurons are able to attach to the nanotube’s surface and the presence of CNTs in 4-hydroxynonenal (4-HNE) promotes the neurite outgrowth and the neurite branching growth of neurons. Thus, it opens up the possibility of incorporating one or more bioactive molecules on CNT’s surface to develop a regulated neuron network on selected matrices. Also, it has been suggested that CNT’s surface charge effects the regulation of neurite outgrowth, length and branching and the neuron cells can
maintain a high viability on the surface of SWCNTs for 10 days\textsuperscript{114}. The possibility of using functionalised MWCNTs with pyrrololidone groups as potential devices to improve neural signal transfer was suggested\textsuperscript{115}. Another study with CNT incorporated neuron cell culture was reported by Matsumoto et al.\textsuperscript{116} to achieve the goal of regenerating the neural network in a certain pattern which can function as desired under \textit{in vivo} conditions. Apart from those cells mentioned earlier, attempts are also being made for directing the growth of stem cells using substrates modified with CNTs\textsuperscript{117}.

Another promising application of CNTs in tissue engineering is the development of new drug and gene delivery systems. For example, cancer treatment represents an enormous challenge for drug delivery in tissue engineering. Because most anti-cancer drugs are harmful to normal tissues as well, it is highly desired to develop a targeted drug delivery system that can distinguish a cancer cell from healthy cells while delivering the drugs\textsuperscript{118}. CNTs are excellent candidates for developing such a system. To use as a cargo for \textit{in vivo} biomolecule delivery, the CNTs need to be chemically modified so that the biomolecules of interest can be readily loaded inside or outside the tubes, which can then be released from the tubes upon reaching the target cells or tissues. As described earlier, CNTs can be functionalised in many different ways depending on their terminal applications. The functionalization helps to integrate various biomolecules such as proteins, nucleic acids, carbohydrates, and other organic chemicals to sidewalls or open-ends of the CNTs.

The ability to incorporate drugs or genes into functionalised CNTs demonstrates a new era in pharmacotherapy for selective delivery of drugs or genes to cells and tissues. A number of studies revealed that different approaches to form DNA-CNT complexes are possible; DNA can wrap around the CNTs\textsuperscript{118}, CNTs can interact directly with DNA through Van der Waals and hydrophobic forces\textsuperscript{119}, and nanotubes can be covalently linked to DNA\textsuperscript{120}, or through the static electronic forces formed between positive-charges on the surface of functionalised CNTs and negatively-charged DNA\textsuperscript{121}. The ability to transport a protein of interest either by spontaneous adsorption or covalently bound to the CNTs is another important application in tissue engineering where such modified nanotubes serve as transporters to deliver the protein into living cells. However, it is of particular importance that the CNT-bound protein remains biologically active during its conjugation with nanotubes. Pantarotto et al.\textsuperscript{122} suggested a promising utility of CNT for vaccine delivery and thus replacing or
complementing the existing vaccine carriers. Also, CNTs are found to facilitate the transportation of proteins into living cells \cite{91}.

Moreover, CNTs have a very high optical absorbance in the near-infrared (NIR) regime (>700 nm), whereas cells and tissues are highly transparent in this range, allowing them to be visualised through the infrared fluorescence microscopy imaging. Thus, continuous NIR irradiation of CNTs will result in heating the cells if the CNTs are internalised inside the cells, resulting thermal destruction of the cells. Also, CNTs can also act as microwave absorbers, allowing microwave based thermal drug release \cite{123}.

**2.3 Bone Cement**

**2.3.1 Introduction**

Total hip and knee joint replacements are now the most common major orthopaedic surgical procedures, with ~1 million performed world-wide annually \cite{124}. Proper fixation to the bones is as important as the design of joint replacement itself. One of the earliest methods adopted for anchoring the artificial joints to the bones is to fix the joint prosthesis into the contiguous cancellous bone using grouting material, called bone cement \cite{125}. The main function of the bone cement, besides immobilising the implant, is to transfer load from the prosthesis to the bone or increase the load carrying capacity of the surgical construct by providing a mechanical interface between native bone and a metallic joint prosthesis. All the commercially available bone cement brands that are used in cemented arthroplasties are based on poly(methylmethacrylate) (PMMA) \cite{126} and, with a few exceptions, have the same constituents, also called acrylic bone cement. PMMA, also known as Plexiglas or Perspex when manufactured for industrial purposes, is an amorphous, glassy polymer at both room and body temperature \cite{127}. There are over 30 commercially available plain acrylic bone cement brands approved, by the relevant regulatory authorities (the Food and Drug Administration, FDA, in the US and the Medical Devices Agency (MHRA) in the UK), for use in cemented arthroplasties \cite{128}. With a few exceptions in the composition of the powder and liquid components as well as initiator/activator ratios, all the brands are remarkably similar in composition as shown in (Appendix I) \cite{126}.
Due to the need for the cement to fill and conform to the cavity inside the bone of a replaced joint, it is normally prepared during surgery several minutes prior to insertion of the prosthesis. The dough-like resin must flow freely enough to achieve interdigitations with cancellous bone and contact with the implant materials. Once the prosthesis has been inserted the resin is allowed to cure in situ. It becomes hard within 10-15 minutes of initial preparation \[129\]. Essentially, bone cement is self-polymerising and consists of two portions: (1) solid powder portion including pre-polymerised methylmethacrylate (PMMA) and initiator, and (2) liquid portion including methylmethacrylate (MMA) monomer and promoter.

When two portions are mixed, polymerisation proceeds as a free radical addition reaction, initiated by benzoyl peroxide contained in the powder. Addition of \(N,N\)-dimethyl-p-toluidine to the monomer liquid is used to activate the free radical decomposition of the benzoyl peroxide initiator. Propagation of the reaction proceeds as additions of individual monomer molecules to the free radical side of the growing polymer chain. An auto-acceleration effect, known as the Trommsdorf or Gel effect, occurring at approximately 20-50% of conversion, causes the reaction to become highly exothermic homogenous cement masses can reach temperatures in the range 50-90°C \[130\]. Tissue damage thresholds have been reported in the range of 50-60°C but sufficiently lower temperatures often occur when the cement cures in contact with a metal implant and circulating blood to prevent thermal necrosis of the bone \[131\]. Also, polymerisation is inhibited by oxygen; this has the effect of decreasing the rate of monomer reaction, exotherm, chain length, and molecular weight.

In addition to reaction related additives, radiopacifiers, e.g. barium sulphate (BaSO\(_4\)), to render the cement visible under x-rays, and antibiotics, e.g. gentamycin, to minimise risk of infection, are also often added. Such fillers can affect fracture properties both positively and negatively since they may act as crack arrestors or initiation sites. Copolymers, e.g. polystyrene which copolymerises readily with MMA, are also sometimes added to bone cement to improve processing characteristics, radiation resistance, or reduced polymerisation exotherm \[126\]. PMMA is also hydrophilic, absorbing up to several weight percent water. Absorbed water acts as a plasticising agent and has been shown to increase fatigue life. Further to the effects of additives, bone cement is prone to ageing as complete conversion of monomer is difficult to achieve under the relatively uncontrolled reaction conditions \[132\].
Residual monomer tends to either polymerise over time, causing ageing phenomena due to increasing molecular weight, or to diffuse into the surrounding tissues where it can lead to necrosis because of its cytotoxicity\textsuperscript{129}.

2.3.2 Applications

Bone cement is widely used in orthopaedics and dentistry. In orthopaedics, bone cement is frequently used as a structural material in total joint arthroplasty and, more recently, in vertebroplasty and kyphoplasty. Bone cement is also used to anchor some dental implants and used to form the bridgework for restorative dental prostheses (dentures).

2.3.2.1 Total Joint Arthroplasty

The adoption of acrylic bone cement in total joint arthroplasty began in the late 1950s. Charnley\textsuperscript{133} first proposed injecting the high viscosity self-polymerising PMMA bone cement into the clean intermedullary canal and insert the metallic stem of the implant into the dough to stabilise the metallic implant during total joint arthroplasty procedures. Within a reasonable amount of time, the bone cement hardens and sets the implant with respect to the neighbouring bone. The hardened cement not only stabilises the implant, but it also provides a buffer zone between the implant and the bone, which have markedly different mechanical properties. The implant, bone cement mantle, and host bone work together as a single unit to restore functionality to the patient’s joint\textsuperscript{134}. At the bone cement-bone interface, the response of the bone tissue to the bone cement varies and several parameters affect the clinical development of this interface, including: chemical necrosis from residual monomer leaching into the bone, thermal necrosis from the exothermic polymerisation, and the micro-motion at the interface, which can produce debris particles that irritate surrounding tissues\textsuperscript{135}. Each of these factors can negatively affect the outcome of the procedure by inducing an inflammatory response, which ultimately leads to the fibrous encapsulation of the bone cement and implant, and thus, to migration and loosening of the implant. Bone-bone cement interface forms a site of good interlocking in the best cementing procedure\textsuperscript{134}. The bone cement-implant interface is the focus of extensive research and is, more often than not, is the reason for re-designing the stem of the metallic implant.

More recently, although implant stems are manufactured with pre-coated surfaces, sintered surfaces, grooved, or serrated patterns to improve the mechanical interlocking between the
implant and the bone cement mantle, to enhance the transfer of body loads from the artificial joint to the surrounding bone; an eternal bond between these two components is unrealistic and some level of de-bonding is likely \cite{135}. Extensive de-bonding can lead to construct failure at this interface, but small amounts of de-bonding appears to minimally affect the lifetime of the construct \cite{136}. Also, wear particles and debris can migrate to the articulating surface of the joint as well as into surrounding tissues \cite{135}. For complete restoration of the joint’s load bearing capabilities, the joint reaction and body forces must be adequately transferred from the implant to the bone. If the dynamic joint reaction forces of the hip and knee attributed to everyday activity and those attributed to body mass are inefficiently distributed, then the host bone may remodel around the construct. If the implant shields the bone from stresses developed from the joint reaction and body forces, then the bone may resorb \cite{135}. Thus, the bone cement aids the distribution of forces and prevents stress shielding; however, loss of mechanical integrity greatly reduces the effectiveness of the bone cement mantle. Mechanical failure of the mantle or de-bonding at either interface directly leads to a loss in stability.

The most popular total joint arthroplasty procedures are knee and hip replacements as well as shoulder and elbow replacements. Clinical failure of the primary implant is corrected with revision surgery and is often the result of fatigue failure of the bone cement mantle; thus, improving the fatigue performance of bone cement is necessary for increasing the clinical life of the implant and reducing healthcare expenditures \cite{134}.

2.3.2.2 Vertebroplasty and Kyphoplasty

Vertebral compression fractures (VCFs) are common among patients with fragile bone structure. A compressed vertebra distorts posture and shifts the centre of gravity away from the spinal column, which negatively alters the biomechanics of the spine. Chronic pain and discomfort may develop from improper healing and spinal kinematics. The current treatments for VCF are vertebroplasty and kyphoplasty \cite{137}. In the vertebroplasty, low viscosity bone cement is percutaneously injected into the collapsed vertebrae to stabilise the fracture. In the kyphoplasty, a balloon is inserted into the fracture site and inflated to restore the height of the vertebral body. Bone cement is then used to stabilise the restored structure of the vertebrae. Low viscosity bone cements leak out of the injection site and into the surrounding
tissues lead to unwarranted health risks such as pulmonary embolism, inflammation, or possible neurological effects [134].

2.3.2.3 Dental Prostheses

In some dental applications, radiolucent bone cement exclusively composed of PMMA is used for structural purposes. Conversely to the orthopaedic application, the polymerisation reaction is induced by heating the monomer phase and the polymerising monomer can be moulded or cast into desired shapes. A metallic framework is constructed to hold the artificial teeth in place and the monomer is polymerised around this framework to create a custom fit for the patient. The role of PMMA in dentures is to provide structural support for the artificial teeth and metalwork. The PMMA also provides mechanical stability and strength to the denture. However, dental PMMA is not immune from mechanical failure. Roughly one out of every three failed prostheses results from midline fractures of the PMMA retainer [134, 138].

2.3.3 Development in Acrylic Bone Cement

Although used universally for many years, PMMA acrylic bone cement is not without its problems, as it has to cure before it sets hence suffering both mechanically and biologically. These problems lead to the failure of the cement and hence the prosthetic it holds in place, via aseptic loosening. Thus, in an effort to prolong the lifetime of the prosthetic, investigations have been carried out to address several drawbacks through the preparation and characterisation of alternative acrylic bone cement formulations. Lewis [128] has published a detailed, comprehensive, and critical review of the open literature on developments in alternative plain acrylic bone cement formulations, and reported the main drawbacks of the commercially available acrylic bone cement brands.

There is a sizeable literature on the results of studies that address the clinic success of prostheses implanted with cement for up to 20 years of follow up. However, despite this and the numerous improvements of the materials used, loosening remains an impediment to the long term success of total joint replacements. Thermal necrosis due the high heat generated during polymerisation and chemical necrosis due to unreacted monomer and other chemicals, the mismatch properties at the interfaces as the cement is orders of magnitude weaker than the bone or implant, as well as, shrinkage during polymerisation, fatigue and fracture of bone cement are some of the major problems found [139].
Various concerns have been raised about a number of constituents in commercially available acrylic bone cement brands, such as toxicity of DMPT accelerator, Madigan et al.\textsuperscript{[140, 141]} prepared a \textit{Reduced-DMPT Content Cements} to diminish these toxicity effects. They stated that reduction of DMPT content for surgical Simplex-P\textsuperscript{®} from 2.5 vol.\% to between 0.8 and 1.4 vol.\%, resulted in cement with acceptable thermal and mechanical properties.

As an admixture formula, the viscosity during curing is initially very high and is practically invariant with time of mixing. Therefore, Hasenwinkel et al.\textsuperscript{[142, 143]} have suggested \textit{Two-Solution Cements}, this means that the two solutions can be mixed simultaneously and delivered into a prepared bone bed using a single closed system. These formulas displayed pore-free and higher flexural strength and modulus, but about the same curing characteristics compare to surgical Simplex-P\textsuperscript{®} as a control.

Another attempt was to develop \textit{Antioxidant Cements} using a natural biological antioxidant such as vitamin E in the liquid monomer to prevent the accumulation of peroxides and protect the cells from being damaged by the residual free radicals in the cured cement \textsuperscript{[144]}. Another option was to use alternative amine accelerators with higher molecular weight than DMPT to develop \textit{Alternative-Accelerator Cements} \textsuperscript{[145]}.

Many deleterious effects of BaSO\textsubscript{4} and ZrO\textsubscript{2} radiopacifier particles on various properties of the cement have been highlighted. Agglomeration of BaSO\textsubscript{4} radiopacifier particles in PMMA matrix, possible involvement of the radiopacifier particles in third-body wear, as well as toxicity effects are among the major problems which affect the cement mechanical and biological properties. In an attempt to disperse the radiopacifier particles uniformly in the cement matrix, nanosized BaSO\textsubscript{4} particles were used. The uniform dispersion of these nanosized particles in the cement hinders its agglomerations within the cured cement matrix, and resulted in significant improvement in compressive modulus, tensile work-to-fracture, and fatigue life for \textit{Uniformly-Dispersed-Radiopacifier Cements} \textsuperscript{[146]}. Also, different materials such as triphenyl bismuth (TPB) \textsuperscript{[147]}, tantalum (Ta) \textsuperscript{[148]}, and bismuth salicylate (BS) \textsuperscript{[149]} have been used as an alternative contrast medium to replace the BaSO\textsubscript{4} particles in the commercially available acrylic bone cement to overcome various undesired effects associated with BaSO\textsubscript{4} particles. These alternative radiopacifiers have changed the properties of the proposal \textit{Alternative-Radiopacifier Cements}, some of these changes are positive; others
are undesirable, while others are unaffected, when compared to cements in which the radiopacity was provided by BaSO$_4$ particles $^{128}$.  

Excessive local contact stress is implicated as an important factor in the initiation of the loosening process after total joint arthroplasties. Therefore, reduced-modulus cements were developed to achieve an even distribution of stresses between the TJR and the bone; and to act as a shear spring between the implant and the cancellous bone and thus to reduce the contact stresses at the cement-bone interface. The elastic modulus at body temperature was one-eighth of standard bone cement for developed cement formula consists of butylmethacrylate beads embedded in a PMMA matrix $^{150}$. The in vivo study model showed a reduction in the rate of loosening of femoral components when compared with cement controls. Also, better resistance to inflammation, lower potential for tissue necrosis, higher attachment of human osteoblast-like were reported when poly(ethyl methacrylate) (PEMA) beads powder and n-butyl methacrylate (n-BMA) liquid monomer were used in another formula $^{151}$.  

Reinforced bone Cements were developed to enhance the mechanical properties of normal acrylic bone cement. The reinforcement phase is incorporated into the cement matrix to carry the bulk of the structural loads imposed on the cement, thus, improve its mechanical defects such as low tensile strength, fracture toughness, and fatigue life. These Reinforced Cements can be classified based on the nature, amount, and geometry of the reinforcement phase. For the first class, long fibres have been blended with the cement powder including: stainless steel fibres, carbon fibres, Kevlar® 29, poly(ethylene terephthalate) fibres, polyethylene fibres; and PE or UHMWPE. These long fibres (typically, 1mm; 1–15 wt.%) are used as-received; or with plain or treated surface $^{152}$. In the second class, short fibres of carbon or titanium have been used to reinforce the cement matrix. These short fibres (typically, 100–250 μm long, 8–20 μm in diameter, and 14 wt.%) are used plain or heat treated $^{153}$. In the third class, particulates (typically, 1–5wt.%) have been blended with the cement powder including: poly(butyl methacrylate) (PBMA) particles, alumina powder, polyisobutylene (PIB) beads, poly($\varepsilon$-caprolactone) (PCL) beads and PCL-toughened PMMA beads, rubber-toughened PMMA beads, montmorillonite (MMT) nanoparticles, and chitosan nanoparticles $^{154}$. It was reported that the interface strength between the filler particulates and the cement matrix is the core factor to determine the mechanical properties in such
composites. Therefore, a sol-gel approach also has been used to create a covalent bond between the particulates and the PMMA matrix instead of a macroscopic interface\textsuperscript{[155, 156]}. The fifth class is a cement formulation in which PMMA matrix is reinforced with carbon nanotubes CNTs (0.5-10 wt.%), refer to section (2.3.5) for more details. Several mechanical properties of these Reinforced Cements, such as fatigue life, $K_{IC}$, UTS, and UCS are an improvement over those of control cements. However, for other properties, such as flexural strength, there was little or no gain\textsuperscript{[157]}.

When partially degradable cement is used to anchor a TJR, the bone will grow around and into the cement in the pores left by the degradation of the cement. Therefore, Partially Degradable Cements that include poly(hydroxyalkenoate), poly(\text{[R]}-3-hydroxybutyrate (PHB), PMMA-graft- PHB or a polyhydroxyalkenoate either in the powder or in the liquid monomer as possible constituents in acrylic bone cements for use in orthopaedic applications were synthesised and the volumetric porosity and compressive strength were about the same as for the control cement\textsuperscript{[158, 159]}. In another approach to overcome the lack of degradability of acrylic bone cement, Biodegradable Cements which contain inactive but biodegradable fillers were suggested. In these cements one or more fillers are blended with the cement powder that is based on PMMA polymer only or based on polymer(s) other than MMA.

Fillers that are blended with a cement that is based on PMMA only include: particles of cancellous bone, nanosized particles of Al$_2$O$_3$, and tricalcium phosphate (TCP) powder. Improved and degraded properties have been reported for these cements, compared to control cements\textsuperscript{[160]}. On the other hand, cements that are based on polymer(s) other than MMA only include: bis-phenol-A-glycidyl methacrylate (Bis-GMA), triethyleneglycol dimethacrylate (TEGDMA), and methylmethacrylate (MMA), PEMA, and DEAEMA polymers. The fillers have been used in these cements include: epoxy-SiO$_2$ sol-gel material, micron- and nano-sized TiO$_2$ particles, and chitosan. Desirable properties of this type cement have been reported\textsuperscript{[156]}.

The thermal necrosis of surrounding bone due to the exothermal reaction of polymerisation which takes place in the direct vicinity of living tissue and the excess of heat generated might contribute to the overall trauma and, consequently lead to prosthesis loosening. To conquer this problem, anaromatic liquid monomers consisted of MMA as the base monomer and acidic co-monomer or alkaline co-monomers were applied to develop Modified-Monomer
Cements. However, these cements have consistently higher residual monomer content and average thermal properties when compared to control cement [161].

Shrinkage of the acrylic bone cement during polymerisation creates gaps at the interfaces between both the bone and implant, which may lose the good load transfer through these interfaces, and is a source of prosthetic loosening. Therefore, copolymers that exhibit the ability to absorb body fluids and swell in a controlled manner to compensate shrinkage of the cement during polymerisation were applied to defeat the poor adhesion between the cement mantle and both the bone and the implant [162]. It was suggested that using cross-linking agents in cement formula provides anchoring points for the PMMA matrix in the cement by forming insoluble network during polymerisation [163]. It has also been reported that the powder/liquid ratio effects the polymerisation shrinkage, thus porosity of bone cements [164].

An increase in the hydrolysis resistance of the implant-bone cement interface may reduce the incidence of osteolysis and, consequently, reduce the potential for aseptic loosening of the prosthesis. Therefore, adding coupling agents to the MMA liquid monomer to improve the tensile bonding strength and the interfacial shear strength between the cement and the implant/bone tissue to develop Improved-Adhesion Cements. However, the interfacial strengths were enhanced compared when control cement was used [165, 166].

Bone-bone cement interface is known as one of the weak-link zones in the (prosthesis-bone cement-bone) construct because it does not adhere to bone due to inertness of the cement. Bioactivation of acrylic bone cement by using bioactive fillers may strengthen bone-bone cement interface. Therefore, Bioactive Cements were developed to overcome the inertness drawback. These cements may be collected into three classes. The first comprises cements in which a bioactive agent is blended with the cement powder of PMMA cement. Examples of fillers are particles of hydroxyapatite (HA), HA+chitosan, strontium-containing HA, apatite wollastonite glass (AWG), and recombinant human growth hormone [167]. In the second class are cements in which one or more bioactive agents is blended with the powder of cement that is based on polymer(s) other than MMA only. Examples of the particles are those of sintered HA, silanated HA, and AWG and the polymers used are bisGMA, PEMA, PEMA-nBMA, META-PMMA, and EAEMA [168]. In the third class, bioactivity is achieved without the incorporation of bioactive filler. An example is one that contains a constituent that provides OH groups and a soluble calcium salt [169]. Although maximum exotherm temperature and
fatigue performance, show an improvement over those of control cements, the results were not satisfactory due to deterioration of the mechanical properties after adding a large quantity of the bioactive particles or lack of bioactivity when small amounts are added\cite{169,170}.

While some of these aforementioned bone cement formulae are designed to address one of the cement drawbacks, others are designed to address two or more. As is the case with most classification schemes, some overlap is unavoidable. Therefore, *Mixed-Action Cements* are developed. Abboud et al.\cite{171} prepared bone cement using pre-treated alumina particles (Al$_2$O$_3$) that are able to act both as radiopacifying and reinforcing agents. Ni GX et al.\cite{172} developed cements from strontium-containing hydroxyapatite (Sr-HA) serves as radiopacifier and also renders the cement bioactive and the *in vivo* study suggested that Sr-HA bioactive bone cement was superior to PMMA bone cement in terms of bone-bonding strength. Morita et al.\cite{173} also utilised alternative monomer (MMA-co-EMA), accelerator (2-5-dimethylhexane-2-5-hydroperoxide), and initiator (tri-nbutyl borane) to prepare a new bone cement formula, (Bonemite®). The mean values of the setting temperature and the elastic modulus of this cement were lower compared to a commercially (CMW®) brand.

Other approaches such as use of acrylic acid based bone cement were used to enhance the mechanical properties of bone cement. It has been reported that by introducing cross-linked poly(methylmethacrylate-acrylic acid-allylmethacrylate) (poly(MMA-AA-AMA)) copolymer into bone cement, the fixation strength in the interface of bone and cement can be improved by the controllable swelling of the modified bone cement, to compensate the shrinkage of the cement during polymerisation\cite{162}.

2.3.4 *In vivo* Performance

Most total joint replacement surgeries use acrylic bone cement as a means of fixation of the prosthesis to bone. The long-term success of this procedure is, to a large extent, attributed to the mechanical integrity of the cement mantle which, in turn, is directly related to the strength of PMMA cement. Although, other factors which are not purely of mechanical origin can lead to failure of the cement mantle\cite{174}.

Many studies have dealt with the determination of the modulus and strength of surgical bone cements and utilised either the direct tension, flexural, or compressive tests to determine modulus. Internationally accepted standards for acrylic bone cement, such as ASTM F 451
and ISO 5833 have been used. Flexural and compressive data are required for market approval of bone cements. Traditionally, these tests have been by compression testing and four-point bend test and to a lesser degree three-point bending \[^{175}\].

Some tests are often considered more applicable to the clinical application, however, there is no absolute measure of modulus that dictates compliance to a standard, but rather there exists a generic modulus based upon all three tests. However, there are many well established techniques to evaluate the flexural or compressive strength of acrylic bone cement and many failure theories that define the extent to which these approaches apply. As an amorphous polymer, PMMA is glassy below its glass transition temperature (114°C) \[^{127}\]. Thus, fractures in a brittle manner at body temperature (37°C).

Flexural strength of a material, also known as bend strength, or fracture strength, is defined as the maximum stress that material can resist before failure when subjected to deformation under bending load, it represents the highest stress experienced within the material at its moment of rupture. The bending load applied or the stress state could be uniaxial or biaxial. Flexural modulus is not to be confused with modulus of rupture, which is also another name for flexural strength. The flexural modulus is the ratio of stress to strain in flexural deformation. The required flexural properties are highly dependent on the clinical applications. Flexural strength is generally considered a meaningful and reliable method to assess the strength of brittle materials as they are much weaker in tension than compression \[^{176}\]. The flexural strength would be the same as the direct tensile strength if the material was homogeneous. In fact, most materials have small or large defects in them which act to concentrate the stresses locally, effectively causing a localised weakness. Therefore, it is common for flexural strengths to be higher than direct tensile strengths for the same material. Conversely, a homogeneous material with defects only on it surfaces (e.g. due to scratches) might have a higher direct tensile strength than flexural strength. Ideally, PMMA composites for use in major load bearing situations would have mechanical properties similar to those of bone, with a tensile strength of at least 50 MPa and fracture toughness of at least \(2 \text{ MPa} \sqrt{\text{m}}\) \[^{76}\]. It should be noted that the most important properties to improve in modified bioactive and reinforced acrylic bone cements are tensile strength and fracture toughness \[^{76}\], and these improvements cannot be evaluated from compressive strength only.
Preclinical evaluation of the mechanical properties of any biological material depends on its physiological conditions, therefore, mechanical testing under realistic loading profiles that include frequency and duration of routine physical activities of patients and the associated contact force as a function of time where the magnitude and the orientation of this contact force varies considerably during routine activities, are required \[177, 178\]. According to Bergmann et al. \[179, 180\], the most common daily activities of patients are: walking, standing, sitting, lying, ascending (stair up) and descending (stair down) stairs, sitting down and getting up from a chair or stumbling. Therefore, it may be agreed that the \textit{in vivo} situation is a very complex loading environment and the nature of physiological loading conditions in the cement mantle in cemented arthroplasty is a combination of compression, direct tension, and flexure loads \[174, 175\], and thus, a given generic modulus representative of dynamic, static and quasi-static loads is acceptable for stress analysis \[177, 178\]. Hence, it could be argued, that cement mantle in cemented arthroplasty would be primarily subjected to biaxial loading during the daily activities cycle \[174\]. If this is the case, then evaluating the cement strength using current traditional uniaxial tests may not provide an accurate characterisation of a bone cements true load-bearing capacity and it would seem appropriate to assess the flexural strength of cement mantle under biaxial rather than uniaxial laboratory conditions.

In a uniaxial flexural test, the flexural loading configuration is defined by how many contact points there are between the specimen and the test fixture. Thus it is termed three-point loading if there are two supports and one loading point, and four-point loading if there are two loading points and two supports.

For biaxial strength testing, a wide variety of test support and loading configurations have been developed and identified in the literature. The support of the specimens is either realised by a ring or by three or more balls (a ring of balls) and the loading by a ring, a ball or a punch resulting in axisymmetric and non axisymmetric loading situations \[181\]. Biaxial strength testing of brittle materials is claimed to have some benefits compared to uniaxial testing (in tension or in bending) \[182\]. The smaller disc specimens utilised for biaxial testing result in an improved representation of the volume and dimension of clinical situation. The maximum tensile stresses occur within the central loading area of the lower face of the plate, eliminating spurious edge failures associated with three-point flexure testing (attributed to edge preparation conditions) and samples are insensitive to specimen geometry and independent of
flaw direction\textsuperscript{[183]}. In other words, the biaxial stress distribution is more searching for defects than a uniaxial distribution \textsuperscript{[181]}. This allows slightly warped specimens to be tested and produces results unaffected by the edge condition of the specimen. It has also been suggested that varying the type of support system does not produce a significant difference in results, providing increased reliability of flexure strength data between different operators and across different test centres \textsuperscript{[183]}. Large variations in strength arise for the same acrylic cement subjected to the same mechanical test due to differences in edge condition for the same surface preparation \textsuperscript{[175]}. Consequently, it is essential to provide reliable evaluation techniques and standards that are experimentally reproducible and clinically relevant.

The most common assemblies for biaxial strength test of discs with an axis-symmetric stress distributions used are ring-on-ring test, ball-on-ring test, and punch-on-ring test. All three methods have the disadvantage that more or less perfect flat discs are required, which might make polishing of the specimens necessary. Any deviation from flatness leads to additional stresses during the loading, so that strength results become hard to interpret. Therefore, testing assemblies for biaxial strength testing of discs with non axis-symmetric stress distributions have been developed. The punch-on-three-ball test tolerates a small “out of flatness” of the disc, since non-planar discs faces are supported stably by three points. In this test assembly only small variations of the geometry are considered which strictly limits its applicability. The most familiar geometry, the ball-on-three-ball test (B3B), is even more tolerant to some out of flatness of the disc than all the other test assemblies, where discs with no special surface finishing can be tested. From experimental results and finite element analysis, it was concluded that only the ball-on-ring and ball-on-three-ball loading methods were capable of accurately determining the flexural strength of brittle dental materials and uncertain facture stresses were found in the other methods, leading to inaccurate results \textsuperscript{[184]}. In the case of the axis-symmetric loading situations the stress field in the disc specimens can be determined relatively easily but in reality, small inaccuracies from the idealised geometry of specimen, support and loading will break the symmetry and can cause large deviations from the idealised solutions. In the case of non axis-symmetric loading situations the determination of the stress field in the specimen is difficult and can only be done properly using numerical methods \textsuperscript{[181]}.
The biaxial flexural test has been used extensively in dental material research to determine the fracture characteristics \cite{184}, but surprisingly only one successful endeavour has been made to establish the efficacy of the biaxial flexural test as a suitable method to determine the strength of acrylic bone cements.

Higgs et al. \cite{174,175} determined the strength of two glass-ionomer dental cements and two acrylic bone cements and compared the calculated results with the analytical representation of the biaxial test and well established biaxial and three-point flexural results for the two types of cements. Furthermore, the calculated theoretical biaxial strength was compared with a value of biaxial strength utilising the finite element method to verify the accuracy of biaxial theory. In their investigation, the analytical approximations for the stress distribution in the disc specimen was based upon the initial work of Bassali \cite{185}, and in more specified way by Kirstein and Woolley \cite{186}. They have verified that the biaxial theory and the equations developed are accurate and valid to use for testing orthopaedic bone cements and their biaxial flexure test results were in agreement with results from compressive and bending tests. These analytical approximations for the stress distribution in the disc specimens used have been reported to be insufficient for two main reasons. Firstly the approximations fail to describe the tensile stresses opposite the loading area in the centre of the disc because they are based on the cylindrical symmetrical thin-plate-theory that predicts infinite tensile stress amplitude opposite to the load transfer point. Secondly the approximations depend on the contact radius between the loading ball and the disc, which predict very different contact radii leading to different results for the tensile stress distribution around the centre of the disc \cite{181}.

The finite element analysis, figure (2-6), of the stress fields in ball on three ball-loaded discs performed by Börger et al.\cite{181} showed, that for brittle materials, the stress state is symmetric everywhere at the axis of the specimen and the maximum tensile stress occurs in the centre of the disc plane opposite the loading ball and can be approximated by point loading for the maximum tensile stress, but to determine the stress field in the whole disc, it is necessary to model the contact area between the balls and the disc.
Figure 2-6: FE-model of the (B3B) test assembly.\textsuperscript{[181]}

The strength of the cement-bone interface is strongly related to cement intrusion into the bone. Thus, considering adding the cement-bone interface strength to those current techniques and standards that have already been used may further increase the predictability of long-term durability of fixation cemented arthroplasties. The bonding strength of the bone cement-bone interface could be conducted by measuring the force required to separate the two phases of standard bonded area in a suitable arthroplasty model (e.g. cemented synthetic solid rigid polyurethane foam proximal femur) using either shear or tensile stress. An additional effort in this research area would entail determining the depth of cement intrusion as a function of the cement-intrusion pressure and the cement composition.

During the daily activity cycle, bone cement is subjected to high forces and repetitive stresses in the “aggressive” internal biological environment of the human body\textsuperscript{[9]}, which may lead to fatigue of the cement mantle and eventual fracture, thus, the influence of body environment on the mechanical properties is considered an important factor in the durability of bone cement\textsuperscript{[176]}; thus, it is important for material formulation and selection. Aging and testing bone cement samples in a physiologically relevant environment (i.e. phosphate buffered saline (PBS), at 37°C) is known to improve the performance of bone cement by enhancing resistance to crack formation and propagation\textsuperscript{[187]}. For example, in MWCNTs-reinforced
bone cement, the PBS may improve the lubricating properties of MWCNTs much in the same way as a humid environment increases the lubricity of graphite \cite{188}. Therefore, the combined plasticisation of the matrix and lubrication of the MWCNTs led to improvements in fatigue performance greater in magnitude than those presented for fatigue testing a similar bone cement nanocomposite in air at room temperature (25°C), thus, the combined effects of the MWCNTs and the testing environment led to elevated fatigue performances of bone cement loaded with MWCNTs \cite{4}. Hence, the bone cement test specimens should be continuously aged and tested in a bio-simulating solution, such as (PBS) solution at 37°C, as stipulated in relevant standards to indicate the acute and instantaneous biological environment in which the biomaterials need to survive \cite{10}.

Surgical-grade PMMA bone cement is formed from an initial mixture of polymer powder and liquid monomer and exothermic during curing. The polymer powder and liquid monomer are hand mixed to obtain a paste capable of being introduced into the cavity of the bone. After 12-15min, the dough sets up into a rigid mass. It is generally accepted that MMA monomer does not reach complete conversion after the cure of the resin, and that a certain amount of residual monomer remains in the hardened material.

Aseptic loosening of cemented arthroplasties, which is a leading cause of their limited in vivo longevity, is a complex phenomenon that has been postulated to be affected not only by mechanical factors, but also by thermal ones, namely thermal injury to the periprosthetic tissues (associated with the high polymerisation heat) and osseous necrosis of those tissues (resulting from the leakage of the residual monomer) \cite{129}. Un-reacted MMA monomer leaks from the cement mantle into the surrounding tissues, impairing bone remodeling and its presence in the hardened resin influences the mechanical properties because it acts as a plasticizer \cite{132}.

Because commercial bone cements consist of a complex mixture of radical initiator, modifiers and inhibitors, it is very difficult to obtain the characteristics of the cure reaction of acrylic bone cements without detailed and extensive chemical analysis. However, a few studies have been performed to investigate the polymerisation of acrylic bone cements and to quantify the amount of monomer present in the cured material using empirical or phenomenological models formulated in terms of degree of cure (or the degree of monomer conversion) \cite{132, 189}.
In the majority of the commercial formulations of bone cement in current clinical use, the polymer powder constituents are gamma-sterilised in air, at a minimum dose of (25 kGy), with the exception of Palacos R®, in which the powder is EtO-sterilised. However, the liquid monomer constituents are sterilised using membrane filtration in all cements [190]. This difference in powder sterilisation methods is important since it is well known that gamma-irradiation at the above-mentioned dose causes chain scission of PMMA [191], which is the primary polymer in bone cement. It has been reported that the flexural fatigue performance of a conventional bone cement formulation, in which the powder constituents were gamma-irradiated (25 kGy), was an order of magnitude worse than the case when unsterilised powder was used [192]. Since bone cement is always supplied in a sterilised condition, results from tests on cement made using unsterilised powder constituents have limited clinical relevance.

Therefore, when studies to compare the properties of different cement formulations are being designed, the method of powder sterilisation should be considered. The time between powder sterilisation and bone cement preparation (shelf-age time) may be an important parameter which affects the polymerisation characteristics of the cement. Also, the possibility of continued degradation of certain cement constituents (notably, benzoyl peroxide) as the package sits on the storeroom shelf should be taken into account when comparing the properties of the fully polymerised cements [190].

When a material is implanted in vivo, it is immediately covered with a thin layer of extracellular fluid. The cells interact with the implant material through this layer and the bonding established varies depending on the forces, namely Van der Waals attractions through to covalent bonding [193, 194]. The surface of implant materials presented to cells can be considered as a foreign chemical species with reactive sites. The end groups of polymer chains may also interact with reactive groups such as protein or carbohydrate molecules in serum [195].

Cells placed in contact with a biomaterial surface may show a range of responses from induction of an inflammatory response through to perception of the material as “tissue-like” invoking no reaction [193]. The nature of the cellular response determines whether the implant becomes encapsulated with fibrous tissue, or initiates bone growth (if in direct contact with bone) [194]. There are four possible responses that may occur; tissue death (toxic materials),
fibrous tissue formation (inert materials), interfacial bond formation (bioactive materials), or replacement by surrounding tissue (biodegradable materials) \[196\]. Cells also react to fillers presented within the material surface topography. Topography can be subdivided into macrotopography dealing with the physical configuration of the implant, and microtopography resulting from surface roughness or texture \[197\]. The material surface can influence cell reaction through changes in the cytoskeleton, a network of protein filaments extending through the cell cytoplasm within eukaryotic cells \[198\]. The actin microfilament cytoskeleton is involved in the formation of cell processes, cell shape, and cell attachment. Cells use topography for orientation, alignment, and migration by recognising surface features and reacting to them.

Surgical-grade PMMA bone cement is still the standard for cement held prostheses, providing immediate structural support. PMMA cements have been described as inert materials, with fibroblastic cells observed at the cement-bone interface \[199\]. Although PMMA can produce good surgical results if used correctly, the bone-cement interface is often considered to be the weak link in cement-held prostheses, providing a barrier to fracture healing. Therefore, when considering implant materials numerous factors have to be considered, including biocompatibility, material surface, the condition of the host bed, surgical technique, and the loading conditions after implant insertion \[193\].

Although there is a very large database on the properties of alternative cement formulations, in the vast majority of studies the focus was on mechanical properties only and, perhaps, more importantly, a variety of approaches have been taken in determining the same property. One of the important prerequisites for the success of a bone tissues substitute and dental or orthopaedic implants is the attachment and proliferation of bone cells on the material surface. The cytocompatibility of these new bone cement composites will require full characterisation. Such tests are considered essential when considering long-term success of bone cement composites in vivo, however it must first be proven that such materials offer enhancements in mechanical performance, internationally accepted standards for biological evaluation of medical devices ISO 10993:2007 are used.

Ideally, in vitro evaluation should be carried out using a model that represents the clinical situation as far as possible. In vitro cell culture techniques have been used to demonstrate the biocompatibility of a range of biomaterials including dental materials, bone substitutes and
cements. Tissues or cells used include neonate rat calvaria, osteoblasts, fibroblasts, bone marrow and osteoclasts. Osteoblasts are mainly responsible for bone formation and remodeling. Though from the point of bone tissue engineering, osteoblasts are best suited, but the first signature of cytocompatibility arrives from the interaction of fibroblast with the host material \cite{200}. It is to be noted that the fibroblast cells are the first cell line, which will come in contact at the implant surface during the implantation and they are easy to grow in a reproducible manner \textit{in vitro}. Once the fibroblast cells adhere on the surface, then signals are transmitted to other cells, including osteoblasts. However, \textit{in vitro} cytocompatibility testing using osteoblast cells are widely recognised as acceptable results to assess the biocompatibility testing \cite{200}.

One consequence of these methodological aforementioned problem situations is that it is difficult to perform true inter-study comparisons for a given material cytotoxicity. Therefore, the issues described above suggest areas for future study as well as matters that should be taken into account in the design of testing protocols.

2.3.5 CNT Reinforced Bone Cement

A new generation of bone cement with mechanical properties significantly higher than that of commercially available bone cements are strongly desired in order to ensure the long term clinical performance of cemented arthroplasty. It is well documented that PMMA acrylic bone cement is a proven polymer having important applications in medicine and dentistry, but this polymer is susceptible to cracking and failure, thus, implant loosening, pain, the need for revision total joint replacement surgery, and substantially increased healthcare expenditures.

Active or over-weight patients with implants fixed with PMMA bone cement are at risk from cement mantle failure. Failure rates of 67% have been recorded after 16 years in patients younger than 45 years \cite{4}. It is also postulated that the annual number of revision total knee arthroplasties performed in the United States will increase 601% to 270,000 by 2030, and the number of total hip arthroplasty revisions will increase 137% to 97,000, with a common cause of implant failure attributed to cement mantle failure.

A variety of materials (especially fibres) have been added to bone cement, in attempts to augment its mechanical strength, to ridge incipient fatigue cracks and arrest their propagation,
but the use of these augmentative additive efforts resulted in limited success due to poor fibre-polymer matrix bonding (and subsequent debonding), increased viscosity, large fibre size (and subsequent filler–damage scale mismatch), inadequate additive dispersion and distribution, ductile fibre deformation and fracture as well as the adverse effects such materials have on the mixing of bone cement [5].

Scale mismatch pertains to the dimensional incompatibility between the diameters of the reinforcing fibres and the size of the fatigue damage in the matrix; fibres that were orders of magnitude larger than the scale of the damage were considered ineffective for preventing or arresting damage accumulation. Scale compatibility is the key motivation in using carbon nanotubes (CNTs) to develop new fibre reinforced bone cement due to their large aspect ratio and high modulus [51]. The small diameter of this nanomaterial is far more comparable to the size of the polymer chains and the scale of fatigue damage compared to the size of traditional graphite fibres commonly used in composite materials. Both single-walled carbon nanotubes (SWCNTs) and multi-walled carbon nanotubes (MWCNTs) offer the potential to augment mechanical, thermal, and electrical properties of polymer systems, while retaining the structural capabilities of the polymer matrix. The long fibre lengths, in conjunction with small diameters, give CNTs with large surface area/volume ratios. This increase the physical interface between the CNTs and the polymer cement matrix, which improves the efficiency of CNTs–matrix stress transfer compared to conventional fibres. These features as well as their extraordinary tensile strength (~0.1TPa) strongly support the use of CNTs for augmenting acrylic bone cement [4]. This use is also supported by prior experimental data in other polymer systems [201-203].

Optimal improvements in mechanical properties can be achieved by ensuring uniform distribution of CNTs within the polymer, thus, maximising the interfacial bond between the CNTs and polymer matrix [4, 8]. It has also been reported that the CNTs must create a well dispersed, overlapping network facilitating the transport of electrons, phonons, and heat energy to improve the thermal properties of a nanocomposite [203].

Many processing techniques have been employed to uniformly disperse CNTs within polymer matrices. These techniques are primarily used; firstly to separate the entanglements and agglomerations of the as-produced CNTs, and secondly to disperse the individual CNTs throughout the matrix. The two most commonly used techniques are: *in situ* dispersion and
high temperature shear mixing \cite{8, 203}. Therefore, the property improvements for CNTs-polymer nanocomposites are a direct result of CNTs type, dispersion, level of weight loading (wt.%), alignment of the CNTs and the polymer matrix. Andrews and Weisenberger \cite{204} stated that the processing techniques produce more favourable results when small concentrations of CNTs are used, however, mixing higher concentrations of CNTs (>5 wt.%) increases the viscosity of the mixture irrespective of the state of the polymer. It was assumed that an elevated viscosity hinders effective dispersion of the CNTs into the polymer matrix, thus, the energy induced into the mixing process must be increased, but, at the risk of shortening the CNTs or irreversibly damaging the matrix material \cite{8}.

Xie et al.\cite{203} reported a significant improvement (≈125\%) in the thermal conductivity of an epoxy based polymer when SWCNTs (1 wt.\%) were added. Choi et al.\cite{201} also observed an increase (≈300\%) in the thermal conductivity of an epoxy polymer when SWNTs (3 wt.\%) were added. Kearns and Shambaugh \cite{202} found that the tensile strength of polypropylene fibres reinforced with CNTs could increase ≈40\%.

There are several studies related to the preparation and characterisation of carbon nanotubes/poly(methylmethacrylate) composites. For example, Zhaoxia et al.\cite{205} studied MWCNTs/PMMA composites fabricated by melt blending and found that the nanotubes were well dispersed in the polymer matrix and the storage modulus of the composites was significantly increased. Stéphan et al.\cite{206} prepared thin films of PMMA-SWCNTs composites by spin coating using different nanotube concentrations and found that the nanotubes were well dispersed in the polymer matrix for low concentration due to the intercalation of polymer. Cooper et al.\cite{207} used a polymer extrusion technique and dry powder mixing method to prepare CNTs mixed in a PMMA matrix and found that the final composite contained well-dispersed CNTs and the tensile modulus was almost insensitive to the presence of either SWCNTs or MWCNTs, whereas the impact strength thus, the fracture toughness was significantly improved by even small amounts of SWCNTs. Also, their proposed method for the dispersion and orientation of CNTs in a polymer matrix showed promise for the preparation of improved engineering composites.

Zhijie et al.\cite{208} prepared PMMA/CNTs composites by an \textit{in situ} process. Their studies show that CNTs could participate in the polymerisation of PMMA initiated by radical initiator AIBN (Azobisisobutyronitrile) and form a strong combining interface between the CNTs and
the PMMA matrix. Increased CNTs led to elevate the mechanical properties and the heat deflection temperatures of composites. The dispersion ratio of CNTs in the PMMA matrix is proportional to the reaction time of polymerising MMA before CNTs were added into the PMMA mixture.

Yu-Hsun and Chiao \cite{125} developed a new type of bone cement by preparing fabricated PMMA/CNTs composites first, and then introduced them as ground powder into commercial OSTEOBOND® bone cement in an attempt to achieve better dispersion of carbon nanotubes in the cement matrix. Their modified bone cement showed potential usage in clinical applications and exhibited excellent tensile and compressive strength properties.

Marrs et al.\cite{4,5} investigated the influence of MWCNTs in the static and dynamic mechanical properties of PMMA based bone cements. The MWNTs (0–10 wt.%) were dispersed throughout the molten matrix of pre-polymerised methylmethacrylate–styrene copolymer (MMA-co-Sty), a principal component of commercial bone cement powder, and combined using high-shear mixing method in heated chamber (220°C). They noted moderate improvements in flexural strength by \(\approx 12\%\) and enhancement in yield stress by \(\approx 13\%\) when 2 wt.% MWCNTs were incorporated into the methylmethacrylate-styrene cement. Bending modulus increased slightly with the smaller concentrations (<5 wt.%), but increased \(\approx 24\%\) in response to the 10 wt.% loading \cite{5}. They also reported that the addition of 2 and 5 wt.% of MWCNTs significantly enhanced the fatigue performance of MMA-co-Sty cement by \(\approx 565\%\) and 592\%, respectively \cite{4}.

Based on the values of these mechanical properties determined, 2 wt.% appears to be the optimum reinforcement mass percentage. There are two bases for this suggestion, with this loading, the nanotubes have their long axis oriented to the plane of the incipient crack; and there is an absence of poorly dispersed nanotubes (and, hence, their agglomerations), as was seen at higher loadings, features that may act as fracture initiation sites \cite{5}. Although the specimens for fatigue testing were aged and tested in Dulbecco’s Phosphate Buffered Saline (PBF) \cite{4}, both studies used non-clinically relevant methods to ensure optimal dispersion of the MWCNTs into the bone cement. Furthermore, the mechanical properties were assessed under uniaxial laboratory conditions where specimens were tested to failure in quasi-static 3-point bending and in 4-point bending fatigue. These traditional uniaxial tests may not provide an accurate characterisation of bone cements true load-bearing capacity \cite{174}.
Ormsby et al.\cite{8} incorporated unfunctionalised and carboxyl (–COOH) functionalised MWCNTs (0.1 wt.%) into commercially available Colacryl\textsuperscript{®} B866 bone cement to investigated its potential augmentation using three different preparation techniques. MWCNTs were either added to the liquid methylemethacrylate component of the cement via magnetic stirring or ultrasonic disintegration or dry blended with the polymer powder component. Improvements in static mechanical properties were reported and attributed to the degree of the MWCNTs agglomerations, which dependent on the type and method of introduction used to incorporate the MWCNTs into the cement. The level of heat produced due to the exothermic polymerisation reaction of the bone cement was significantly reduced when functionalised MWCNTs were added.

Despite these improvements, the influence of other functional groups and the effects of higher wt.% loadings of MWCNTs which could manipulate the dispersion and interfacial bonding of the MWCNTs within the PMMA cement matrix, limited their study. Therefore, Ormsby et al.\cite{6} conducted another study to investigate the incorporation of various concentrations of MWCNTs (0.1–1.0 wt.%) to PMMA bone cement with differing functional groups (carboxyl and amine) to address some of these limitations. They reported that the mechanical properties were influenced by the type and wt.% loading of MWCNTs used. The extent of the effect was dictated by the chemical functional groups added to the MWCNT, and the level of loading used. Low loadings of MWCNTs (≤ 0.25 wt.%) to PMMA bone cement improved the mechanical properties of the resultant nanocomposite while higher loadings (≥ 0.5 wt.%) provided lesser improvements, and in some cases significant reductions in the mechanical properties. Those improvements were attributed to the MWCNTs well dispersion within the PMMA cement, thereby arresting or retarding crack propagation through the cement. The dispersion of MWCNTs within the cement matrix was dependent on the weight fraction and functionality of MWCNTs incorporated into the cement and enhanced by adding chemical functional groups, with the carboxyl functionalised MWCNTs providing the most significant improvements in mechanical integrity. Similar to the studies conducted by Marrs et al.\cite{4, 5}, the mechanical properties of the bone cement composite were evaluated by compression and 4-point bending tests, which may not provide an accurate characterisation of bone cements true load-bearing capacity. In addition, even though the test specimens in both experiments were prepared using contemporary cement
mixing and delivery techniques, as per current clinical practice for joint replacement surgery, the test specimens were stored and tested in ambient laboratory conditions, which is clearly non-physiological.

In a recent study, Ormsby et al.\cite{7} reported that incorporation of MWCNTs to acrylic bone cement significantly altered the polymerisation reaction and kinetics of resultant bone cement composites. The reduced rate of reaction allowed for up to 34% in the peak exotherm during polymerisation, thus, decreasing in the thermal necrosis index (TNI) values for the respective composites (between 3% and 99%), which could preventing the likelihood of the polymerising PMMA cement causing thermally-induced bone tissue necrosis. Despite these improvements in the thermal properties, there were limitations to this study. For example, the TNI assessment under ambient conditions (22±1°C) was not representative to \textit{in vivo} temperature (37°C) in which the cement is contained; thus, disregard the heat transfer rate from the maximum temperature ($T_{\text{max}}$) to the body’s temperature. Also, it should be noted, the produced values of TNI were several orders of magnitude different to any other records.

For new generation biomedical applications materials, Singh et al.\cite{209} synthesised a novel hybrid nanocomposite bioactive bone cement using COOH-functionalised MWCNTs (0.01, 0.1, 0.5, and 1 wt.%) mixed with a composite of commercially available PMMA and HA particles in 1:2 ratio by weight percentage, via freeze-granulation technique to increase material homogeneity and enhance the dispersion of MWCNTs in the composite matrix. Their results indicated that 0.1 wt.% concentration of MWCNTs in the PMMA-modified HA nanocomposite cement yields the best mechanical properties. This novel nanocomposite material could be specifically used in bone cement that requires high strength and bone repair due to the ability of HA to bond chemically with living bone tissues caused by its similar chemical composition and crystal structure to apatite in the human skeletal system.

Notwithstanding aforementioned enhancements in mechanical performance of acrylic bone cements by incorporating low loadings of MWCNTs, no information exists on the cytocompatibility of these bone cement nanocomposites. Although studies to employ CNTs in biomaterials are increasing rapidly, the use of CNTs in biomaterials in contact with bone is still in its infancy.
Because CNTs are already used for industrial products and not for biomaterials, several studies have been instituted worldwide focused on the inhalation risk from CNTs. Therefore, pulmonary toxicity of CNTs is low by these studies and further testing must be performed \[^{[210]}\]. Concerns have been raised that CNTs may have adverse effects on living cells. To date, no definitive studies regarding these concerns have appeared in the literature. However, only one clinical study has been reported on utilising CNTs to develop reinforced bone cement, where the cytotoxicity of bone cement based on functionalised CNTs reinforced PMMA/HA was tested against Gram-negative bacteria Escherichia coli JM109 \[^{[211]}\]. The use of non-relevant cell line, for example, was the major limitation for this study. Also, some important aspects were consistently absent from studies referenced in the literature for similar composites such as the influence of sterilisation methods and conditions of the cement on its monomer release, and thus, its biocompatibility.

### 2.4 Aims and Objectives

In this work, novel nanocomposite materials were made comprising various concentrations (0.0, 0.1, 0.2, and 0.5 wt.%) of multi-walled carbon nanotubes (MWCNTs) with different chemical functionality (–OH, and –COOH groups) and Simplex-P® commercial bone cement powder. The MWCNTs were incorporated via a clinically relevant method. A series of experiments were performed to address the following objectives:

- To examine the applicability of the biaxial flexure test as a suitable method to determine the strength of acrylic bone cement composites. For this purpose, a finite element analysis was performed to verify the analytical biaxial theory and to inspect the quality and accuracy of analytical approximations used to estimate the correct equivalent contact radius between the balls and the disc specimen.

- To assess the influence of MWCNTs chemical functionality and loadings on the mechanical properties of bone cement composites. For this purpose, the biaxial properties were evaluated using a ball-on-three-ball biaxial flexural strength test. Furthermore, ring-on-ring biaxial flexural test and four-point bend uniaxial flexural test tests were conducted to compare the results determined.
To assess the effect of MWCNTs chemical functionality and loading on the reliability of bone cement composites by considering the associated Weibull moduli of the strength data for the cement composites.

To assess the effect of MWCNTs chemical functionality and loading on the bond strength between stem-cement and cement-bone interfaces. For this purpose, a single-lap joint test was used to characterise the mechanics of stem-cement and cement-bone interfaces failure.

To assess the effect of MWCNTs chemical functionality and loading on the degree of monomer conversion attainable and the amount of heat released under isothermal polymerisation conditions at 37°C, (the temperature in the bone bed during a cemented arthroplasty). For this purpose, the polymerisation kinetics were monitored and analysed by differential scanning calorimetry (DSC) in isothermal and non-isothermal conditions, while the temperature caused by the highly exothermic reaction during the polymerisation was measured using a thermocouple to determine the cumulative thermal necrosis index (TNI).

To evaluate the cytocompatibility of these MWCNTs reinforced Simplex-P® bone cement nanocomposites to determine their potential as a bone cement material for use in bone tissue engineering. For this purpose, the influence of MWCNTs chemical functionality and loadings on the cellular adhesion, growing, and viability in these new composites of an in vitro model of human osteoblasts cells was investigated using MTS assay, Scanning Electronic Microscope (SEM), Laser Scanning Confocal Microscope (LSCM). A new real time cell monitoring technique was also employed to understand the response and behaviour in human bone cell cultures.

All tests were conducted in a physiologically relevant environment (i.e. phosphate buffered saline, 37°C) rather than laboratory conditions (i.e. dry air at room temperature 25°C) to overcome some limitations of previous studies in evaluating the mechanical and thermal properties.
3 Experimental
3.1 Materials

The materials used in these studied were:

- Commercial surgical Simplex-P® radiopaque bone cement (*Stryker Orthopaedics, Limerick, Ireland*), is widely available and used as total hip replacement surgery cement. The cement is a two-component system and each package consisting of a 40 g of powder polymer and a 20 ml liquid monomer; as shown in table (3-1). The methacrylate styrene copolymer provides resistance against degradation of the bone cement during gamma radiation sterilisation; improve processing characteristics; and possibly reduces temperature rise during polymerisation. BaSO₄ imparts radiation opacity and improves the fracture toughness of the bone cement.

<table>
<thead>
<tr>
<th>Powder</th>
<th>Liquid</th>
</tr>
</thead>
<tbody>
<tr>
<td>30.0 g Poly(methyl methacrylate, styrene)</td>
<td>18.31 g (19.5 ml) methyl methacrylate</td>
</tr>
<tr>
<td>6.0 g Poly(methyl methacrylate)</td>
<td>0.48 g (0.5 ml) N,N-dimethyl-para-toluidine</td>
</tr>
<tr>
<td>4.0 g Barium Sulphate U.S.P BaSO₄</td>
<td>75±15 ppm hydroquinone</td>
</tr>
<tr>
<td>0.6 g Benzoyl Peroxide BPO</td>
<td></td>
</tr>
<tr>
<td>40.0 g</td>
<td>18.79 g (20 ml)</td>
</tr>
</tbody>
</table>

- Multi-walled Carbon Nanotubes MWCNTs, hydroxyl group (−OH) functionalised nanotubes MWCNTs−OH (contain 1.6% OH groups), and carboxyl group (−COOH) functionalised nanotubes MWCNTs−COOH (contain 1.2% COOH groups) were purchased from (*Cheap Tubes Inc., Vermont, USA*) and used as received without any further treatment in this study. Table (3-2) and figure (3-1) showed the properties and the Raman spectra of MWCNTs, respectively. Figure (3-2) shows TEM image of the MWCNTs used in this study.
Chapter 3  Experimental

Figure 3-1: Raman spectra of MWCNTs with 20-30 nm OD.[212]

Table 3-2: Properties of MWCNTs used.[212]

<table>
<thead>
<tr>
<th>Property</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Outer Diameter</td>
<td>20-30 nm</td>
</tr>
<tr>
<td>Inside Diameter</td>
<td>5-10 nm</td>
</tr>
<tr>
<td>Length</td>
<td>10-30 μm</td>
</tr>
<tr>
<td>Ash</td>
<td>&lt;1.5 wt.%</td>
</tr>
<tr>
<td>Purity</td>
<td>&gt;95 wt.%</td>
</tr>
<tr>
<td>Components Contents (%)</td>
<td>C 98.35; Cl 0.45</td>
</tr>
<tr>
<td></td>
<td>Fe 0.26; Ni 0.94</td>
</tr>
<tr>
<td>Specific Surface Area</td>
<td>60 m$^2$/g</td>
</tr>
<tr>
<td>Electrical Conductivity</td>
<td>&gt;100 S/cm</td>
</tr>
<tr>
<td>Bulk density</td>
<td>0.28 g/cm$^3$</td>
</tr>
<tr>
<td>True density</td>
<td>~2.1 g/cm$^3$</td>
</tr>
<tr>
<td>Manufacturing Method</td>
<td>CCVD</td>
</tr>
</tbody>
</table>
Solid rigid polyurethane foam 20 PCF, an alternative test medium for human cancellous bone for comparative testing of bones screws and other medical devices and instruments, was purchased from Sawbones® Europe AB, (Malmö, Sweden). The polyurethane foam has a closed cell content ranging from 96.0–99.9 %, and density of 0.32 g/cm³. The average mechanical properties are shown in table (3-3). Foam density designate per ASTM F-1839-08 standard specification [213].

Table 3-3: Average Material Properties of solid rigid polyurethane foam 20 PCF.

<table>
<thead>
<tr>
<th></th>
<th>Compressive</th>
<th>Tensile</th>
<th>Shear</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Strength MPa</td>
<td>Modulus MPa</td>
<td>Strength MPa</td>
</tr>
<tr>
<td></td>
<td>8.4</td>
<td>210</td>
<td>5.6</td>
</tr>
</tbody>
</table>


Phosphate Buffered Saline (PBS) solution, 1X, was purchased from Fisher (Fisher BioReagents®, Dublin, Ireland).

3.2 Sample Preparation

Six formulae of new reinforced bone cement nanocomposites were prepared by incorporating various concentrations of MWCNTs (0.0, 0.1, 0.2, and 0.5 wt.%) with different chemical functionality (−OH, and −COOH groups) to commercial Simplex-P® bone cement powder. This low weight loading was chosen as it has been reported that due to MWCNTs high surface area, small loadings significantly improve mechanical reinforcement [8]. Also, lower
levels of MWCNTs can reduce the tendency for agglomerations and prevent chemical or physical interference during the polymerisation reaction.

To increase material homogeneity and also enhance the dispersion of MWCNTs in the cement composite matrix, the amount of different MWCNTs were incorporated into the commercial bone cement powder using ball milling blending technique for 2 h. The composition of the liquid portion of the bone cement was the same in all composites; bone cements were mixed at the liquid to powder ratio (L/P) of 0.5 ml/g.

To manufacture each bone cement specimen, the powder cement blend and the liquid monomer were measured and hand mixed for 45 s to reduce the formation of air bubbles. Once the bone cement reached the doughing time the dough was transferred into multi-cavity polytetrafluoroethylene (PTFE) split moulds to cast the specimens in a designed shape of defined dimensions. The moulds were initially lubricated with a thin film of silicon release spray. The paste was then packed to excess in the mould, covered with a glass plate and allowed to cure under pressure.

A clamping pressure was applied directly to the mould and released after 25 min. The bone cement was mixed in air at a standard operation room environment held at a nominal temperature of 23±1°C and relative humidity ranging from 40-60% in agreement with ISO 5833:02 [214] recommendations to simulate clinical conditions.

Once the bone cement became hard, specimens were separated from the moulds, and flash was removed with a scalpel blade. Specimens then were inspected and checked in order to reject any specimens with visible defects indicative of ineffective filling in the working region. The edges of specimens were smoothed using a wet 400-grit silicon carbide paper to adjust the thickness to the desired value. The parallelism and the flatness of opposing surfaces were verified with a micrometer (Moore and Wright, Sheffield, England) to tolerance within ±0.05 mm. Since the bone cement will function at body temperature, after the polymerisation, specimens were immersed in saline solution (0.9 wt.%) at 37°C to simulate body environment until testing.

3.3 Samples Notation

Depending on the chemical functionality of the MWCNTs, the new Simplex-P® bone cement composite reinforced with unfunctionalised MWCNTs is denoted SUNF, the composite reinforced with MWCNTs functionalised with hydroxyl groups is denoted SOH, and the
Chapter 3

Experimental

composite reinforced with MWCNTs functionalised with carboxyl groups is denoted \textbf{SCOOH}. Furthermore, based on the weight percentage of MWCNTs in the new bone cement composites, the numbers 1, 2, and 5 are considered for 0.1, 0.2 and 0.5 wt.%.

Sample notation and composition of the bone cement in each nanocomposite, (40 g), are shown in table (3-4).

Table 3-4: Notation and compositions of bone cement nanocomposites.

<table>
<thead>
<tr>
<th>Powder portion (g)</th>
<th>CNTs (wt.%)</th>
<th>Simplex powder</th>
<th>CNTs</th>
<th>CNTs-OH</th>
<th>CNTs-COOH</th>
<th>Commercial liquid portion (ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Control</strong></td>
<td>0.0</td>
<td>40.00</td>
<td>0.00</td>
<td>—</td>
<td>—</td>
<td>20</td>
</tr>
<tr>
<td><strong>SUNF1</strong></td>
<td>0.1</td>
<td>39.96</td>
<td>0.04</td>
<td>—</td>
<td>—</td>
<td>20</td>
</tr>
<tr>
<td><strong>SUNF2</strong></td>
<td>0.2</td>
<td>39.92</td>
<td>0.08</td>
<td>—</td>
<td>—</td>
<td>20</td>
</tr>
<tr>
<td><strong>SUNF5</strong></td>
<td>0.5</td>
<td>39.80</td>
<td>0.20</td>
<td>—</td>
<td>—</td>
<td>20</td>
</tr>
<tr>
<td><strong>SOH1</strong></td>
<td>0.1</td>
<td>39.96</td>
<td>—</td>
<td>0.04</td>
<td>—</td>
<td>20</td>
</tr>
<tr>
<td><strong>SCOH1</strong></td>
<td>0.1</td>
<td>39.96</td>
<td>—</td>
<td>—</td>
<td>0.04</td>
<td>20</td>
</tr>
</tbody>
</table>

3.4 Mechanical Characterisation

The mechanical properties of nanocomposite bone cements resulted from the incorporation of various concentrations of MWCNTs with differing functional groups to acrylic Simplex-P® bone cement were characterised using flexural strength test. The objective of this study was to establish the utility of the biaxial flexural test on the evaluation of the strength and modulus of surgical bone cements. The accuracy of the biaxial theory using the most common approximations found in literature for the contact radius of the central loading ball was investigated to address some of the limitations of the previous studies. To verify the accuracy of the equivalent contact radius, thus, the contact area, a finite element analysis was performed. Different flexural strength tests were also conducted to compare the results determined.

3.4.1 Flexural Strength

Flexural strength of each bone cement composite was evaluated using ball-on-three-ball (B3B) test using three different approaches to calculate the contact radius \((r_0)\), of the central loading ball. The flexural strength of a material is the maximum stress that it can resist before
failure when subjected to bending load. The flexural modulus is the ratio of stress to strain in flexural deformation and it can be determined from the slope of a stress-strain curve produced under bend load. In addition, two other standardised flexural test methods, non-axis-symmetric equibiaxial ring-on-ring test (ASTM C 1499-09 [215]), and uniaxial four-point bend test (ISO 5833 Standard [214]) were used to compare the obtained results and investigate the accuracy of using the biaxial test to determine the acrylic bone cement properties.

3.4.1.1 Ball-On-Three-Ball Test

Minimum of twelve disc-shaped specimens of each bone cement composite with a diameter of 15 mm and thickness of 1.8±0.1 mm were prepared and subjected to a biaxial flexural test using the appropriate biaxial testing rig [174], figure (3-3).

![Figure 3-3: Biaxial flexural test setup.](image)

The test rig of the ball-on-three-ball (B3B) biaxial test held in an environmental chamber made of clear polycarbonate container filled with a continuous flow of phosphate buffered saline solution (9 wt.%), fluid was over the specimens throughout testing and maintained at 37±1°C using a digitally controlled stirred water baths and circulators (*Grant GD120, Grant Instruments, Cambridge, England*). The schematic of the experimental setup is illustrated in figure (3-4).
The lower face of a disc specimen was placed centrally and supported by three stainless-steel ball bearings equidistant and positioned 120° apart from its centre, on a support circle of 11.5 mm diameter, while the upper face of the specimen was axially loaded via a fourth central ball until failure occurred, all balls used were 4 mm in diameter.

To minimise regional stresses, the ball bearings were freely supported on three drilled holes of 1 mm. To ensure applying symmetrical and concentric loading; the biaxial testing fixture was aligned with respect to the piston prior to the test. Loads were applied to the specimens using a screw-driven universal testing machine (Instron, Model 4302, Buckinghamshire, England) with a 1 kN load cell. Load-displacement data and the maximum load (F) exerted on the specimen prior to fracture were recorded. In order to negate any strain-rate affects; a low cross-head speed of 0.5 mm/min was used causing fracture in 65±25 s.

The calculation of the biaxial modulus is somewhat more complicated than that for compression and flexural testing \[^{175}\]. The calculation is based upon the initial work of Bassali \[^{185}\], and later in a more specified way by Kirstein and Woolley \[^{186}\] which suggested a simple relationship between center deflection and modulus, although this was not established experimentally \[^{175}\].
According to Kirstein & Woolley, the expression for the center deflection of the disc is \[^{[186]}\] :

\[
\omega_c = \beta_c \frac{FR_a^2}{Et^3}
\]  

(3-1)

in which \((F)\) is the applied load, \((R_a)\) is the radius of the support circle, \((t)\) is the disc specimen thickness at fracture origin, \((E)\) is the elastic modulus of the disc material, and the centre deflection function \((\beta_c)\) is given by \[^{[175]}\] :

\[
\beta_c = -0.0642 - 2.19 \, m^{-3} + (0.5687 + 3.254 \, m^{-3})(1 - \nu^2) \\
+ [-0.3793 + 11.0513 \, m^{-3} + (0.5223 - 7.8535 \, m^{-3})(1 - \nu^2)] \tau^3
\]  

(3-2)

where \((m)\) is the number of equally spaced supports; \((\tau)\) is the ratio of the support radius to the radius of the disc specimen and \((\nu)\) is the Poisson's ratio for the material of the disc which is defined as the lateral contraction per unit breadth divided by the longitudinal extension per unit length.

Figure (3-5) shows the dependency of the centre deflection function \((\beta_c)\) on the number of supports \((m)\) and the ratio of support radius to specimen radius \((\tau)\).

![Figure 3-5: Relationship of the theoretical deflection to number of supports.\[^{[175]}\]](image-url)
If the centre deflection and specimen dimensions are known, then correspondingly, the modulus of the disc specimen can be determined from Equation (3-1). Experimentally, it is the slope of the load-displacement curve, \( \frac{dF}{d\omega_c} \), from which the modulus \((E)\), can be reliably determined, namely;

\[
\frac{dF}{d\omega_c} = E \frac{t^3}{\beta_c R_a^2} = E \left( \frac{t^3}{\beta_c R_a^2} \right)
\]

(3-3)

The strength is defined to be the maximum principal tensile stress in the disc, which occurs on the disc surface opposite the centred loading ball. Of course the stress field in the disc depends on the applied load, on the geometric set-up of the test, namely the thickness and the diameter of the disc and the size and the position of the balls, and also on the elastic properties of ball and disc materials [181]. Different approaches have been made for the analytical calculation of the stress distribution in centrally loaded biaxial disc tests.

According to Higgs et al. [174, 175] the maximum biaxial flexural strength \((\sigma_{\text{biaxial}})\) in the centre of the disc face is approximately independent of the number of support points and can be described by [174]:

\[
\sigma_{\text{biaxial}} = \frac{3 F (1 + v)}{4 \pi t^2} \left[ 1 + 2 \ln \frac{R_a}{r_0^*} + \frac{1 - v}{1 + v} \left( \frac{2 R_a^2 - r_0^{*2}}{2 R^2} \right) \right]
\]

(3-4)

where \((t)\) is the specimen thickness, \((r_0^*)\) is the radius of loading contact area; \((R)\) is the radius of the disc specimen; \((R_a)\) is the radius of the support circle.

To apply this solution to a ball loaded disc, the elastic body interaction whereby the contact, or more specifically, the equivalent radius of contact \((r_0^*)\) of the central loading ball has to be known, taking into account the radius of the ball \((R_b)\), the load at failure \((F)\), and the material properties of the indenter ball and the disc specimen [174]. Various approximations have been found in literature to determine this parameter [181]. The first approximation for the contact equivalent radius, \((1^{\text{st}} r_0^*)\), of the most three common approximations is [216]:

\[
1^{\text{st}} r_0^* = \sqrt{(1.6 * R_b^2 + t^2)} - 0.675 \ t
\]

(3-5)

This formula was given by Westergaard [216] and applies to a plate of any form for all values of \((0 \leq R_b \leq 1.742 \ t)\); for larger values the actual \((R_b)\) may be used.
The second approximate expression for this equivalent radius, \(2^{nd} r_0^*\), was given by Shetty \[217\] as:

\[
2^{nd} r_0^* = \frac{t}{3}
\]

(3-6)

The third common approximation for the contact equivalent radius, \(3^{rd} r_0^*\), was suggested by Godfrey \[218\] and given as:

\[
3^{rd} r_0^* = 0.721 \times \left( 2 \times F \times R_a \left[ \frac{1-\nu_1^2}{E_1} + \frac{1-\nu_2^2}{E_2} \right] \right)^{1/3}
\]

(3-7)

in which \(E_1\), \(\nu_1\), \(E_2\) and \(\nu_2\) are the modulus and Poisson's ratio of the loading ball and specimen, respectively.

The use of the equivalent contact radius of the central loading ball makes possible the calculation of the finite maximum stresses produced by a (nominal) point loading; whereas the ordinary formulae would indicate that the stresses at the loading site were infinite. The results obtained with the use of (B3B) biaxial testing are shown in section (4.1.1).

3.4.1.2 Ring-On-Ring Test

Six disc-shaped specimens of each of the bone cement-MWCNTs composites with a diameter of 37 mm and thickness of 2.3±0.2 mm were prepared and subjected to an equibiaxial flexural test. Flexural strength was determined in accordance with ASTM C 1499-09 \[215\] using the appropriate equibiaxial testing rig. The test rig was held in an environmental chamber made of clear polycarbonate container filled with continues flow of saline solution (0.9 wt.%), fluid was circulated to over the specimens throughout testing maintained at 37±1°C using same digitally controlled stirred water baths and circulators as above.

In a ring-on-ring testing assembly, the disc is supported by a ring and loaded from the opposite side by another smaller concentric ring. In the area underneath the smaller ring, an equibiaxial tensile stress state exists where initialisation of fracture is expected \[181\]. The experimental setup is shown schematically in figure (3-6) \[215\].
The lower face of a disc specimen was placed centrally and supported by a support (outer) ring with a nominal diameter of \(D_S = 30\) mm, while the upper face of the specimen was loaded with a loading (inner) ring with a nominal diameter of \(D_L = 12\) mm until failure occurred. The load and support fixtures were made of hardened steel. The tip radius \(r\) of the cross sections of the load and support rings was 2 mm. The loading and support rings were checked for microscopic damage to the bearing surfaces throughout testing. Prior to testing, specimens were concentrically aligned with the support ring, and load was applied through the centre of the test piece with the loading ring. The test fixture was self aligning; therefore, no compliant materials were required between the disk specimen and the load ring. The specimens were loaded in the same screw-driven universal testing machine as before with a 1 kN load cell, at cross-head speed of 0.5 mm/min causing fracture in 210±30 s. The displacement rate, load, and support rings diameters had have been determined from the specimen thickness by referring to the ASTM C1499-09 standard used. The recorded fracture load \(N\) was used in conjunction with the following equation to give the flexural strength \([215]\):

\[
\sigma_{\text{equibiaxial}} = \frac{3F}{2\pi t^2} \left[ (1 - \nu) \frac{D_S^2 - D_L^2}{2D^2} + (1 + \nu) \ln \frac{D_S}{D_L} \right] \quad (3-8)
\]

where \(F\) is the braking load (N); \(t\) is the specimen thickness (mm); \(D_L\) is the load ring diameter (mm); \(D_S\) is the support ring diameter (mm); \(D\) is the specimen diameter (mm);
and \((v)\) is the Poisson's ratio for the material of the disc. The results of this test are shown in section (4.1.1).

### 3.4.1.3 Four-Point Bending Test

In comparison with other commonly used mechanical test methods such as tension, plane bending and torsion, four-point bending has its own advantages for characterising the mechanical properties of materials. Firstly, it produces a uniform moment between the two inner loading rollers in the specimen which gives rise to a uniform maximum tensile stress in the specimen surface. Secondly, no special sample gripping is needed for the four-point bend test, which makes it possible to test brittle materials in tension and makes sample preparation relatively simple since a specimen with a uniform rectangular cross-section is usually used in the test. Thirdly, sample mounting and dismounting are fairly straightforward in four-point bend. Furthermore, using four-point bend a pure shear stress can be applied to a test piece by asymmetrically loading the specimen. Because of these advantages, four-point bending is traditionally often used to characterise the flexure strength of a specimen. It is also sometimes applied to fatigue studies [219]. Figure (3-7) shows a schematic of the experimental setup used.

![Figure 3-7: Four-point bend test rig][214]

Six beam-shaped specimens of each bone cement composite with a rectangular cross-section with average geometry of 75 mm length; 10 mm width; 3.3 mm thickness were prepared and bent until fracture using the appropriate flexural testing rig in accordance with ISO 5833.
The standard deviation in the measured specimens’ dimensions was negligible. The test rig of four-point bending test held in an environmental chamber made of clear polycarbonate container filled with a continuous flow of saline solution (0.9 wt.%), fluid was maintained at 37±1°C over the specimens throughout testing using the same digitally controlled stirred water baths and circulators.

When a specimen of bone cement is bent, it experiences a range of stresses across its depth. At the edge of the specimen on the inside of the bend (concave face) the stress will be at its maximum compressive stress value. At the outside of the bend (convex face) the stress will be at its maximum tensile value. Most materials fail under tensile stress before they fail under compressive stress, so the maximum tensile stress value that can be sustained before the beam fails is its flexural strength. The lower face of a disc specimen was placed on two outer loading points, while the upper face of the specimen was loaded with two inner loading points. The distance between outer loading points is 60 mm. The distance between outer and inner loading points is 20 mm. To minimise the localised deformation of the specimen during loading, supporting and loading points were made of hardened steel with a tip radius of cross sections of 2 mm. The four-point bend rig was compact and self-aligning upon loading; therefore, no compliant materials were required between the disk specimen and the load points. The specimens were loaded in the same screw-driven universal testing machine with a 1 kN load cell, at cross-head speed of 5.0 mm/min.

The bending strength, \(\sigma_{\text{bend}}\) (MPa) was computed from the maximum force at break \(F\) (N), using the expression \(^{[214]}\):

\[
\sigma_{\text{bend}} = \frac{3Fa}{b_t^2} \tag{3-9}
\]

and bending modulus, \(E_{\text{bend}}\) (MPa) from the equation \(^{[214]}\):

\[
E_{\text{bend}} = \frac{\Delta Fa}{4fbt^3} (3l^2 - 4a^2) \tag{3-10}
\]

where \((f)\) is the difference between the deflections under the loads of 15 N and 50 N (mm); \((b)\) is the average measured width of specimen (mm); \((t)\) is the average measured thickness of specimen (mm); \((l)\) is the distance between the outer loading points (60 mm); \((\Delta F)\) is the load range (15 N-50 N = 35 N); \((a)\) is the distance between the inner and outer loading points.
(20 mm). The flexural properties determined with the use of 4-point bend testing are shown in section (4.1.1).

3.4.2 Single Lap-Shear Test

Mechanical failure of the stem-cement and cement-bone interfaces as well as fracture of the cement mantle have been proposed as initiation sites for eventual clinical loosening of cemented hip components [220]. The first step towards greater use of bone cement is the characterisation of those interfaces found typically in these joints in order to improve the knowledge of the behaviour of these joints and increase the amount of data available to the biomaterials design engineer. Since a single-lap joint is widely used to characterise bond strength, the testing programme was used to investigate the mode of failure in lap joint geometry of stem-cement and cement-bone interfaces, and to compare the interfacial bond strength of these joints. The single-lap joint may be loaded in tension or in bending (sometimes both together) and so only tension loading condition was considered here.

Although lap joints are easy to make and test, because of the distribution of stress, their behaviour is quite complicated. The single-lap shear test is commonly used for testing joints made from rigid adherends and adhesives to compare shear strengths of adhesives, as detailed in BS EN 1465:2009 Standard [221]. To have a reasonable chance of obtaining reproducible joint strengths, there are a large number of variables to be set and then controlled, namely;

- Size of the adherends and amounts of overlap
- Control of the thickness of the adhesive layer (bond-line thickness)
- Conditions of cure, such as time, temperature, and pressure
- Ageing of joints prior to testing

For all test specimens, adherends were machined from aluminium sheet and solid rigid polyurethane foam block using precision cutting tools, and an assembling jig was developed for the purpose. Each single lap joint was assembled from two identical adherend specimens joint together with bone cement mantle. The dimensions of the adherend specimens are (100±1 mm length, 25±0.5 mm width, 2 mm thickness) and shown diagrammatically in figure (3-8).
Chapter 3  Experimental

Figure 3-8: schematic of single lap shear joint.

Every aspect of the experimental procedure was standardised to minimise variations. The square end type of geometry was used. Two series of bonded joints were prepared using aluminium and polyurethane foam adherends plate to simulate the stem and the bone, respectively. The assembly jig consisting of 1mm thick rubber sheet between two 2 mm sheets and was used to ensure making a lap shear joint with overlap or shear area of (12±0.5 mm length, 25±0.5 mm width, 1 mm thickness) by locating the adherends accurately above and underneath the cement mantle, maintaining the 1 mm gap between the two adherends and bolting the bonded joint components together until testing. An adherend specimen is placed in its slot in the lower (2 mm) rubber sheet of the assembly jig, slight excess of adhesive (bone cement) inserted, the second adherend accurately positioned in its slot in the upper (2 mm) rubber sheet of the assembly jig, on the top of the adhesive layer, light finger pressure is exerted to reduce the bond line to the thickness controlled by the middle (1mm) rubber sheet of the assembly jig. After hardening, the bonded joints were removed from the assembly jig and stored in air at room conditions (23±1°C and 50±5% relative humidity) for 24 h. Two bonded specimens per bone cement composite for each series were loaded in tension to failure using the same screw-driven universal testing machine with a 1 kN load cell, and cross head rate of 1mm/min causing fracture in 45±20 s. The results of this test are shown in section (4.1.3).

3.4.3 Finite Element Analysis

To examine the applicability of the biaxial flexure test and verify the analytical biaxial theory results presented by Kirstein and Wooley [186], a finite element analysis was performed using Pro/ENGINEER Wildfire 5.0 integrated with Pro/MECHHANICA structure analysis Software
package. A three-dimensional model of the biaxial disc specimen was analysed for the case of a concentrated and uniformly distributed load, which acts over an equivalent radius of contact and the contacts between the balls and the disc were modelled by surface-to-surface contact elements. It has previously been reported that friction effects on the stress distribution are minor [181] and therefore are neglected in this analysis. The applied loads were of a magnitude equivalent to cause failure of the disc specimen experimentally. The finite element model is more refined in regions of the support and loading points to allow more accurate representation of the high stress gradients at these sites. In order to describe the behaviour of the balls in the test assembly, the centre of the support balls (lower balls) are fixed in their position and their radii are equal to the loading ball and made of the same material, also the centre of the loading ball (upper ball) is allowed to move only perpendicular to the disc surface (Y-direction) to apply the load on the disc. Since acrylic bone cements are well described as isotropic brittle materials [175], the disc was modelled as isotropic, homogeneous and linear-elastic continua defined by Young’s modulus and Poisson’s ratio. The finite element analysis was undertaken using a geometric model representing the biaxial specimen with typical values for Simplex-P® acrylic bone cement and stainless steel ball bearings, these chosen parameters for the standard model being (2.5 and 193 GPa) elastic modulus, (0.33 and 0.3) Poisson’s ratio, and (1.18 and 7.74 g/cm³) density, respectively. Non-linear contact mechanical analysis approach was assumed and the maximum principal stress, displacement magnitude, stress energy and contact pressure were measured for the bottom face (tensile) and the top face (compression) at the centre of the disc specimen to determine the centre deflection at the centre of the biaxial specimen, stress distribution field, and the contact areas. These parameters could be extrapolated when the experimental failure load is known. Multi-pass adaptive method for 10 equally spaced load intervals at maximum polynomial order of 9 and 10% convergence on displacement, strain energy and stress was used. The results of FE-analysis are shown in section (4.1.2).

3.4.4 Statistical Analysis

The biaxial flexure strength and modulus data for each material group were ranked in ascending order and a Weibull analysis was performed on the resultant data. The basic form of the Weibull distribution is shown as:
\[
P_f = 1 - \exp \left( -V \left( \frac{\sigma - \sigma_u}{\sigma_o} \right)^m \right)
\]

where \((\sigma)\) is the failure stress (MPa) and \((\sigma_u), (\sigma_o)\) and \((m)\) are all constants. \((m)\) is known as the Weibull modulus and was given physical meaning as characterising the ‘brittleness’ of a material \([183]\). A close grouping of the flexure stress data was manifested as a higher value of \((m)\); whilst decreased values represented a larger scatter in the flexure stress data. \((\sigma_u)\) is the stress at which the failure probability approaches zero and is known as the threshold stress (MPa), \((\sigma_o)\), normally referred to as the normalising or scaling constant and \((V)\) is the specimen volume. \((P_f)\) is the probability of failure, which varies from 0 to 1 and is calculated from \([183]\):

\[
P_f = \left( \frac{n - 0.3}{N^* + 0.4} \right)
\]

where \((N^*)\) is the total number of specimens and \((n)\) is the ranking number of the specimen, the specimens being ranked in ascending order. As the sample size and specimen volume remained constant throughout the study, the volume term was ignored. Also, it was reported that \((\sigma_u = 0)\) can be assumed as a safe stress level for brittle materials since there is always a finite probability that a critical flaw may be present in the material under investigation before it is stressed \([183]\) which reduced Eq. (3-11) to the form

\[
1 - P_f = 1 - \left( 1 - \exp \left[ - \left( \frac{\sigma}{\sigma_o} \right)^m \right] \right)
\]

This equation may be simplified further using logarithms to the straight line equation as:

\[
\ln \ln \left( \frac{1}{P_s} \right) = m \ln(\sigma) - m \ln(\sigma_o)
\]

where \((P_s)\) is the probability of survival \(P_s = 1 - P_f\) and the intercept at the Y-axis is \([-m \ln(\sigma_o)]\) and the slope is \((m)\). The number of nominally identical disc-shaped specimens used in the experiment to determine the Weibull constants \((m)\) and \((\sigma_o)\) for brittle materials determines the confidence in the accuracy of these predictions. The confidence limits for the specimen groups under investigation were calculated and the differences between test groups subjected to Weibull analysis were considered to be significant when the confidence intervals did not overlap.
Regression analysis of the Weibull data was conducted to provide \( (R^2) \) values that represented the scatter of flexure strength data along the line of best fit.

The survival probability curves were also examined in an attempt to assess the distribution of flexure strengths to identify if failures at low stress levels are caused by the same defects as those causing failure at high stress levels. The Weibull analytical results are shown in section (4.1.1).

### 3.5 Thermal Characterisation

To determine the degree of conversion attainable and the amount of heat released under isothermal polymerisation conditions at 37°C, a variety of experimental techniques have been used. In the first part, the reaction kinetics are analysed by differential scanning calorimetry (DSC) and its data was used for the quantitative determination of the rates of polymerisation in isothermal and non-isothermal conditions. The residual monomer content was evaluated by comparing the heat generated during isothermal polymerisation at 37°C with the heat that has been generated when complete polymerisation had been achieved in a dynamic scan from 20–180°C at 10°C/min. In the second part, the temperature increase caused by the highly exothermic reaction during the polymerisation of the bone cements at 37°C was measured using a thermocouple to mimic the clinic conditions and consequently determine the cumulative thermal necrosis index (TNI).

#### 3.5.1 DSC Measurements

Differential scanning calorimetry (DSC) is a widely used technique for studies of reaction kinetics. For each of the bone cement composite, the powder component was prepared separately by mixing the required substances in the desired proportions. The appropriate amounts of the powder and the liquid monomer in the proportion suggested by the manufacturer at \((L/P)\) ratio of 0.5 were manually mixed for 30 s in a polyethylene bowl, open to the ambient laboratory atmosphere, with stirring to obtain a uniform mixture.

To avoid complete polymerisation before the DSC test, sample weights ranged from 10–20 mg of that mixture were transferred very quickly (less than 30 s) from the bowl using a small spatula, and placed at the centre of an aluminium sample pan that was situated in a Perkin-Elmer DSC6 (Perkin Elmer Inc., Cambridge, UK) operating under a nitrogen purge,
with a constant flow rate of 20 ml/min, figure (3-9). The DSC cell was calibrated for heat of fusion using the indium standard and an empty pan served as the reference.

The DSC instrument can be used isothermally at any predetermined temperature. In the case of a chemical reaction carried out at a constant temperature, a plot of heat output rate versus time is obtained. Alternatively, the instrument can be programmed to scan between two preset temperatures at a constant heating rate in a dynamic (non-isothermal) measurement to provide useful information over a broad temperature range. In this case, a plot of heat output rate versus temperature is obtained. In both modes, the DSC measurement can be used for determining the progress of bone cement polymerisation by assuming that the heat evolved during the polymerisation reaction is directly proportional to the overall extent of the reaction (the area under the exothermic peak), thus, to the amount of monomer reacted, given by the fraction of reactive groups consumed.

Therefore, from each thermogram, heat evolved as a function of time $\Delta H(t)$ or temperature $\Delta H(T)$ was calculated from the area under the peak of the exotherm. Using this approach, the fractional conversion of reaction, $x$, is defined as:

$$x = \frac{\Delta H(t)}{\Delta H_{(tot)}} = \frac{\Delta H(T)}{\Delta H_{(tot)}}$$  \hspace{1cm} (3-15)
where $\Delta H_{(\text{tot})}$ is the total heat developed which is calculated by integrating the total area of the exotherm under the DSC curve in a non-isothermal experiment. The reaction rate, $\left(\frac{dx}{dt}\right)$, is thus obtained from the heat flow, $\left(\frac{dH}{dt}\right)$, as follows:

$$\frac{dx}{dt} = \frac{1}{\Delta H_{(\text{tot})}} \left(\frac{dH}{dt}\right)$$ \hspace{1cm} (3-16)

Isothermal DSC experiments show that the developed heat, $\Delta H_{(\text{iso})}$, is lower than $\Delta H_{(\text{tot})}$, thus indicating the presence of unreacted monomer. Therefore, a maximum degree of conversion, $x_{\text{max}}$, is introduced$^{[222]}$:

$$x_{\text{max}} = \frac{\Delta H_{(\text{iso})}}{\Delta H_{(\text{tot})}}$$ \hspace{1cm} (3-17)

A phenomenological kinetic model for the reaction in free radical polymerisation of unsaturated thermosetting resins that exhibits autocatalytic behaviour was proposed by Kamal and Sourour$^{[223]}$ and has been successfully used. The polymerisation reaction can be divided into two steps. In the first step the reaction exhibits autocatalytic behaviour as follows$^{[189]}$:

$$\frac{dx}{dt} = k x(1 - x)$$ \hspace{1cm} (3-18)

where $k$ is the reaction rate constant that is a function of temperature only. A plot of $\left(\frac{dx}{dt}\right)$ against $x(1 - x)$ from the first part of the reaction peak yields a straight line of slope equal to constant reaction rate ($k$). In the second part of polymerisation the reaction rate is described by the following relationship:

$$\frac{dx}{dt} = k^* x(1 - x)^2$$ \hspace{1cm} (3-19)

A plot of $\left(\frac{dx}{dt}\right)$ against $x(1 - x)^2$ from the second part of the reaction peak yields a straight line of slope equal to constant reaction rate ($k^*$).

To determine the presence of unreacted monomer in the hardened cement as well as the effect of MWCNTs chemical functionality and loadings on polymerisation reaction rate constants,
samples were polymerised inside the DSC cell isothermally at 37°C for 30 min. Once the isothermal polymerisation was completed, the samples were heated from 37°C to 150°C at a heating rate of 10°C/min, cooled from 150°C to 37°C at a cooling rate of 20°C/min, and finally reheated again from 37°C to 150°C at a heating rate of 10°C/min.

The variation of the rate of heat output as a function of time and temperature was obtained and recorded using Pyris DCS Manager Software.

In order to determine the maximum degree of conversion that could be attained, dynamic scans at heating rate of 10°C/min were also conducted from the initial temperature of the dough to the final temperature of the cement of 180°C. The initial temperature was taken to be the temperature of the air in the room in which the calorimeter was housed, which was 20°C; relative humidity of air 65%. Triplicate DSC runs were performed; thus, for each bone cement composites, 6 runs were made. The samples, weighed after each experiment, showed negligible evaporation of monomer (less than 2%). The DSC thermograms and results are shown in section (4.2.1).

3.5.2 Thermal Necrosis Evaluation

Necrosis of the bone is a function of time and temperature. In this study, the necrosis index was calculated at the centre of each bone cement specimen. The cumulative thermal necrosis index (TNI) allowed for further characterisation of the exothermic reaction of the bone cements, providing an index value for each bone cement composite, which represents the likelihood of the polymerising acrylic bone cement causing cellular damage. For each temperature and each time step in the analysis, a necrosis index increment was calculated by dividing the time step size by the time to thermal damage at temperature \( T \), where \( T \) is the average temperature in the sample over the time step. The time to thermal damage at temperature \( T \) was calculated using an exponential function fitted to the data of Moritz and Henriques \[^{130, 224}\].

\[
f(T) = \frac{\text{Time tissue is held at temperature } (T)}{\text{Time to thermal damage threshold for temperature } (T)} \tag{3-20}
\]

It has been reported that the temperature threshold for impaired bone regeneration is 44-47°C for an exposure time of 60 s, and only 30 s for exposures to >55°C are required for irreparable damage to living tissue to occur \[^{130}\]. Therefore, temperatures above 55°C were assumed to cause a necrosis index increment of 1 for the time step, and temperatures below
Chapter 3  Experimental

44°C a necrosis index increment of 0. Finally, the necrosis index increments were summed at two temperature levels (>44°C and >55°C) to calculate the TNI, using Eq.(3-20) \[^{[130]}\] .

\[
TNI = \int_{T_1}^{T_2} f(T) \, dt  \tag{3-21}
\]

If the summation \( f(T) \) values at the end of the analysis exceeds one, then there is the probability of irreparable damage to living tissue occurring.

The temperature reached during the polymerisation of PMMA-based bone cements depends on the balance between the rate of heat production and the rate of heat transfer within the medium. Therefore, the temperature increase caused by the highly exothermic reaction during the cure of the bone cements was measured in a customised mould. This was based on the accepted international standard for acrylic bone cement (ISO 5833:02 \[^{[214]}\] ), but with some modifications; cylindrical moulds consisted of polypropylene disposable tubes of 55 mm length and internal diameters of 20 mm, to keep the same size of the sample as in the ISO standard. Additionally, the cement specimen preparation was performed at 20°C using a constant mixing time of 1 min. The samples were transferred to the mould, a k-type thermocouple wire was inserted into the exact centre of each cement specimen before the cement was set, and the standard mould was kept in an oven at a constant temperature of 37°C. The overall time, from the contact of the monomer with the bone cement powder to the beginning of the test recording, was \( \approx 2 \) min. This was intended to mimic the temperature reached during the bone cement polymerisation and the dissipation of heat away from the bone cement at human body’s temperature.

The thermocouple wires were attached to a digital reader (\emph{dataTaker DT85 recorder}), and the thermal profile and peak exotherm temperatures were recorded. Temperature data were recorded at regular intervals of 1 s for a period of 30 min using \emph{Delooger 5.0™} Software. A graph was plotted of elapsed time from mixing against exotherm temperature. The measurements were performed in duplicate.

Additionally, the maximum temperature (\( T_{\text{max}} \)), and setting time (\( t_{\text{set}} \)) for each bone cement composite was measured as defined by ISO 5833:02 \[^{[214]}\].

\[
t_{\text{set}} = \frac{T_{\text{maximum}} + T_{\text{ambient}}}{2}  \tag{3-22}
\]
The rate of polymerisation was also determined by calculating the gradient of the linear portion of the temperature vs. time plot at the setting time \( t_{\text{set}} \). The TNI results are shown in section (4.2.2).

### 3.6 Cytotoxicity Studies

The influence of multi-walled carbon nanotubes chemical functionality and loadings in bone cement composites on the adhesion and viability of an \textit{in vitro} model of human osteoblasts cells was investigated. This was conducted to determine its potential as a bone cement material for use in bone tissue engineering.

In this study, the biocompatibility evaluations were determined in accordance with ISO 10993:2009\textsuperscript{[225]}, but with some modifications to the sample sterilisation procedure; a second series of the powder component of each bone cement composite was prepared separately by mixing the required substances in the desired proportions. These powders were sterilised prior to mixing and bone cement samples were prepared in the lab and utilised directly in biocompatibility tests, avoiding any effects of time between sterilisation and testing on the test samples, and to simulate the clinical environment.

Also, real time cell viability was studied using a new flexible real-time cell monitoring technique xCELLigce\textsuperscript{®} RTCA Instrument, (Roche, Penzberg, Germany) to understand the real-time cell response and behaviour in human bone cell cultures.

The cytotoxic effect of the material was assessed by the MTS assay by determining cell viability and by confocal microscopy. Both direct and indirect contact cytotoxicity tests were conducted. Cell-material interface and adhesion were analysed by scanning electron microscopy (SEM).

#### 3.6.1 \textit{In vitro} Sample Preparation

**Direct contact test samples:** ten disc-shaped specimens of each bone cement composite with a diameter of 15 mm and thickness of 1.8±1 mm were prepared for the biocompatibility test, using the ratio and composition given in section (3.3). After samples hardened, six discs of each bone cement were randomly chosen and cleaned with acetone and left until fully dry in air at ambient temperature.
Once dry, all discs were packaged and sterilised by gamma irradiation treatment with minimum irradiation dose of 25 kGy (Isotron Westport Ltd, Mayo, Ireland) in accordance with ISO 13485:2003 Quality system-Medical Devices, DM76165. The resulting samples were stored in a desiccator at room temperature until used for MTS assay studies.

**Indirect contact test samples:** the powder component of each bone cement composite was sterilised by gamma irradiation treatment with minimum irradiation dose of 25 kGy (Isotron Westport Ltd, Mayo, Ireland) in accordance with ISO 13485:2003 Quality system-Medical Devices, DM76165. The pre-sterilised powder of each bone cement composite and the liquid monomer were measured and hand mixed at (2 g/ml) ratio for 45 s and poured in sterile rubber mould and left to harden in air at normal room conditions (23±1°C and relative humidity 50±5 %). Once the bone cement reached the doughing time the dough was transferred into sterile, closed glass extraction vessels with caps. The extract solution was prepared by incubation of the resulted disc samples in PromoCell® growth medium at a defined concentration of 0.2 g/ml for (72±2 h) at (37±1°C). The disc-shaped samples were further used for SEM and confocal microscopy evaluations.

The individual components of the aforementioned bone cement composites were also analysed via indirect contact testing to examine their individual influence of either viability or cytotoxicity. These components include the Simplex-P® cement powder; the MWCNTs powder and also the liquid monomer (10 % v.v). All sample preparation was conducted under a biological fume hood in a sterile lab environment.

### 3.6.2 In vitro Test Materials

- Normal Human Osteoblast Cells (HOB-c) (cat.# C-12720) were grown in Complete Osteoblast Growth Medium (cat.# C-27001) which consisted of one bottle of Basal Medium (cat.# C-27001B) and one vial of SupplementMix (cat.# C-39615) from (PromoCell GmbH, Heidelberg, Germany). PromoCell® Human Osteoblasts are mononuclear cells of mesenchymal origin, isolated from normal femoral bone tissue from the knee and the hip joint region. Shortly after isolation, HOB-c cells were cryopreserved at passage two (P2) using HOB-c cell freezing medium. Each cryo vial contains more than 500,000 viable cells after thawing. Proliferating cell cultures are made from 500,000 cryopreserved cells that have been thawed and cultured for three days and tested positive to alkaline phosphatase, and osteocalcin, at PromoCell’s cell culture facility.
Cell culture plastic ware was purchased from Fisher (*Fisher BioReagents®, Dublin, Ireland*). All other chemicals were of the highest purity available from standard laboratory suppliers, and were generously provided by Centre for Applied Biomedical Engineering Research (CABER) (*University of Limerick, Limerick, Ireland*).

### 3.6.3 Culture of Osteoblast Cells

Human osteoblastsic cell monolayers were routinely grown in culture in complete supplemented PromoCell® growth medium at 37°C in a humidified atmosphere of 5% CO₂ in air. Cells were subcultured once they have reached ≈ 90% confluency, using Trypsin, according to the subcultivation protocol (Appendix II). Medium was completely renewed and confluence was observed every two days. For all experiments, human osteoblasts were used at the fifth passage (P5).

### 3.6.4 Seeding of Osteoblasts on Samples

When osteoblastsic cell monolayers (P5) reached confluency (yielding ≈ 2x10⁶ cells/ml), the cells were enzymatically lifted from the flask using trypsin. The cells were concentrated by centrifugation and resuspended in fresh complete media containing supplement. Cells were counted using a hemocytometer grid with Trypan blue staining. Aliquots of cell suspension at density of 5000 cells/well (2500 cells/cm²) were seeded directly onto the bone cement sample surfaces, and onto polystyrene surface of a 24-well plate that served as a positive control, with three replicates. The negative control consisted of growth medium without cells onto the polystyrene surface. The control and test samples were cultured in an incubator for predefined periods of time (1, 3, and 7 days) at 37°C in a humidified atmosphere of 5% CO₂ in air, to allow the cells to attach to the samples and grow.

### 3.6.5 Cell Viability

The MTS-assay was used to investigate the adherence and viability of osteoblast HOB-c cells that were directly seeded on various bone cements samples, and indirectly tested for the cytotoxicity of bone cement' leaching. Viability of the cells adherent on bone cement surface was also evaluated using a live/dead fluorescent staining assay using Laser Scanning Confocal Microscopy (LSCM).
3.6.5.1 MTS-assay

Cell adherence and viability were analysed after 1, 3, and 7 days, using MTS-assay (CellTiter 96® AQueous Non-Radioactive Cell Proliferation Assay, Cat.# G-5421, Promega, Hampshire, UK). This test is a colorimetric method for determining the cells proliferation. The concept is based on the enzymatic reduction of a tetrazolium salt. The conversion of methyl thiazol sulphate (MTS) into aqueous soluble formazan is accomplished by dehydrogenase enzymes found in metabolically active cells and hence the intensity of the colour observed is proportional to the number of living cells in culture and can be measured at a wavelength of 540 nm.

Briefly, human osteoblast HOB-c cells were cultured either onto the various bone cement discs or in the absence of material (for control tests) in 24 multi-well plates at a density of 5000 cells/well (2500 cells/cm²). After indicated times, spent culture media was removed, cells were washed, and 100 μl of MTS solution was pipetted into each well of the 24-well plates containing 0.5 ml fresh culture medium. The plate was incubated for 3 h at 37°C in a humidified atmosphere of 5% CO₂ in air, according to the manufacturer’s instructions. 100 μl of the cells/MTS suspension was then aliquoted into 96-well plates with three replicates. For indirect contact MTS-assay test, cells were incubated at a density of 5000 cells/well (15625 cells/cm²) into 96-well culture plates with 100 μl of culture medium at 37°C in a humidified atmosphere of 5% CO₂ in air for 24 h. The medium was removed and replaced by 100 μl of prepared dilutions of the leaching solution, and the plate was incubated for 24 h. The spent culture media was removed and 100 μl of MTS/fresh culture medium solution was pipetted into each well of the 96-well plates with three replicates. Another 100 μl of culture medium was added for the negative control and the 96-well plates were placed in the incubator at 37°C in a humidified atmosphere of 5% CO₂ in air for 3 h.

Finally, the absorbance of formazan salt was determined at 540 nm in a plate reader (TriStar Multimode Microplate Reader, Model LB491, Berthold Technologies GmbH, Bad Wildbad, Germany). Cell number was determined from standard curve of a six point serial dilution obtained using cells from the same original culture. Again replicates of three were employed. The amount of attached cells was calculated.

Results were expressed as relative MTS activity as compared to control conditions (cells cultured in the absence of materials). The MTS-assay cell viability and adhesion results are shown in sections (4.3.1) & (4.3.2).
3.6.5.2 Laser Scanning Confocal Microscopy

The cell cultures were imaged by using Carl Zeiss Laser Scanning Confocal Microscope, with Zen 2008 Software, (Model LSM 710; Carl Zeiss, Germany) based on inverted optical microscope AxioObserver II. In-house microscopic glass chamber were specifically manufactured to hold samples and obtain images without compression or addition/disruption of the sample surface and structure, figure (3-10).

![Figure 3-10: Laser Scanning Confocal Microscope.](image)

Cellular viability was assessed using calcein and ethidium homodimer Live/Dead® Viability/Cytotoxicity Kit (Invitrogen, Paisley, Scotland). The assay kit provides a two-colour fluorescence cell viability assay that is based on the simultaneous determination of live and dead cells with two probes that measure recognised parameters of cell viability. Calcein AM and ethidium homodimer (EthD-1) are optimal dyes for this application. The kit is suitable for use with fluorescence microscopes. The assay principles are general and applicable to most eukaryotic cell types, including adherent cells. It is faster, less expensive, safer and a more sensitive indicator of cytotoxic events than alternative methods. Live cells are distinguished by the presence of ubiquitous intracellular esterase activity, determined by the enzymatic conversion of the virtually nonfluorescent cell-permeant to the intensely fluorescent calcein. The polyanionic dye calcein is well retained within live cells, producing an intense uniform green fluorescence in live cells (~495 nm/~515 nm). EthD-1 enters cells with damaged membranes and undergoes a 40-fold enhancement of fluorescence upon binding to nucleic acids, thereby producing a bright red fluorescence in
dead cells. EthD-1 is excluded by the intact plasma membrane of live cells. Determination of cell viability depends on the physical and biochemical properties of cells.

Attachments to the biomaterials was visualised by cell nuclei staining (DAPI Vector). Excitation of the fluorophores were performed using three lasers with spectral properties are optimal and recommended for maximum recovery of the fluorescent signal of applied fluorophores. Spectra detection system (Meta 710, Carl Zeiss) were used in order to detect emission signal produced by fluorophores. Optical sectioning (5 mkm) were performed in order to localise structural components through whole depth of the samples. Nuclei are shown blue by excitation of the DAPI. Samples for Laser Scanning Confocal Microscopy (LSCM) were prepared as described in a previous section (3.6.1). After culturing HOB-c Cells (2500 cells/cm²) for 3 h on various bone cement specimens in 24-well plates, cells were fixed, the nuclei were stained using Live/Dead® Viability/Cytotoxicity Kit, according to the manufacturer’s instructions, LSCM was applied to observe cell morphology and the cytoskeletal arrangement. The live-dead LSCM images are shown in section (4.3.2).

3.6.6 Real-Time Cell Monitoring

The xCELLigence system monitors cellular processes events such as cell adhesion, viability, proliferation, invasion, migration, and cytotoxicity in real time using electronic cell sensor array technology. The xCELLigence system measures a relative change in electrical impedance across interdigitated micro-electrodes integrated on the bottom of tissue culture E-Plates. The impedance change is derived to represent cell biological status in a number of cell-based assays, and termed Cell Index (CI) [226].

The RTCA DP Instrument, figure (3-11), is composed of three main components: RTCA DP Analyser; RTCA Control Unit; and E-Plate16.

The RTCA DP analyser has three integrated stations for E-Plates16. It is located inside a tissue culture incubator. Each of the three 16-well plate holders can be used independently under the RTCA Software. The RTCA DP analyser is located inside a tissue culture incubator and can automatically select wells for measurement and continuously transfer measured impedance data to a computer [226]. Cell Index values are continuously displayed on the software user interface. xCELLigence is a trademark of Roche and E-Plate16 is registered trademarks of ACEA Biosciences, Inc. in the USA.
The electrical impedance depends on the cells presence on top of the electrodes, which affect the local ionic environment at the cell suspension/electrode interface, and the quality of the cell interaction with the electrodes surface. The more cells are attached or spread on the electrodes, the larger the increases in electrode impedance. Therefore, change in the biological status of the cells, including cell number, viability, and morphology will lead to a change in electrode impedance, which is displayed as cell index (CI) values, figure (3-12).
The xCELLigence system can be used to dynamically and quantitatively to monitor cellular quality control in terms of adhesion and proliferation. Primary cells display very distinct attachment and growth kinetics depending on their inherent attachment quality, morphology and rate of cell division; figure (3-13).

E-Plate16 is a single use, disposable device used for performing cell-based assays on the xCELLigence RTCA DP Instrument. In order for cells inside each well to be monitored and assayed, each individual well on an E-Plate contains an interdigitated gold electrode with a "string of pearls" shape covers ~80% of the bottom area of the well, figure (3-14). The plate lid is designed to ensure low evaporation.
Briefly, cell line characterisation/profiling and determination of the growth curve of the HOB-c osteoblasts cell line over time are important and necessary when starting working with the xCELLigence system. Therefore, four HOB-c cells densities of 750, 1500, 3000 and 6000 cells/well were cultured in complete media for 100 h and were fed after 20 h, in order to get enough information to be able to choose the right time point for stimulation or challenge (addition of compounds/cells) and the appropriate number of cells to be seeded.

Also, cells of a density of 3000 cells/well were cultured in complete osteoblasts media and challenged after 3 h with different extract solution concentration (20, 30, 40%v.v) of Simplex bone cement control and SUNF5 samples by diluting the extract solution in the osteoblasts growth media to study the effect of MWCNTs contents on the cell viability and growth. Dynamic cell viability was monitored in 15 min intervals from the time of plating until the end of the experiments. Cell Index values for all cell lines were calculated and plotted on the graph. Standard deviation of duplicates of wells for corresponding cell lines with different cell numbers were analysed with the RTCA Software. The xCELLigence RTCA DP instrument and E-Plates16 were generously provided by Roche Ireland (Cork, Ireland). The real time dynamic monitoring results are shown in section (4.3.1).

### 3.6.7 Morphological Characterisation

Samples for scanning electron microscopy (SEM) were prepared as described in section (3.6.1). HOB-c osteoblastic cells were seeded onto various bone cement samples in 24-well plates at a final density of 5000 cells/well. After 3 h and 24 h culture period, media were removed and specimens were placed in 0.3% paraformaldehyde; 1% formamide in cacodylate buffer (0.1M) in PIPES [piperazine-N,N-bis (2-ethanesulphonic acid)] buffer
(100 mM, pH 7.4). The samples were then fixed with 4% (w/v) aqueous Osmium tetroxide (OsO₄) in cacodylate buffer (0.1M) in order to preserve and maintain the original structure without any loss structural components in future staining and analysis steps. Samples were then dehydrated stepwise in a graded series of ethanol solutions (0%, 20%, 30%, 40%, 50%, 60%, 80%, 95%, and finally, 100%) for 15 min in each step. Test samples were subsequently air-dried in the fume hood environment, placed onto copper stumps, and sputter-coated with gold to a depth of ~80 nm. After coating with gold, the microstructure was observed in precise details by a (Hitachi, SU-70, Hitachi, Japan) scanning electron microscope (SEM). The scanning electron micrographs are shown in section (4.3.2).
4 Results
4.1 Mechanical Studies

4.1.1 Flexural Properties

The typical force-displacement curves determined with the use of the ISO5833:02 uniaxial four-point bend test, ASTM C 1499-09 biaxial ring-on-ring test, and biaxial ball-on-three-ball (B3B) test methods of all cements tested are presented in figure (4-1).

![Figure 4-1: Load-displacement curves of various bone cement composites when tested under (a) 4-point bend, (b) ring-on-ring, and (c) B3B biaxial test methods.](image-url)
The failure behaviour of all bone cement composites under the three different flexural tests can be described as classically brittle as the load-deflection curves showed liner relation between load and displacement, although this relation in 4-point bending test were not as linear as those obtained in the biaxial tests, suggesting some degree of yielding before the brittle failure. The resistance to flexural loading, biaxial modulus ($E_{\text{biaxial}}$) determined by the biaxial flexural test was obtained by calculating the centre deflection function ($\beta_c$), Eq.(3-2), the measured slope of the force-displacement curve ($\frac{dF}{d\omega_c}$), and the recorded fracture force ($F$) using Eq.(3-3). The flexural or bending modulus ($E_{\text{bend}}$), obtained with the use of the four-point bend test was calculated using the recorded fracture load ($F$) in conjunction with Eq.(3-10). The resulting moduli are summarised in table (4-1) as mean modulus±standard deviations.

Table 4-1: Biaxial and Bending modulus of all bone cement composites.

<table>
<thead>
<tr>
<th>CNTs Content wt.%</th>
<th>Modulus $E$ (GPa)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>biaxial</td>
</tr>
<tr>
<td>Control</td>
<td>0.99±0.09</td>
</tr>
<tr>
<td>SUNF1</td>
<td>1.14±0.08</td>
</tr>
<tr>
<td>SUNF2</td>
<td>1.03±0.09</td>
</tr>
<tr>
<td>SUNF5</td>
<td>0.91±0.08</td>
</tr>
<tr>
<td>SOH1</td>
<td>0.98±0.08</td>
</tr>
<tr>
<td>SCOOH1</td>
<td>1.10±0.12</td>
</tr>
</tbody>
</table>

![Figure 4-2: Biaxial and Bending modulus of bone cement composites.](image-url)
Chapter 4  
Results

The tests results, as shown in figure (4-2), demonstrated that the SUNF1 bone cement composite was optimal for enhancing the modulus of bone cement in both tests.

This loading produced enhancement of >15% in biaxial and ≈20% in bending modulus compared to the control bone cement. Incorporating 0.2 wt.% (SUNF2 bone cement composite) did not have a significant effect on the bone cement biaxial (≈4%) and bending (<2%) modulus compare to the control bone cement. However, reductions in both moduli (≈8%) were also observed in SUNF5 bone cement composite. Alternatively, the incorporation of 0.1 wt.% of functionalised MWCNTs showed different effect on the bone cements moduli depending on the functional groups used, the hydroxyl (-OH) groups modified SOH1 bone cement composite showed a negative effect when a small reductions (≈1% and 2%) were observed, while the carboxyl (-COOH) groups used in SCOOH1 bone cement composite showed an improvement of ≈11% and 6% compare to the control bone cement in the flexural and bending modulus respectively.

The biaxial and bending tests were conducted at different strain rates of 0.5 and 5.0 mm/min, respectively. When comparing the results determined by the biaxial test with the results obtained from uniaxial four-point bending test, considering non-equivalent strain rates, a difference between the two tests values was found, and the mean values were the same among the six bone cement composites. The biaxial modulus was considerably (≈65%) lower than the modulus presented for bending test for all bone cement composites.

To compute the correct biaxial strength (σ_{biaxial}) with the use of (B3B) biaxial flexural test, three most common equivalent contact radii were used; i.e (1st \( r_0^* \)) Eq.(3-5), (2nd \( r_0^* \)) Eq.(3-6), and (3rd \( r_0^* \)) Eq.(3-7). The modulus obtained from Eq.(3-3) was used to calculate (3rd \( r_0^* \)). The recorded fracture load from the biaxial testing (\( F \)) was used in conjunction with the calculated equivalent radii (\( r_0^* \)) and the Eq.(3-4) to give the biaxial flexural strength (σ_{biaxial}).

The mean strengths ± standard deviations and Weibull parameters for the six acrylic bone cement nanocomposites under investigation are summarised and tabulated in table (4-2).

The mean biaxial flexural strengths and the Weibull characteristics yielded using different radii, which predict different contact radii leading to different results for the tensile stress distribution around the centre of the disc, and were in the order of (1st \( r_0^* \)) < (3rd \( r_0^* \)) < (2nd \( r_0^* \)). The ratio, however (1st \( r_0^* \)) : (3rd \( r_0^* \)) : (2nd \( r_0^* \)) of 1.636 : 1.004 : 1 remained relatively unchanged.
Table 4-2: The mean biaxial flexural strength and Weibull analytical results for all bone cement composites.

<table>
<thead>
<tr>
<th></th>
<th>Mean Flexural Strength ($\sigma_{\text{biaxial}}$) (MPa)</th>
<th>Characteristic Strength ($\sigma$) (MPa)</th>
<th>Weibull Modulus ($m$)</th>
<th>95% Confidence intervals (%)</th>
<th>95% Reliability Level (MPa)</th>
<th>$R^2$-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Control</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1st $r_0^*$</td>
<td>60.0 ± 2.7</td>
<td>61.3</td>
<td>24.1</td>
<td>19.3 – 28.9</td>
<td>64.2</td>
<td>0.93</td>
</tr>
<tr>
<td>2nd $r_0^*$</td>
<td>97.9 ± 4.2</td>
<td>99.8</td>
<td>25.5</td>
<td>21.9 – 29.1</td>
<td>104.2</td>
<td>0.96</td>
</tr>
<tr>
<td>3rd $r_0^*$</td>
<td>97.4 ± 4.5</td>
<td>99.5</td>
<td>23.6</td>
<td>20.7 – 26.6</td>
<td>104.2</td>
<td>0.97</td>
</tr>
<tr>
<td><strong>SUNF1</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1st $r_0^*$</td>
<td>65.6 ± 3.1</td>
<td>67.0</td>
<td>22.8</td>
<td>17.4 – 28.2</td>
<td>70.3</td>
<td>0.90</td>
</tr>
<tr>
<td>2nd $r_0^*$</td>
<td>107.4 ± 5.1</td>
<td>109.9</td>
<td>22.7</td>
<td>17.5 – 27.9</td>
<td>115.3</td>
<td>0.90</td>
</tr>
<tr>
<td>3rd $r_0^*$</td>
<td>107.2 ± 5.1</td>
<td>109.6</td>
<td>23.1</td>
<td>18.6 – 27.5</td>
<td>114.9</td>
<td>0.93</td>
</tr>
<tr>
<td><strong>SUNF2</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1st $r_0^*$</td>
<td>59.1 ± 3.8</td>
<td>60.9</td>
<td>16.7</td>
<td>13.1 – 20.2</td>
<td>65.1</td>
<td>0.92</td>
</tr>
<tr>
<td>2nd $r_0^*$</td>
<td>96.9 ± 6.6</td>
<td>100.0</td>
<td>16.0</td>
<td>13.1 – 19.0</td>
<td>107.0</td>
<td>0.94</td>
</tr>
<tr>
<td>3rd $r_0^*$</td>
<td>96.8 ± 6.3</td>
<td>99.7</td>
<td>16.8</td>
<td>14.3 – 19.3</td>
<td>106.5</td>
<td>0.96</td>
</tr>
<tr>
<td><strong>SUNF5</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1st $r_0^*$</td>
<td>55.7 ± 3.5</td>
<td>57.3</td>
<td>17.5</td>
<td>15.5 – 19.5</td>
<td>61.0</td>
<td>0.97</td>
</tr>
<tr>
<td>2nd $r_0^*$</td>
<td>90.8 ± 5.7</td>
<td>93.4</td>
<td>17.4</td>
<td>15.3 – 19.5</td>
<td>99.5</td>
<td>0.97</td>
</tr>
<tr>
<td>3rd $r_0^*$</td>
<td>90.2 ± 5.3</td>
<td>92.6</td>
<td>18.9</td>
<td>16.8 – 20.9</td>
<td>98.1</td>
<td>0.98</td>
</tr>
<tr>
<td><strong>SOH1</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1st $r_0^*$</td>
<td>59.9 ± 2.6</td>
<td>61.1</td>
<td>25.3</td>
<td>22.4 – 28.3</td>
<td>65.1</td>
<td>0.97</td>
</tr>
<tr>
<td>2nd $r_0^*$</td>
<td>98.0 ± 4.1</td>
<td>99.9</td>
<td>26.4</td>
<td>22.9 – 30.0</td>
<td>104.2</td>
<td>0.97</td>
</tr>
<tr>
<td>3rd $r_0^*$</td>
<td>97.4 ± 3.6</td>
<td>99.0</td>
<td>29.6</td>
<td>26.5 – 32.7</td>
<td>102.8</td>
<td>0.98</td>
</tr>
<tr>
<td><strong>SCOOH1</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1st $r_0^*$</td>
<td>64.2 ± 4.4</td>
<td>66.2</td>
<td>16.0</td>
<td>12.3 – 19.6</td>
<td>70.9</td>
<td>0.90</td>
</tr>
<tr>
<td>2nd $r_0^*$</td>
<td>105.3 ± 7.2</td>
<td>108.7</td>
<td>16.0</td>
<td>12.3 – 19.6</td>
<td>116.4</td>
<td>0.90</td>
</tr>
<tr>
<td>3rd $r_0^*$</td>
<td>105.2 ± 6.7</td>
<td>108.3</td>
<td>17.1</td>
<td>13.7 – 20.5</td>
<td>115.5</td>
<td>0.93</td>
</tr>
</tbody>
</table>
Comparing these three sets of data obtained using different radii of contact areas for the six cement composites showed that the differences were significant between cement treatments and there is significant effect of contact radius ($r_0^*$) on the resulting values. However, there is no interaction between cement reinforcement loadings and modifications and the contact radius used. In other words, the response to contact radius does not depend on the cement reinforcement, or vice-versa.

![Figure 4-3: The mean flexural strength obtained with different equivalent contact radii.](image)

The tests results, as shown in figure (4-3), demonstrated that the SUNF1 bone cement composite was optimal for enhancing the biaxial flexural strength ($\sigma_{\text{biaxial}}$) of bone cement. This loading produced $\approx 10\%$ enhancement in biaxial flexural strength than the control bone cement. However, a reduction in the biaxial flexural strength of ($\approx 1.5\%$) and ($\approx 7\%$) was observed in SUNF2 and SUNF5 bone cement composites, respectively. Alternatively, the chemical functionality showed different effects on the bone cements flexural strengths, depending on the functional group used, the hydroxyl (-OH) groups in SOH1 bone cement composite did not substantially change the cement biaxial flexural strength, while the carboxyl (-COOH) groups in SCOOG1 bone cement composite showed an improvement of $\approx 8\%$ compare to the control bone cement.

Figure (4-4) shows Weibull modulus between cements determined with the use of the (B3B) test method using ($3^{rd} r_0^*$) contact radius approximations.
The gradients, that is, the Weibull \( (m) \) moduli are to some extent close and the highest value was for SOH1 bone cement composite \((m=29.6)\) and the lowest was for SUNF2 composite \((m=16.8)\). Addition of 0.1 wt.% unfunctionalised MWCNTs shows no effect on the Weibull modulus compare to the control bone cement, while 0.2 and 0.5 wt.% showed small negative effect. The functionalisation with carboxyl \((-\text{COOH})\) groups showed an improvement of \(\approx 26\%\) while the hydroxyl \((-\text{OH})\) groups substantially reduce the Weibull modulus by 27\% compared to the control cement. However, the Weibull moduli within all bone cement composites are similar for the three contact radii (table 4-2 and figure 4-5).
The characteristic strength results, as shown in figure (4-6), demonstrated that the SUNF1 and SCOOH1 cement composites produced $\approx 10\%$ and $9\%$ improvement in the characteristic biaxial strength compared to the control bone cement. However, while SUNF2 and SOH1 bone cement composites had no effects, SUNF5 bone cement composites had a negative effect by reducing the characteristic strength by $\approx 6\%$ compare to the control bone cement.

Figure 4-6: The characteristic flexural strength acquired with different equivalent contact radii.

Another way to look at this data is by using a box and whisker plot diagram. It is a way of graphically depicting groups of numerical data considering the median of a data set (the middle number when the set is sorted in numerical order) rather than the arithmetic mean, and the spacings between the different parts of the box (quartiles) help indicate the degree of dispersion and skewness in the data. Therefore, the distributions of each level of biaxial flexural strengths among all bone cement composites under investigation in terms of MWCNTs loadings and chemical functionality effects are shown in figure (4-7).

The results show that the SUNF2 and SOH1 composites are close to the control bone cement and had no effect on its properties. Also, SUNF1 and SCOOH1 composites are to some extent close, producing a positive enhancement in the biaxial strength in terms of strength values and distributions. Based on this analysis, if we were aiming for a biaxial strength of around 105 MPa, the 0.1 wt.% unfunctionalised (SUNF1 composite) might be more cost effective than the 0.1 wt.% (-COOH) functionalised (SCOOH1 composite).
Figure 4-7: The variation and central tendency of biaxial flexural strength distributions in terms of filler loadings and chemical functionality for all bone cement composites.

The best way to compare the reliability of all bone cement composites is perhaps by using a survival graph. This line graph depicts the survival probabilities of each bone cement composite at various biaxial flexural strengths. The resulting survival graph looks like figure (4-8), which allows a comprehensive comparison of the MWCNTs loadings and functionalisation effect of all bone cements’ survival rates.

Figure 4-8: The effect of MWCNTs loadings and chemical functionality on the survival rates.
In figure (4-8), at flexural strength of 95 MPa, about 96% of SUNF1 bone cement has survived, whereas only about 70% of control bone cement, 65% of SUNF2 bone cement composite and 20% of SUNF5 bone cement composite have survived. Therefore, for the stated reliability goal of \( R(95) \geq 0.95 \), SUNF1 bone cement composite is clearly superior. Also, about 86% of SUNF1 bone cement composite survives to 100 MPa strength whereas only about 32%, 36% and less than 2% of control, SUNF2, and SUNF5 bone cement composites had survived, respectively. On the other hand, about 90% of SCOOPH1 bone cement composite and 76% of SOH1 bone cement composite have survived at flexural strength of 95 MPa. However, about 70% of SUNF1 bone cement composite survives to 105 MPa strength whereas only about 55% of SCOOPH1 composite and less than 3% of the control bone cement had survived. At very high biaxial strength, only 6% of SCOOPH1 bone cement and 4% of SUNF1 bone cement composites would survive.

The survival probability distributions were also plotted against the ranked biaxial flexural strength data obtained by using the \( (3^{rd} r_0^*) \) approximations in ascending order for each bone cement composite, in an attempt to identify possible differences in the underlying failure mechanisms, as shown in figure (4-9).

The survival probability distributions illustrated that flexure strength data showed different scattering or uniform defect distribution at different stress levels; figure (4-9a). This was further emphasised in the survival probability distributions for the flexure strength data for the control and SOH1 bone cement composites; figure (4-9b), which appeared to exhibit an asymmetry at higher stress levels compared with the lower levels, although they have identical \( R^2 \)-value, table (4-2). SUNF1 and SCOOPH1 bone cement composites had the same \( R^2 \)-values and showed different symmetry at higher and lower stress levels.

The flexural strength determined with the use of the four-point bend test, ring-on-ring test, and (B3B) (using \( 3^{rd} r_0^* \)) test methods are tabulated in table (4-3).
Figure 4-9: Survival distributions from the ranked biaxial flexural strength data.

Table 4-3: Means bending and biaxial strengths results.

<table>
<thead>
<tr>
<th>CNTs Content wt.%</th>
<th>Bending</th>
<th>Equibiaxil</th>
<th>Biaxial</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control 0.0</td>
<td>58.0 ± 9.1</td>
<td>97.7 ± 11.8</td>
<td>97.4 ± 4.5</td>
</tr>
<tr>
<td>SUNF1 0.1</td>
<td>58.0 ± 11.8</td>
<td>99.6 ± 13.0</td>
<td>107.2 ± 5.1</td>
</tr>
<tr>
<td>SUNF2 0.2</td>
<td>50.0 ± 2.7</td>
<td>97.2 ± 17.0</td>
<td>96.8 ± 6.3</td>
</tr>
<tr>
<td>SUNF5 0.5</td>
<td>53.2 ± 3.8</td>
<td>84.8 ± 12.8</td>
<td>90.2 ± 5.3</td>
</tr>
<tr>
<td>SOH1 0.1(-OH)</td>
<td>54.2 ± 3.0</td>
<td>86.5 ± 10.4</td>
<td>97.4 ± 3.6</td>
</tr>
<tr>
<td>SCOOH1 0.1(-COOH)</td>
<td>55.1 ± 5.0</td>
<td>87.3 ± 11.0</td>
<td>105.2 ± 6.7</td>
</tr>
</tbody>
</table>
Chapter 4  Results

The (B3B), ring-on-ring, and four-point tests were conducted at different strain rates, 0.5, 0.5 and 5.0 mm/min, respectively. Considering non-equivalent strain rates, the resulting values showed that there are differences among these three sets of results determined by three different tests. However, the results for the two biaxial tests conducted at the same strain rate, also showed no significant difference between the test methods or among the bone cement composites. Considering non-equivalent strain rates, comparing the bending and biaxial results showed significant difference between the two tests.

The mean flexural strengths yielded using three different tests were in different magnitude and were in order of \( \sigma_{\text{biaxial}} > \sigma_{\text{equibiaxial}} > \sigma_{\text{bend}} \). The ratio, however, remained relatively unchanged; figure (4-10).

![Mean flexural strength](image)

Figure 4-10: Bending, equibiaxial and biaxial flexural strengths.

The locations of the fracture origin were examined to prove whether the fracture origin occurs in the theoretical predicted area of maximum stress, which is in the centre of the tensile loaded surface of the disc. In all fractured discs, figure (4-11), failure with a threefold symmetry dominates was observed and the fracture initiated in the tensile surface plane underneath the loading ball.
In no case, did the fracture start from the area around the loading ball were tensile stresses occurred. The fracture origin was at or very near to the tensile surface in all investigated cases. Also, the fracture origin showed no significant defects were the initiations sites for fracture.

### 4.1.2 Finite Element Analysis

Assembly of a finite element -model of an acrylic bone cement disc deforming under flexural load using the (B3B) biaxial test assembly is shown in figure (4-12).
Assuming the midpoint of the support balls are fixed and the midpoint of the centred loading ball is only allowed to move vertically; the radii of the supporting balls and the loading ball are chosen to be equal ($R_b=2$ mm). The disc specimen was 1.8 mm in thickness ($t=1.8$); 15 mm diameter ($R=7.5$ mm), and was supported centrally by three stainless-steel ball bearings positioned $120\degree$ apart from its centre on a support circle of 11.5 mm diameter ($R_a=5.75$ mm). The displacement distribution in a disc for a typical loading condition (190 N) in a (B3B) test using the finite element analysis model is shown in figure (4-13).

Figure 4-13: The displacement magnitude and distribution in a disc for a typical loading condition in a (B3B) test; (a) top, (b) top excluding ball, and (c) bottom overviews.
The disc central deformation determined by the finite element analysis method was 0.4714 mm for a cement modulus of 2.5 GPa and failure load of 190 N, while the experimentally determined centre deflection measured from the load-displacement curve and its gradient was 0.48±0.02 mm. By substituting the FE-disc central deformation into Eq.(3-1), the analytical biaxial modulus of the cement was 1.37 GPa. However, the experimentally obtained biaxial modulus of the Simplex-P® bone cement (control) was about 1.00 GPa. The deviation in the modulus and the centre deflection as determined with this method were 45% for a modulus and 6% for the centre deflection compared to the analytical results obtained using Eq.(3-1) and Eq.(3-2), respectively.

Using the finite element solver, table (4-4) shows the parametric study results for moduli determined from Eq.(3-1), correspond with different disc thickness ($t$), loading ball diameter ($R_b$), and loading force ($F$) input into the finite element solver.

Table 4-4: Relationship between FE load-displacement gradient ($\frac{dF}{d\omega_c}$), deflection function ($\beta_c$), loading force ($F$), loading ball diameter ($R_b$), and sample thickness ($t$) for a FE modulus of 2.5 GPa.

<table>
<thead>
<tr>
<th>$t$ (mm)</th>
<th>$\beta_c$</th>
<th>$R_a$ (mm)</th>
<th>$R_b$ (mm)</th>
<th>Load (N)</th>
<th>$\omega_c$</th>
<th>$\frac{dF}{d\omega_c}$</th>
<th>$E_{calc}$ (GPa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.70</td>
<td>0.5753057</td>
<td>5.75</td>
<td>2.0</td>
<td>190</td>
<td>0.49536</td>
<td>383.563</td>
<td>1.48</td>
</tr>
<tr>
<td>1.75</td>
<td>0.5753057</td>
<td>5.75</td>
<td>2.0</td>
<td>190</td>
<td>0.47142</td>
<td>403.038</td>
<td>1.43</td>
</tr>
<tr>
<td>1.80</td>
<td>0.5753057</td>
<td>5.75</td>
<td>2.0</td>
<td>190</td>
<td>0.45125</td>
<td>421.053</td>
<td>1.37</td>
</tr>
<tr>
<td>1.83</td>
<td>0.5753057</td>
<td>5.75</td>
<td>2.0</td>
<td>190</td>
<td>0.44020</td>
<td>431.619</td>
<td>1.34</td>
</tr>
<tr>
<td>1.85</td>
<td>0.5753057</td>
<td>5.75</td>
<td>2.0</td>
<td>190</td>
<td>0.43208</td>
<td>439.733</td>
<td>1.32</td>
</tr>
<tr>
<td>1.88</td>
<td>0.5753057</td>
<td>5.75</td>
<td>2.0</td>
<td>190</td>
<td>0.42121</td>
<td>451.081</td>
<td>1.29</td>
</tr>
<tr>
<td>1.92</td>
<td>0.5753057</td>
<td>5.75</td>
<td>2.0</td>
<td>190</td>
<td>0.40914</td>
<td>464.393</td>
<td>1.25</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Deviation in modulus 46%</td>
<td>AVG 1.355</td>
</tr>
<tr>
<td>1.80</td>
<td>0.5753057</td>
<td>5.75</td>
<td>1.5</td>
<td>190</td>
<td>0.46053</td>
<td>412.565</td>
<td>1.35</td>
</tr>
<tr>
<td>1.80</td>
<td>0.5753057</td>
<td>5.75</td>
<td>2.0</td>
<td>190</td>
<td>0.45125</td>
<td>421.053</td>
<td>1.37</td>
</tr>
<tr>
<td>1.80</td>
<td>0.5753057</td>
<td>5.75</td>
<td>2.5</td>
<td>190</td>
<td>0.44250</td>
<td>429.379</td>
<td>1.40</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Deviation in modulus 45%</td>
<td>AVG 1.373</td>
</tr>
<tr>
<td>1.80</td>
<td>0.5753057</td>
<td>5.75</td>
<td>2.0</td>
<td>170</td>
<td>0.40794</td>
<td>416.729</td>
<td>1.36</td>
</tr>
<tr>
<td>1.80</td>
<td>0.5753057</td>
<td>5.75</td>
<td>2.0</td>
<td>180</td>
<td>0.42964</td>
<td>418.959</td>
<td>1.37</td>
</tr>
<tr>
<td>1.80</td>
<td>0.5753057</td>
<td>5.75</td>
<td>2.0</td>
<td>190</td>
<td>0.45125</td>
<td>421.053</td>
<td>1.37</td>
</tr>
<tr>
<td>1.80</td>
<td>0.5753057</td>
<td>5.75</td>
<td>2.0</td>
<td>210</td>
<td>0.49449</td>
<td>424.683</td>
<td>1.39</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Deviation in modulus 45%</td>
<td>AVG 1.370</td>
</tr>
</tbody>
</table>
The finite element analysis, table (4-4), demonstrates a constant deviation in the biaxial modulus values of (≈45%) for the range of parameters investigated.

Example of a stress field distribution in a disc for a typical loading condition (190 N and 4 mm balls diameter) in a ball-on-three-ball test at the tensile side is shown in figure (4-14).

Figure 4-14: Stress field in a disc for a typical loading condition in a (B3B) test; (a) top, (b) top excluding ball, and (c) bottom views of the surfaces of the disc.
Chapter 4

It is assumed that friction effects only slightly change the stress distribution and is therefore not taken into account in this study. These finite element analysis colour-coded overview pictures of the maximum principal stress distribution have demonstrated some of the main features of this test:

- The region of greatest tensile stress is located directly underneath the load point, on the tensile surface (bottom surface) of the disc specimen (red area).
- The stress field shows a threefold symmetry, caused by the three supporting balls.
- The maximum tensile stress region is small.
- The maximum tensile stress gradient is small and as such only a small volume of the material is experiencing large stress.
- Close to the upper loading point the compressive stresses are very large (blue areas)
- Large compressive stresses arise in the contact area between support balls and the disc.

These results suggest that the maximum tensile stress component occurs in the centre of the surface plane of the disc underneath the loading ball, where the stress state is purely biaxial and that the stress state scales with the maximum tensile stress component in the region around this maximum.

Using the finite element solver, figure (4-15) shows the effects of loading force \( F \), disc thickness \( t \), and loading ball diameter \( r_b \) on the centre deflection of a disc specimen, the contact area radius and thus the biaxial flexural strength within the range of parameters investigated. The thickness was normalised with the disc radius value.

As expected, it can clearly be seen, that – in the parameter range investigated – for a given combination of geometrical parameters the biaxial strength increases with the applied force, for all the analytical approximations and FEA results; figure (4-15a). Surprisingly, the loading force has no effects on the contact radius as it remains the same but differs in the radius magnitude for different approximations except a very small gradient for \( (3^{rd} r_0) \) and FEA results; figure (4-15b).

Centre deflection or disc deformation was proportional to the applied force in both analytical and FEA results; figure (4-15c). The biaxial strength was also inversely proportional to the disc thickness; figure (4-15d), and it can clearly be seen, that the approximations using
(2\textsuperscript{nd} $r_0^*$) and (3\textsuperscript{rd} $r_0^*$) almost match and show a slight deviation at larger thicknesses of specimen in predicting the contact radius. However, all results have the same trends.

However, for the influence of the loading ball radius, only the analytical results obtained from (1\textsuperscript{st} $r_0^*$) approximation are highly influence by the loading ball radius; figure (4-15g,h). Also, FEA result show a small dependency of contact area radius on the loading ball radius. Again, the approximations using (2\textsuperscript{nd} $r_0^*$) and (3\textsuperscript{rd} $r_0^*$) almost match in predicting the biaxial strength and the contact radius and have the same trends.

Comparison of the analytical values for the contact radius and the biaxial strength, with the results of the FE-calculations postulates that the results obtained using (2\textsuperscript{nd} $r_0^*$) and (3\textsuperscript{rd} $r_0^*$) expressions are converging and almost coincide or overlap with the FEA evaluation within a few percents of deviation. However, the values obtained using (1\textsuperscript{st} $r_0^*$) expression deviated away from the other values, suggesting inaccuracy in the contact radius evaluation using this expression. The contact radius obtained from the FEA under typical loading conditions ($R=7.5$ mm, $R_b=2$ mm, $R_a=5.75$ mm, $t=1.8$ mm, and $F=190$ N), shows that the (3\textsuperscript{rd} $r_0^*$) approximations describe the maximum tensile stress component in the disc fairly well, the results deviate from the FE-solutions less than 13\%, and the deviation was much higher 20\% when (2\textsuperscript{nd} $r_0^*$) approximations was used. Conversely, (1\textsuperscript{st} $r_0^*$) approximations were less precise where the deviations from the FE-calculations exceed 275\%.
Figure 4-15: Effects of loading force, disc thickness, and loading ball radius on the (a) biaxial strength, (b) contact radius, and (c) displacement of a disc loaded in (B3B) test.
Observation of the top surface of the biaxial specimens; figure (4-16), shows an indentation of area equivalent to the equivalent radius of contact ($r_0$).

![Figure 4-16: top surface of fractured biaxial specimens.](image)

The equivalent contact radius determined using the FEA at the typical loading conditions was 0.504 mm. Analytical results at the same loading conditions showed a contact radius of 0.57 mm. Experimentally, the top surface of the biaxial specimens measured a contact radius of 0.60 mm. This result is identical to the ($2^{nd}$ $r_0^*$) contact radius approximations (0.60 mm), and with deviations of 5% and 16% to the analytical and FEA values respectively ($r_0^*$), confirming the efficiency of using the ($2^{nd}$ $r_0^*$) and ($3^{rd}$ $r_0^*$) contact radius approximations to determine the biaxial strength of a disc loaded in (B3B) test.

### 4.1.3 Single Lap Shear Test

Single lap shear test was conducted according to BS EN 1465:09 [221] standard to assess joint bond strength and durability. Joints were made of aluminium sheet adherends and acrylic bone cement mantle to represent stem-cement joint and from solid rigid polyurethane foam adherends and acrylic bone cement mantle to represent cement-bone joint. The adherend plates were detached from the bone cement surface by a direct tensile force. The test results were expressed in detaching stress by dividing the load required to detach the plate from the
bone cement surface by the shear area of the valid test specimens and shown in table (4-5) and figure (4-17).

Table 4-5: Tensile lap-shear strength.

<table>
<thead>
<tr>
<th>CNTs content wt.%</th>
<th>( \sigma_{\text{Polyurethane}} ) (MPa)</th>
<th>( \sigma_{\text{Aluminium}} ) (MPa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.0</td>
<td>451 ± 24</td>
</tr>
<tr>
<td>SUNF1</td>
<td>0.1</td>
<td>434 ± 18</td>
</tr>
<tr>
<td>SUNF2</td>
<td>0.2</td>
<td>464 ± 19</td>
</tr>
<tr>
<td>SUNF5</td>
<td>0.5</td>
<td>372 ± 26</td>
</tr>
<tr>
<td>SOH1</td>
<td>0.1(-OH)</td>
<td>424 ± 16</td>
</tr>
<tr>
<td>SCOOH1</td>
<td>0.1(-COOH)</td>
<td>463 ± 17</td>
</tr>
</tbody>
</table>

Figure 4-17: Single lap shear strength of various joint systems.

The results shown in figure (4-17) showed that the bonding strength in all systems is quite good and ranged between 342–470 MPa and incorporation of MWCNTs improved the bonding strength between the aluminium sheet adherends and the bone cement composites. Reinforcement with 0.1, 0.2, and 0.5 wt.% of as received MWCNTs improved the bonding strength by 30%, 37%, and 24% respectively compare to the control bone cement. The functionalisation of the MWCNTs had lower improvement of 12% and 2.5% when hydroxyl and carboxyl groups were used, respectively.
Failure of all aluminium-cement joints occurred at the cement/aluminium interface, showing interfacial mode of failure; figure (4-18), indicating relatively poor bonding strength between the acrylic bone cement and the aluminium sheets.

![Figure 4-18: Failure surfaces of Aluminium-bone cement joints.](image)

However, cohesive mode of failure occurred within the rigid polyurethane foam adherends, as seen in figure (4-19), indicating good bonding between the cement and the polyurethane foam.

![Figure 4-19: Failure surface of the rigid polyurethane foam-bone cement joints.](image)
4.2 Thermal Studies

4.2.1 DSC Measurement

Figure (4-20) displays typical DSC thermograms recorded during the polymerisation reaction of the control Simplex bone cement.

Figure 4-20: DSC thermograms for the control samples subjected to (a) isothermal polymerisation at 37°C, (b) first scan at 10°C/min, and (c) second scan at 10°C/min.

Figure (4-20a) is a plot of the heat flow vs. time of the control sample polymerised isothermally at 37°C. The reaction started after an induction time. The time required for the
completion of the polymerisation was determined when the rate curve levelled off at the baseline very close to the initial baseline. Figure (4-20b) is the thermogram of second scan from 37°C to 150°C at 10°C/min in which the presence of a residual exotherm is observed. The residual peak was overlapped with the \( T_g \) of the cement powder particles which made it impossible to accurately estimate the residual heat of the remaining reaction. Figure (4-20c) shows a third scan of the sample from 37°C to 150°C at a heating rate of 10°C/min. The absence of a residual exothermic peak is observed here as well as the inflection corresponding to the \( T_g \) of the polymerised sample.

Figure (4-21) shows typical dynamic DSC thermograms recorded for fully polymerisation reaction of bone cement composites.

![DSC thermograms](image)

Figure 4-21: Example of typical DSC thermograms for fully polymerised control bone cement and SUNF5 samples obtained at 10°C/min.

The heat evolved for fully polymerised bone cement samples as a function of temperature \( \Delta H_{(tot)} \) was calculated by integrating the total area of the exotherm under the DSC curve in a dynamic (non-isothermal) scan from 20°C to 180°C at 10°C/min from the initial temperature \( (T_i) \) to the final temperature \( (T_f) \). The heat evolved \( \Delta H_{(tot)} \) for the bone cement composites were calculated and summarised in table (4-6).
Table 4-6: Heat evolved for fully polymerised bone cement composites (dynamic conditions).

<table>
<thead>
<tr>
<th>CNTs Content</th>
<th>ΔH_{(tot)} (J/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(wt.%)</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>179.6±4.6</td>
</tr>
<tr>
<td>SUNF1</td>
<td>162.7±2.8</td>
</tr>
<tr>
<td>SUNF2</td>
<td>158.2±4.1</td>
</tr>
<tr>
<td>SUNF5</td>
<td>169.1±3.3</td>
</tr>
<tr>
<td>SOH1</td>
<td>181.5±4.2</td>
</tr>
<tr>
<td>SCOOH1</td>
<td>180.7±4.2</td>
</tr>
</tbody>
</table>

Figure (4-22) shows the effect of different MWCNTs chemical functionality and loadings on the DSC thermograms of variety of bone cements composites recorded during isothermal polymerisation reaction at 37°C. The area of the exotherm gives the amount of heat released [ΔH_{(iso)}] during the polymerisation reaction.

Figure 4-22: The effect of MWCNTs loadings and chemical functionality on DSC thermograms for bone cement obtained during isothermal polymerisation at 37°C.

The polymerisation reaction characteristics, such as heat of the polymerisation reaction (ΔH_{(iso)}), initial polymerisation time (t_i), peak time (t_p), time of completion (t_f) and the polymerisation range (Δt = t_f - t_i) of bone cement with different chemical functionality and
loadings of MWCNTs were derived from the thermograms of figure (4-22) and are summarised in table (4-7).

Table 4-7: The effect of different MWCNTs chemical functionality and loadings on $\Delta H$, $t_i$, $t_p$, $t_f$, and $\Delta t$ at 37°C (isothermal conditions).

<table>
<thead>
<tr>
<th>CNTs Content (wt.%)</th>
<th>$\Delta H_{(iso)}$ (J/g)</th>
<th>$t_i$ (min)</th>
<th>$t_p$ (min)</th>
<th>$t_f$ (min)</th>
<th>$\Delta t$ (min)</th>
<th>$\Delta H/\Delta t$ (J/g.min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.0</td>
<td>137.2±3.8</td>
<td>2.4±0.6</td>
<td>4.4±0.8</td>
<td>5.9±0.6</td>
<td>3.4±0.5</td>
</tr>
<tr>
<td>SUNF1</td>
<td>0.1</td>
<td>122.8±4.1</td>
<td>2.5±0.3</td>
<td>4.3±0.4</td>
<td>6.0±0.5</td>
<td>3.5±0.7</td>
</tr>
<tr>
<td>SUNF2</td>
<td>0.2</td>
<td>116.1±3.7</td>
<td>2.6±0.4</td>
<td>4.3±0.6</td>
<td>6.3±0.7</td>
<td>3.7±0.3</td>
</tr>
<tr>
<td>SUNF5</td>
<td>0.5</td>
<td>110.6±5.3</td>
<td>2.8±0.5</td>
<td>5.2±0.8</td>
<td>6.9±0.6</td>
<td>4.1±0.6</td>
</tr>
<tr>
<td>SOH1 (-OH)</td>
<td>0.1</td>
<td>140.9±4.3</td>
<td>2.2±0.4</td>
<td>3.7±0.6</td>
<td>4.8±0.4</td>
<td>2.6±0.4</td>
</tr>
<tr>
<td>SCOOH1 (-COOH)</td>
<td>0.1</td>
<td>106.8±3.5</td>
<td>2.4±0.5</td>
<td>3.9±0.6</td>
<td>5.3±0.6</td>
<td>2.9±0.4</td>
</tr>
</tbody>
</table>

From figure (4-22) and table (4-7), it was found that the total amount of heat released decreased with increased MWCNT concentration. This indicates that the bone cement composites containing MWCNTs have lower extents of reaction than that of control bone cement. However, the functionalisation of MWCNTs with (-OH) groups increased the amount of heat released $\approx 28\%$ and $\approx 15\%$ compared to control and SUNF1 samples, respectively. The (-COOH) groups decreased the released heat by $\approx 18\%$ and $\approx 14\%$ compared to the control and SUNF1 samples, respectively. SOH1 bone cement composites released the maximum heat and SCOOH1 bone cement composites released the minimum heat during isothermal polymerisation at 37°C. Also, the incorporation of MWCNTs has a small effect on the initial time ($t_i$), peak time ($t_p$), time of completion ($t_f$) and as a result the polymerisation range ($\Delta t$). This phenomenon is significant when the MWCNTs loading is 0.5wt.% (SUNF5 cement composites). The functionality of MWCNTs resulted in smaller values of initial time ($t_i$) and peak time ($t_p$) than even the control sample. The values of the apparent heat release rate ($\Delta H/\Delta t$) of the bone cement decreased with increase MWCNTs concentration. However, SOH1 bone cement composite showed the highest heat release rate.

The relative fractional conversion of reaction, $(x)$, as a function of time for the bone cement composites containing different MWCNTs loadings were calculated using Eq.(3-16) and are shown in figure (4-23).
The maximum degree of conversion of each bone cement composite, \( x_{\text{max}} \), which represents the reacted monomer was calculated using Eq.(3-17) and presented in table (4-8) as well as the final degree of conversion of un-reacted monomer (1-x).

Table 4-8: The degree of MAA monomer conversion of bone cement composites polymerised isothermally at 37°C.

<table>
<thead>
<tr>
<th>CNTs Content (wt.%c)</th>
<th>( x )</th>
<th>1-x</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.0</td>
<td>0.764</td>
</tr>
<tr>
<td>SUNF1</td>
<td>0.1</td>
<td>0.756</td>
</tr>
<tr>
<td>SUNF2</td>
<td>0.2</td>
<td>0.734</td>
</tr>
<tr>
<td>SUNF5</td>
<td>0.5</td>
<td>0.654</td>
</tr>
<tr>
<td>SOH1</td>
<td>0.1(-OH)</td>
<td>0.776</td>
</tr>
<tr>
<td>SCOOH1</td>
<td>0.1(-COOH)</td>
<td>0.591</td>
</tr>
</tbody>
</table>

Figure 4-23: The conversion of variety of acrylic bone cement composites with different MWCNTs contents polymerised at 37°C (isothermal conditions).
It is clear from figure (4-23) and table (4-8) that the polymerisation curve shifts to the right with increasing MWCNTs content, and the maximum degree of conversion decreased with increase MWCNTs concentration, thus, there is an increase in the amount of the un-reacted monomer in the composite’s matrix. However, the polymerisation curve shifts to the left when the MWCNTs functionalised with (-OH) and (-COOH) groups compare to the control sample. The (-OH) groups in SOH1 bone cement composite increased the monomer conversion compared to unfunctionalised MWCNTs in SUNF1 and control composites. On the other hand, (-COOH) groups in SCOOH1 composite decreased the monomer conversion to the minimum value among all bone cement composites.

The phenomenological kinetic model and theory for the curing reaction of thermosetting resins was applied to study the kinetics of the polymerisation reaction and the effect of MWCNTs chemical functionality and loadings on the reaction kinetics. The rate constant $k$ of the chemistry-controlled regime and rate constant $k^*$ of the diffusion-controlled regime for bone cement composites polymerised isothermally at 37°C were determined using Eq.(3-18) and Eq.(3-19), respectively. Example plots of $dx/dt$ vs. $x(1-x)$ and $dx/dt$ vs. $x(1-x)^2$ are displayed in figure (4-24a) and (4-24b), respectively.

![Figure 4-24:](image)

Figure 4-24: Experimental curves for the determination of (a) the rate constant $k$ and (b) the rate constant $k^*$ for SUNF5 bone cement composite polymerised isothermally at 37°C.

Straight lines were obtained, showing that the polymerisation reaction of bone cement is a first order reaction and there are two kinds of reaction constants for the polymerisation reaction, which can be determined from the slopes. The constant reaction rate values ($k$) and
(\(k^*\)) were found and calculated before and after the peak time (\(t_p\)), respectively, and are shown in table (4-9).

<table>
<thead>
<tr>
<th>CNTs Content (wt.%)</th>
<th>(k) (\pm 0.170)</th>
<th>(k^*) (\pm 0.012)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.0</td>
<td>1.201</td>
</tr>
<tr>
<td>SUNF1</td>
<td>0.1</td>
<td>1.162 ±0.097</td>
</tr>
<tr>
<td>SUNF2</td>
<td>0.2</td>
<td>1.014 ±0.118</td>
</tr>
<tr>
<td>SUNF5</td>
<td>0.5</td>
<td>1.040 ±0.118</td>
</tr>
<tr>
<td>SOH1</td>
<td>0.1(-OH)</td>
<td>1.234 ±0.153</td>
</tr>
<tr>
<td>SCOOH1</td>
<td>0.1(-COOH)</td>
<td>1.245 ±0.144</td>
</tr>
</tbody>
</table>

Table (4-9) demonstrates that there are two kinds of reaction rate constants (\(k\) and \(k^*\)) for the polymerisation reaction. The lower values of the rate constant (\(k\)) were found before the peak time, while the higher values of the rate constant (\(k^*\)) were found after the peak time. The existence of the two rate constants means that the polymerisation reaction took place at a slower reaction rate at the beginning, as there was more monomer of low viscosity.

After the peak time, the faster rate of polymerisation reaction was attained during polymerisation reaction with high viscosity. However, MWCNTs chemical functionality seems to have greater influence on the reaction rate constants than the MWCNTs loadings.

4.2.2 Thermal Necrosis Evaluation

Figure (4-25) displays example of temperature vs. time plots related to the exothermic heat generated during polymerisation for bone cement composites as a function of the MWCNTs loadings and chemical functionality.
The maximum temperature ($T_{\text{max}}$), and setting time ($T_{\text{set}}$) for each bone cement composite were measured in accordance with the international standard for acrylic resin cements (ISO 5833:02), using Eq.(3-22). The rate of polymerisation was also derived from figure (4-25) by calculating the gradient of the linear portion of the temperature vs. time plot at the setting time as defined by the ISO standard. The exothermic polymerisation characteristics are summarised in table (4-10).

Table 4-10: The exothermic polymerisation characteristics for variety of acrylic bone cements.

<table>
<thead>
<tr>
<th>CNTs Content (wt.%%)</th>
<th>$t_{\text{max}}$ (min)</th>
<th>$T_{\text{max}}$ (°C)</th>
<th>$t_{\text{set}}$ (min)</th>
<th>$T_{\text{set}}$ (°C)</th>
<th>$k$ (°C/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.0</td>
<td>7.3±1.2</td>
<td>119.0±1.6</td>
<td>6.3±1.8</td>
<td>78.0±1.8</td>
</tr>
<tr>
<td>SUNF1</td>
<td>0.1</td>
<td>7.5±1.9</td>
<td>116.1±2.5</td>
<td>6.3±1.4</td>
<td>76.5±1.3</td>
</tr>
<tr>
<td>SUNF2</td>
<td>0.2</td>
<td>7.7±1.6</td>
<td>95.4±2.7</td>
<td>6.8±1.6</td>
<td>66.2±1.4</td>
</tr>
<tr>
<td>SUNF5</td>
<td>0.5</td>
<td>8.0±0.9</td>
<td>94.7±1.6</td>
<td>7.4±1.8</td>
<td>65.9±1.7</td>
</tr>
<tr>
<td>SOH1</td>
<td>0.1(-OH)</td>
<td>6.8±1.3</td>
<td>99.7±2.4</td>
<td>6.7±1.6</td>
<td>68.4±1.4</td>
</tr>
<tr>
<td>SCOOH1</td>
<td>0.1(-COOH)</td>
<td>7.0±1.4</td>
<td>99.4±1.2</td>
<td>6.9±1.3</td>
<td>68.2±1.5</td>
</tr>
</tbody>
</table>

Figure (4-25) and table (4-10) demonstrate that the maximum temperature ($T_{\text{max}}$) decreased with increasing MWCNTs concentration. This indicates that the control bone cement composite has higher ($T_{\text{max}}$) than those MWCNTs reinforced bone cement composites.
However, the functionality of MWCNTs with (-OH) and (-COOH) groups have significant effect on the \( (T_{max}) \) compare to the cement reinforced with unfunctionalised MWCNTs (SUNF1). The type of chemical functionalisation used did not have a significant effect on the \( (T_{max}) \).

The highest polymerisation reaction rates \( (k) \) were observed for bone cement composites containing MWCNTs with (-OH) and (-COOH) groups, SOH1 and SCOOH1 composites. On the other hand, MWCNTs loadings did not have a significant effect on the reaction rate compared to the control cement.

The cumulative thermal necrosis index (TNI) was determined using figure (4-25) in conjunction with Eq.(3-20) and Eq.(3-21). The TNI was calculated for two temperature levels (>44°C and >55°C), figure (4-26).

![Thermal Necrosis Index (TNI)](chart.png)

Figure 4-26: The effect of MWCNTs chemical functionality and loading on the Thermal Necrosis Index for variety of acrylic bone cement composites.

It can be observed from the results in figure (4-26) that all bone cement composites including the control bone cement had produced a cumulative thermal necrosis damage of greater than one. However, the TNI value decreased with increase MWCNTs concentration whereby the maximum TNI value was recorded for the control cement. However, the minimum values were for SOH1 and SCOOH1 bone cement composites with reduction of \( \approx 20\% \) and \( \approx 25\% \) in TNI values at >44°C and >55°C, respectively, compared to the control cement.
4.3 Biocompatibility Studies

4.3.1 Cytotoxicity Assessments

**MTS-assay Evaluation**

In order to evaluate the cytocompatibility of MWCNTs-reinforced Simplex-P® bone cement composites, an osteoblast viability study using HOB-c osteoblast cells was conducted. Indirect contact investigation was performed to quantify the cell viability after 24 h of culturing using the MTS-assay. The cytotoxic effects of the bone cement composites main constituents (Simplex-P® powder, MWCNTs powder, and liquid monomer) were assessed individually; figure (4-27). The population of cells counted on the 96-wells plate as positive control culture (cells cultured in the absence of materials) was normalised to 100%.

![Cellular Population](image)

Figure 4-27: Percentage of HOB-c cell population after 24 h exposure to extracts of the main constituents of bone cement composites.

Figure (4-27) shows that all three components had reduced cellular population compared to the positive control. MWCNTs powder showed ≈25% reduction, while the Simplex-P® powder and the liquid monomer showed reduction of ≈18 and 15%, respectively. This indicates that the MWCNTs are directly the most toxic among the bone cement composite’s components.

The cytotoxic effects of possible substances that could leach out of the acrylic bone cement composites as function of MWCNTs loadings were also evaluated by quantifying the cellular
population after 24 h of culturing using the MTS-assay; figure (4-28). The population of cells counted on the 96-wells plate as positive control culture was again normalised to 100%.

![Cellular Population](image)

Figure 4-28: Percentage of HOB-c cell population after 24 h exposure to extracts of varies bone cement composites.

Figure (4-28) shows that bone cement composites containing MWCNTs showed higher cell population than of that of Simplex control bone cement. Incorporation of 0.1 and 0.2 wt% of MWCNTs increases the cell quantity by ≈15% and 40%, respectively, while incorporation of 0.5 wt% of MWCNTs did not have a significant effect (only ≈3% increase) on the cell population compared to the Simplex control bone cement.

Also, dose response was observed due to dilution when the extracts from all composites were subjected to a two-fold dilution. A negative correlation between extract concentration and cell population was found, indicating that leaching has a negative effect on cells. Again, the diluted extracts for SUNF1 and SUNF2 induced an increase of ≈ 4% and 18%, respectively, while SUNF5 extract showed no influence on the cell population compared to the Simplex control bone cement.

**xCELLigence Evaluation**

In order to determine the optimum cell concentration for cell attachment and viability measurements, dynamic cell attachment and viability were monitored in real-time using xCELLigence system.
Osteoblasts HOB-c cells at densities of 6000, 3000, 1500, and 750 cells/well were seeded in the E-Plate16 and the impedance cell index (CI) determined during 100 h of observation; figure (4-29).

![Graph](image)

Figure 4-29: Dynamic monitoring of HOB-c osteoblast cell line at different densities observed during 100 h.

As shown in figure (4-29) the impedance cell index CI increased proportionally to cell number and the CI of each cell concentration sharply increased after seeding up to its maximum at 12.5 h. Thereafter the CI of each cell concentration slowly decreased to reach a minimum at 20 h when the cells were fed by replacing the spent media with fresh. After feeding, the CI of 3000, and 1500 cells/well increase again slowly to a maximum at 100 h. However, the CI of 6000 and 750 cells/well did not match this correlation. The CI of 6000 cells/well sharply decreased after feeding to reach its minimum at 100 h while CI reaches a plateau value after a feeding time for the 750 cells/well.

The CI increases depending on the number of attached cells on the electrodes, but the CI of the lowest cell concentration failed to display changes during the incubation time, suggesting a confluence response by the cells on attachment. The steep increase of the CI of each cell concentration of the HOB-c cells up to 12.5 h may be characterised by its similar adhesion as well as the time.

After 12.5 h in the wells HOB-c with 6000 and 3000 cells/well entered a lag phase up to feeding time 20 h, in which they most likely fully spread but were not actively proliferating.
While HOB-c with 1500 and 750 cells/well assumed growth after 12.5 h, the cells with 3000 cells/well remained in the lag phase up to feeding time, 20h, and only thereafter entered the growth phase.

In contrast, the HOB-c with 6000 cells/well remained in the lag phase and never entered growth phase. This response of the 6000 cells/well suggested a saturation effect likely by contact inhibition of the cell cycle by densely attaching among the HOB-c cells in the E-Plate16. Hence, this cell concentration was excluded for further experiments. Interestingly, cell concentrations of 3000 and 1500 cells/well displayed similar growth phases after feeding time. The slowed growth response of the 750 cells/well could be attributed to the confluence status of the cells.

Overall, the results clearly demonstrate that the response seen in the 1500 and 3000 cells/well experiments reflects cell cycle effects, and are the optimum concentrations for a dynamic monitoring of the attached cells’ biological status by the xCELLigence system, and can be applied as a conventional end-point in \textit{in vitro} assays.

The concentration of 3000 cells/well was chosen to be used in the xCELLigence assay to examine the cytotoxic effects of possible substances that leach out of the acrylic bone cement composite in real-time, as this concentration displayed a slow gradient presenting reasonable cell growth phase. The osteoblast HOB-c cells at density of 3000 cells/well were cultured in complete osteoblasts media and challenged after 3 h with extract solution (40\%v.v) of Simplex control and SUNF5 (the highest loading weight of MWCNTs 0.5 wt.\%) composite samples by diluting the extract solution in the osteoblasts growth media. The HOB-c cells were fed after 24 h by replacing the spent media with fresh; figure (4-30).

Figure (4-30) demonstrated that incorporation of 0.5 wt\% of MWCNTs did not have a significant effect on the cells population compared to the Simplex control bone cement. This result was consistent with previous indirect MTS-assay result; figure (4-28). The CI of both bone cement composites gradually decreased after challenging the HOB-c cell suspensions with the extracts to reach its minimum at feeding time (24 h). Thereafter the CI of both bone cement composites slowly increased with time over the duration of experiment. Also, CI for SUNF5 shows a higher value compared to the CI for Simplex control sample after feeding point indicating higher cellular growth rate.
Cellular Adhesion and Morphology

**MTS-assay Evaluation**

For an implant material to be successful it must first promote cellular attachment. Therefore, cellular attachment of human osteoblast cells onto the surface of Simplex control bone cement and its composites was assessed as a function of the MWCNTs chemical functionality and loadings. Direct contact investigation was performed to quantify the initial adhesion of human osteoblasts cells onto the surfaces of the bone cement composites after 24 h of culturing using the MTS-assay; figure (4-31). The population of cells counted on the Simplex control bone cement sample cultures was normalised to 100%.

Figure (4-31) shows significant differences between the numbers of osteoblast cells attached to MWCNTs–bone cement composites compared into the Simplex control after a 24 h attachment period. The number of osteoblast cells attached onto the surface of all MWCNTs–bone cement composites was lower than cells attached to the Simplex control.
Chapter 4  Results

Figure 4-31: Cellular population of HOB-c osteoblast cells cultured onto the surfaces of bone cement-MWCNTs composite samples for 24 h.

A reduction of cells population of ≈40% was observed for SUNF1 sample, and ≈25% reductions was recorded for both SUNF2 and SUNF5 bone cement composites compared to the Simplex control. Although the numbers of attached cells were lower than the Simplex control sample, the cell number increased with increasing the MWCNTs weight loadings.

The results also showed that the numbers of osteoblast cells attached were affected by the chemical functionality of the MWCNTs. Although the numbers of attached cells remained lower than the Simplex control sample, the modification with hydroxyl groups (SOH1 samples) had increased the numbers of cells by ≈30% while modification with carboxyl groups (SCOOH1 samples) induced ≈ 40% increase in comparison to the same weight loading percentage (1wt.%) of unfunctionalised MWCNTs (SUNF1 samples).

SEM Evaluation

Attempts have also been made to investigate the suitability of MWCNTs as fillers in bone cement biomaterials by examining the adhesion and function of osteoblast cells on these bone cement composites in culture. Therefore, the morphology of HOB-c osteoblast cells (5000 cells/well) cultured for 3 and 24 h onto Simplex control, the highest MWCNTs loadings (0.5 wt.%) SUNF5, hydroxyl functionalised MWCNTs (SOH1), and carboxyl functionalised MWCNTs (SCOOH1) bone cement composite surfaces, was investigated using SEM assessment. For comparison, the results are shown in figure (4-32).
Figure 4-32: Scanning electron micrographs for HOB-c cultured on (a, b) Simplex control, (c, d) SUNF5, (e, f) SOH1, and (g, h) SCOOH1 bone cement samples for 3 h (a, c, e, g) and 24 h (b, d, f, h).
After 3 h in culture, a flattened osteoblast morphology (black arrows) was seen on the Simplex control sample; figure (4-32a), whereas globular osteoblast morphology (blue arrows) was noted on all the MWCNTs bone cement composites, indicating normal HOB-c cell attachment and growth.

Morphological investigation also showed preferential cell attachment to the MWCNTs cement composite when compared to the Simplex control cement by a network of fibrous cell extensions (red arrows) coming out from the cell body, for anchoring to the cement surface; figure (4-32 c, e, g). Protruding HOB-c fibres initiate contacts with surrounding cells to obtain a confluent layer on top of the cement surface. However, SCOOH1 bone cement composite exhibited a higher number of cells attached to its surface and the HOB-c cells were also seen to overlap and anchor to other surrounding cells; figure (4-32 g).

Figure (4-32 b, d, f, h) elicits an overall spread of flattened osteoblast cells on all cement surfaces and complete confluence was observed after 24 h of culturing. These results suggested that MWCNTs have no obvious influence on the size and shape of the osteoblast cells since they did not vary between samples. Thus, high numbers of focal adhesions were observed and the cell cytoskeleton was seen to be clearly organised on all cement surface cultures, linking to good adhesions.

**LSCM Evaluation**

The cell live-dead viability was also observed using a confocal microscopy (LSCM) assessment after culturing osteoblast HOB-c cells (5000 cells/well) for 3 h on various bone cement specimens. Live cells (green) stained with calcein; dead cells (red) stained with ethidium homodimer (EthD-1); and combination of live and dead cells are represented by top, middle, and bottom images, respectively; figures (4-33).

The results in figure (4-33) show that the population of cells was seen to increase with MWCNTs incorporation into the Simplex bone cement, with significant differences between plain Simplex control and Simplex bone cement with 0.1 and 0.2 wt.% unfunctionalised MWCNTs incorporation. Although, no significant differences were seen for cell viability (living cells based on a total number of cells sample) between the control Simplex cement and its composites (≈80–90% viability), it is clearly evident that the incorporation of 0.1 and 0.2 wt.% MWCNTs does not have any negative impact.
However, on the Simplex control culture the osteoblasts showing a circular and lenticular cellular morphology of the cytoskeleton. The osteoblasts exhibit a smaller rounded shape as well as some cells with flat morphology when the MWCNTs were incorporated (0.1 wt.%). As the concentration of the MWCNTs increases (0.2 wt.%), the osteoblast cells showed more big flattened and spread cell morphology indicated that cell the cytoskeleton is more adherent and oriented along the nanostructure of the cement composites. Also, the rounded cell spheres in the presence of flattened cells are a sign of cell division and active proliferation.

Figure (4-34) shows the effect of chemical functionality of MWCNTs on the viability of osteoblast HOB-c cells.
This live-dead confocal microscopy (LSCM) assessment, figure (4-34), shows that functionalisation of MWCNTs can have a negative effect on the number of live cells population compare to the unfunctionalised MWCNTs. Although, by looking at the number of dead cells, MWCNTs functionalised with hydroxyl group has the lowest amount of dead cells while the MWCNTs functionalised with carboxyl group showed the greatest number of dead cells among all composites. However, live-dead assessment had show that functionalisation of MWCNTs has a negative effect on the osteoblast cells shape and, thus, cells adherence. Big unspread and rounded cellular morphology was dominantly seen when the hydroxyl and carboxyl groups functionalised MWCNTs were incorporated compared to the unfunctionalised MWCNTs where flat cellular morphology was observed.

Figure 4-34: Live-dead LSCM images for (a) SUNF1; (b) SOH1; (c) SCO0H1 bone cement samples. (Top: live cells, Middle: dead cells, Bottom: companied live and dead cells).
4.3.3 Cell Viability

HOB-c osteoblasts cell viability on the Simplex control bone cement and its composites was assessed as a function of the MWCNTs chemical functionality and loadings. Direct contact investigation was conducted on each bone cement composite and compared after 3 and 7 days of culturing using MTS-assay on live osteoblast cells. The amount of osteoblast cells counted on the Simplex control bone cement culture after 1 day was normalised to 100%. The results were interpreted as early and late stage of cell viability; figure (4-35).

Figure 4-35: Effects of (a) MWCNTs loading and (b) chemical functionality on cell viability of HOB-c osteoblasts cultured for 1, 3, and 7 days on various bone cement composites.
Early stage of cell viability (1-3days):

After 3 days of culturing (stage of early cell viability), figure (4-35a) shows that SUNF5 had the greatest cell count with \(\approx 8\%\) greater cell quantity than Simplex control while SUNF1 and SUNF2 samples reduced the cell quantity by \(\approx 21\%\) and \(\approx 9\%\) compared to the Simplex control. However, cell viability increased with increase the MWCNTs concentration in the composites regardless of the initial cell population on the Simplex control sample. On the other hand, the cells were grown at different growth rates. The cell growth rate on the Simplex control was \(\approx 34\%\), while the growth rate on SUNF1, SUNF2, and SUNF5 bone cement composites were \(\approx 83\%\), \(\approx 66\%\), and \(\approx 92\%\), respectively.

Figure (4-35b) shows the influence of MWCNTs chemical functionality on osteoblast cells at the early cell viability stage. The modification of MWCNTs with hydroxyl groups (SOH1 samples) had increased cell quantity by \(\approx 13\%\) while carboxyl groups (SCOOH1 samples) had small negative effect (\(\approx 2\%\)) compared to the unfunctionalised MWCNTs (SUNF1 samples). However, the cellular growth rate on SOH1 and SCOOH1 bone cement composites were \(\approx 40\%\) and \(\approx 50\%\), respectively, compared to \(\approx 83\%\) for SUNF1 bone cement composites.

Overall, these results demonstrate that incorporation of 0.5% of unfunctionalised MWCNTs into Simplex control bone cement did not only show the highest cell viability but resulted in the highest cell growth rate among all tested composites. Therefore, it can be concluded, that SUNF5 bone cement composite is best support for enhancement osteoblast cells during early viability stage.

Late stage of cell viability (3-7 days):

In contrast, figure (4-35a) shows that after 7 days in culture (stage of late cell viability), an increase and enhancement in osteoblasts cell quantity of \(\approx 25\%\) and \(\approx 8\%\) was measured on SUNF1 and SUNF2 bone cement composites, respectively, compared to Simplex control bone cement. On the other hand, SUNF5 composite with higher concentration of MWCNTs was less efficient in enhancing viability of osteoblast cells at this stage. However, the osteoblasts cell growth rate on SUNF1, SUNF2 and SUNF5 bone cement composites were \(\approx 114\%\), \(\approx 68\%\) and \(\approx 31\%\), respectively, compared to \(\approx 42\%\) for Simplex control bone cement.

The influence of MWCNTs chemical functionality on osteoblast cells at the late cell viability stage is shown in figure (4-35b). SUNF1 bone cement composite with unfunctionalised MWCNTs had the greatest osteoblast cell count with \(\approx 6\%\) greater cell quantity that
MWCNTs chemical functionalised SOH1 and SCOOH1 bone cement composites. The cell growth rate on SOH1 and SCOOH1 bone cement composites were ≈103% and ≈77%, respectively, compared to ≈114% for SUNF1 bone cement composite. Although modification of MWCNTs with hydroxyl and carboxyl groups had shown no effect on cell population at the late stage of cell viability, the cell growth rate was significantly affected by these chemical modifications. The cell growth rate on SOH1 bone cement composite was 1.34 times higher than on SCOOH1 bone cement composite.

Overall results demonstrated that, despite lower levels of initial cellular attachment, incorporation of 0.1 wt.% of unfunctionalised MWCNTs into Simplex control bone cement presented the highest cell viability and cell growth rate among all tested composites. Thus, SUNF1 bone cement composite is best support for enhancement osteoblast cells during late viability stage.
5 Discussion
5.1 Mechanical Analysis

Although there are many more complex mechanical and clinical factors affecting in vivo bone cement performance, the bone cement mantle is subjected to complex forces with a considerable amount of flexural stresses, primary biaxial loading, during the daily activities cycle \(^{[174]}\). Therefore, estimating the bone cement strength using current traditional uniaxial tests may not provide an accurate characterisation of bone cements true load-bearing capacity and it is appropriate to evaluate the strength of bone cement mantle under biaxial rather than uniaxial laboratory conditions.

The advantages of ball-on-three-ball (B3B) loading configuration employed here are; its test fixtures and specimen geometry simplicity, minimum requirements for alignment, and its ability to predict the stresses at the centre of the specimen accurately using the developed analytical solutions as compared to other methods \(^{[184]}\). It has been hypothesised that the biaxial flexural test method is only suitable for testing of brittle materials with minimal deflection.

On reviewing the mechanical test data, it was thought that the (B3B) biaxial flexure testing is an easy, useful, and more reliable test method than ISO ring-on-ring and four-point bending tests in evaluation the mechanical properties of orthopaedic bone cement materials. The finite element analysis have verified that the (B3B) biaxial flexure test is representative of strength and stiffness for this type of material and the biaxial theory is accurate in determining the mechanical properties values. The biaxial test offers several advantages over the others because it minimises the volume or the surface area investigated and also the edge effect. It has been established that the ratio of biaxial to uniaxial flexural strength is the same for all acrylic bone cement composites tested. The difference in strength between the (B3B), ring-on-ring biaxial and four-point bending tests was explained on the basis of the stress gradient through the cement specimen.

Also, the addition of multi-walled carbon nanotubes substantially improves the mechanical performance of acrylic bone cement nanocomposites. The effectiveness of MWCNTs reinforcement is dependent on the concentration and dispersion of MWCNTs. Furthermore, concentration of 0.1 wt.% unfunctionalised MWCNTs performs the best reinforcement for the PMMA/MWCNTs nanocomposites. Beyond this limit, further addition of MWCNTs to the Simplex matrix yields a considerable decrease of the biaxial strength and stiffness.
Functionalisation of the MWCNTs surfaces with Hydroxyl (-OH) or Carboxyl (-COOH) groups did not enhance the dispersion of MWCNTs within the cement matrix, thus, did not improve the mechanical integrity. Therefore, it is unclear whether the improvements in performance of these cement nanocomposites are a direct consequence of good MWCNTs dispersion within the PMMA matrix, or is due to a chemical interaction between the MWCNTs and PMMA matrix through surface chemical modification.

The force-displacement curves of all bone cement composites under the three different flexural tests, figure (4-1), showed a linear relation between load and displacement in both (B3B) and ring-on-ring biaxial tests, indicating a classic brittle failure behaviour. Thus, the (B3B) biaxial test is valid to determine the mechanical properties for bone cement composites. However, the load-displacement curves obtained in the 4-point bending test were not as linear as those obtained in the biaxial tests, suggesting some degree of yielding before the brittle failure. This non-linearity was attributed to the difference in the distance of the support spans with respect to the loading. When the specimen is subjected to bending load in the 4-point bending test, the support span of 60 mm allows greater flexibility and higher deflection. On the contrary, the materials behaved more stiffly in the (B3B) biaxial test, which had a much smaller circular support span of only 5.75 mm.

The evaluation of the biaxial properties were based upon the relationship between the applied force, the centre deflection and the maximum tensile stress distribution of the disc specimen that was suggested by Kirstein and Woolley\textsuperscript{[186]}. In order to examine the applicability of the (B3B) biaxial flexural test for this type of bone cement composite, all assumptions when biaxial stress equations are used, Eq.(3-1) & Eq.(3-2), should be satisfied: (a) the central deflection should not exceed half the specimen thickness at fracture, (b) the maximum tensile stress at failure develops at the centre of the support surface and the spurious edge failure is limited, and (c) the correct radius of the contact area should be used\textsuperscript{[184]}.

The deformation parameters of the disc; centre deflection ($\omega_c$), and the centre deflection function ($\beta_c$), which depend on the number of supports and the ratio of the support radius to the radius of the disc, Eq.(3-1) and Eq.(3-2), were examined using finite element analysis to verify the analytical biaxial theory. The disc specimen was 1.8 mm in thickness ($t=1.8$ mm); 15 mm diameter ($R=7.5$ mm), and was supported centrally by three stainless-steel ball bearings positioned 120° apart from its centre on a support circle of 11.5 mm diameter ($R_d=5.75$ mm). Assuming the midpoint of the support balls are fixed and the midpoint of the
centred loading ball is only allowed to move vertically; the radii of the supporting balls and the loading ball are chosen to be equal ($R_b = 2$ mm). A three-dimensional non-linear analysis of the biaxial flexure test was conducted to determine the central deformation response to the failure load at the centre of the biaxial disc specimen for a given modulus of cement. The central deformation data was then substituted into Eq. (3-1) to yield the modulus of the acrylic bone cement. If the modulus, as determined from the finite element analysis central deformation, is equivalent to the modulus as determined by the analytical centre deflection, Eq. (3-1), then the biaxial theory is applicable.

The disc central deformation determined in the finite element analysis method was 0.4714 mm (≈26% of the original thickness) for a cement modulus of 2.5 GPa and failure load of 190 N, figure (4-13). However, the experimentally centre deflection measured from the load-displacement curve and its gradient was 0.48±0.02 mm. The deflection of each specimen tested did not exceed half its original thickness and therefore the linear theory of elasticity applies [227].

By substituting the FE-disc central deformation data into Eq. (3-1), the analytically biaxial modulus of the cement was 1.37 GPa. However, the experimentally obtained biaxial modulus of the Simplex bone cement was about 1.00 GPa. The deviation in the centre deflection as determined with FEA method was 6% compared to the analytical results obtained using the equations postulated by Kirstein and Woolley [186]. However, table (4-4), demonstrates that the biaxial modulus shows a constant deviation in the modulus values of (≈45%) for all range of parameters investigated. In another words, the equations of Kirstein and Woolley [186] characterise a precise description of the centre deflection ($\omega_c$) and centre deflection function ($\beta_c$) and, consequently, modulus of the biaxial disc specimen, regardless the scale of the modulus value.

These results supported the verification made by Higgs et al. [175] using the gradient of load-displacement data obtained from two-dimensional finite element nonlinear analysis for different modulus of cements to confirm the biaxial theory. They studied the biaxial modulus with the same test method as the current study and have established the applicability of using biaxial flexural test and measured the modulus of acrylic bone cement of 2.43 GPa at accuracy of 2.6% for FE modulus of 2.5 GPa and 2.09 GPa experimentally for Simplex bone cement.
The strength is defined to be the maximum principal tensile stress in the disc \[^{181}\], which occurs on the disc surface opposite the centred loading ball. The stress field in the disc depends on the applied load, on the geometric set-up of the test, and also on the elastic properties of ball and disc materials. The calculation of the biaxial strength was based upon the relationship between applied load and the maximum tensile stress distribution in the disc. Various approximations have been suggested to describe the tensile stresses opposite the loading area in the centre of the disc. These approximations also depend on the contact radius between the loading ball and the disc and predict very different contact radii leading to different results for the tensile stress distribution around the centre of the disc \[^{181}\].

The stresses at contact caused by the pressure between the load ball and disc specimen are of importance and must be addressed when calculating the biaxial strength. This parameter has to be determined because when a load is applied over a small area the stress can reach very high values as the radius of the loaded area (contact area) approaches zero. The actual maximum tensile stress produced on the lower surface by a load concentrated on a very small area, of radius \(r_0\), can be found by replacing the actual radius of contact area \(R_b\) by a so-called equivalent radius, \(r_0^c\). This equivalent radius depends largely upon the thickness of the disc \(t\) and to a lesser degree on its transverse dimension \[^{175}\].

A three-dimensional non-linear FE-analysis was conducted to determine the maximum principal tensile stress distribution in (B3B) loaded disc and the accurate expression in predicting the contact radius \(r_0^c\) between the loading ball and the disc, thus, the correct biaxial flexural strength for an acrylic bone cement.

These results suggest that the maximum tensile stress region is located directly underneath the loading ball on the centre of the bottom surface of the disc specimen where the stress state is purely biaxial. This region of the maximum stress is very small and its gradient in this region is very small indicating that only a small volume of the disc material is experiencing this large stress, figure (4-14). It also has been confirmed from finite element analysis that the stresses at the edge of the biaxial specimen are minimal and it may be argued that the edges do not play a role in strength determination.

With this FE-model, a parametric study was carried out in order to verify the quality of analytical approximations used in this study to estimate the equivalent contact radius \(r_0^c\). This investigation intended to measure the biaxial strength of acrylic bone cement composites.
using the three different expressions and compare the FEA and the analytical results in order to confirm the applicability and accuracy of these approximations and stress equations used to validate the biaxial theory presented by Kirsten and Wooley \[186\], which was the foundation of predicting the biaxial strength in this study.

Using the finite element solver, table (4-4) and figure (4-15) showed that overall comparison of the analytical approximations for the contact radius, thus, stress distribution in the disc specimen, with the results of the FE-calculations postulates that the results obtained using (2\textsuperscript{nd} $r_0^*$) and (3\textsuperscript{rd} $r_0^*$) expressions are converging and almost coincide or overlap with the FEA evaluation within a few percents of deviation at larger thicknesses of specimen in predicting the contact radius, this may attributed to the higher load required for thicker specimen. However, the solutions using (1\textsuperscript{st} $r_0^*$) expression deviated away from the other values. The contact radius obtained from the FEA under typical loading conditions ($R$=7.5 mm, $R_b$= 2 mm, $R_a$=5.75 mm, $t$=1.8 mm, and $F$=190 N), shows that the (3\textsuperscript{rd} $r_0^*$) approximations describe the maximum tensile stress component in the disc fairly well, the results deviate from the FE-solutions less than 13\%, and the deviations was much higher 20\% when (2\textsuperscript{nd} $r_0^*$) approximations was used. Conversely, (1\textsuperscript{st} $r_0^*$) approximations were less precise where the deviations from the FE-calculations exceed 275\%.

Experimentally, the top surface of the biaxial specimens; figure (4-16), showed an indentation of area equivalent to the equivalent radius of contact ($r_0^*$) and measured a contact radius of about 0.6 mm. This result is matching the (2\textsuperscript{nd} $r_0^*$) contact radius approximation (0.60 mm), and with deviation of 5\% and 16\% compared to the analytical and FEA results respectively, thus, confirming the efficiency of using the (2\textsuperscript{nd} $r_0^*$) and (3\textsuperscript{rd} $r_0^*$) contact radius approximations to determine the biaxial strength of a disc loaded in (B3B) test.

The possible sources for the analytical stress and centre deflection deviations from the FEA idealised solution could be attributed to disregarding the influence of friction forces between the disc and the support balls, possible buckling of the disc and geometric inaccuracies. Also, for a proper data evaluation, the Poisson’s ratio of the tested material has to be known with an accuracy of 5\%, which is not the case for any material \[181\]. Furthermore, the size distribution and density of pores, which are crucial factors in the failure of acrylic bone cement composites, were neglected in this analysis, thus, virtual bone cement FEA model with the respective pore size distribution should be use to simulate the test specimen and minimise these differences.
It has been reported that the dimensions of the specimens and the loading ball must be determined carefully to ensure the validity of the use of the analytical solutions for the (B3B) biaxial test [174]. Figure (4-15) clearly supported this fact and confirmed that accurate measurement of the thickness of the specimen is the most important factor in the biaxial test, as it affects the degree of deflection and distribution of the stresses. However, for the influence of the loading ball radius, only the analytical results obtained from \( (1^{st} r_0) \) approximation are highly influenced by the loading ball radius and the FEA result also showed small dependency of contact area radius on the loading ball radius.

The results of the biaxial and bending modulus of all bone cement composites, table (4-1) and figure (4-2), demonstrate that increasing MWCNTs concentration in the Simplex bone cement matrix significantly reduced the stiffness of the nanocomposites. The maximum reduction in modulus was for SUNF5 composite, which contained 0.5 wt.% MWCNTs. This unexpected result can be due to extensive agglomeration of MWCNTs in the matrix, which has led to uncontrolled microstructure and possibly resulted in residual porosity.

However, considering the constant reduction of 45% in the biaxial modulus of our results, for the range of the materials tested in both tests, the result values fall within the range of moduli determined for the flexural tests and are consistent with published data [5, 6, 8, 126, 175, 228].

Ormsby et al. [6, 8] found bending moduli were 3.57, 3.50, and 3.13 GPa when 0.1 wt.% unfunctionalised, carboxyl functionalised and amine functionalised MWCNTs were incorporated respectively compared to 3.01 GPa for Colacryl B866 bone cement as control, and recorded modulus value as low as 0.496 GPa when 0.5 wt.% of unfunctionalised MWCNTs were added. Contrary to our results, Marrs et al. [5] reported that the bending modulus increases with increasing the MWCNTs loadings and measured 3.4 GPa for control cement sample compared to 3.4, 3.8, and 4.2 GPa for 0.5, 5.0, and 10 wt.% loadings respectively.

Also, the results of the biaxial and bending modulus indicate that the respective magnitudes of the moduli are consistent with the well-known strain rate characteristics of acrylic surgical bone cements. In this respect, the cement acts like a spring and dashpot connected in series. When a mechanical force is applied, a velocity-dependent resisting force is developed.

Higgs et al. [175] demonstrated the strain rate dependence of acrylic bone cements and reported that compressive modulus of Simplex cement had increased with strain rate and similar
responses of strain rate on other modes of loading like bending and shear was stated. On the examination of the flexural properties of PMMA, Pal and Saha \cite{44} reported an increase of 23% in the flexural modulus for a strain rate of 0.00486/min to 0.486/min.

Figure (4-3), demonstrated that the SUNF1 bone cement composite was optimal for enhancing the biaxial flexural strength ($\sigma_{\text{biaxial}}$) of bone cement. However, a reduction in the biaxial flexural strength was observed in SUNF2 and SUNF5 bone cement nanocomposites. On the other hand, the hydroxyl (-OH) groups in SOH1 bone cement nanocomposite did not substantially change the cement biaxial flexural strength, while the carboxyl (-COOH) groups in SCOOH1 bone cement nanocomposite showed an improvement compared to the control Simplex bone cement.

During the loading the central ball and the disc deform so that a circular contact area of radius ($r_0^\ast$) builds up under the loading ball and a smaller contact area builds up over each of the three supporting balls. This contact area increases with increasing the applied force. This change in the loading geometry may also influence the tensile stresses in the disc. The result values obtained using ($2^{\text{nd}} r_0^\ast$) and ($3^{\text{rd}} r_0^\ast$) approximations fall within the range of flexural strength for MWCNTs–acrylic bone cement nanocomposites determined with the use of three-point bending test and are consistent with published data \cite{5, 174}. However, the result values obtained using ($1^{\text{st}} r_0^\ast$) fall within the range of flexural strength for MWCNTs–acrylic bone cement nanocomposites determined with the use of four-point bending test \cite{6, 8}.

Higgs et al. \cite{174} found the flexural strength of 72.6 and 64.8 MPa for Simplex and CMW3 acrylic bone cements when test in three-point bend test and 98.4 and 89.4 MPa when tested using the same method for the current study, (B3B) biaxial test. Marrs et al. \cite{5} measured flexural strength of 85.7 MPa when reinforced an acrylic commercial bone cement had flexural strength of 85.3 MPa with 0.5 wt.% of as produced MWCNTs. Ormsby et al. \cite{6, 8} also reported flexural strength of 56.5, 68.8, and 54.1 MPa when 0.1 wt.% unfunctionalised, carboxyl functionalised and amine functionalised MWCNTs were incorporated respectively, compare to 56.4 MPa for Colacryl B866 bone cement as control. Also, a small value of bending strength of 19.4 MPa was recorded for reinforcement with 0.5 wt.% of unfunctionalised MWCNTs.

It has been reported that the most important key requirements for an optimal fibre reinforced polymer composite system, namely \cite{209}. 

140
• The fibres aspect ratio should be high so that the stress developed within them is significantly larger than the nominal stress on the composite.

• The cross-sectional area of the fibre should be as small as possible, as the strength of the fibre is inversely proportional to the square root of its maximum flaw size; therefore, for smaller cross-sectional areas, the appearance of flaws is reduced.

• The spatial arrangement of the fibres in the matrix has to be of a significant order to ensure a unidirectional, maximal reinforcement.

Also, it has previously been reported that the addition of MWCNTs significantly improves the static properties and fatigue performance of PMMA and the efficacy of MWCNT reinforcement is largely dependent on the level of loadings, the dispersion of these MWCNTs and the peak stress of dynamic loading cycle [4-6, 8, 125].

Ormsby et al.[6, 8] investigated the mechanical properties of acrylic bone cement–MWCNTs nanocomposites with different weight loadings and functionalisation groups using different methods of incorporation. They have reported that adding MWCNTs at higher (≥ 0.5 wt.%) loadings provided a negative effect on the mechanical performance of the nanocomposites cement. Also, they claimed that the 0.1 wt.% MWCNTs concentration was optimal for enhancing the mechanical properties of bone cement matrix. These findings were concurred with our results and this was attributed to poor dispersion of high load of MWCNTs resulting in agglomerations forming within the cement matrix. These agglomerations acted as stress concentrations within the cement microstructure, providing a mechanism for premature failure of the cement when subjected to load. In contrast, low loadings of MWCNTs were more readily disentangled and homogenously dispersed in the resulting nanocomposite. Also, the reduced agglomerations tendency for MWCNTs at lower level loadings can prevent chemical and physical interference during the polymerisation reaction of PMMA cement.

Additionally, it has been assumed that functionalisation of MWCNTs could potentially improve the dispersion and interfacial bonding of the MWCNTs within the cement matrix, thereby leading to improvements in the static and dynamic properties of the resultant bone cement nanocomposites, but to date this has not been proven [4, 5]. Gojny et al.[229] reported that the addition of chemical functional groups to the MWCNTs can provide a negative charge to the MWCNTs and thus reduced agglomeration and improve interaction between the
nanotubes and the host polymer. Pande et al.\textsuperscript{230} stated improvement in mechanical properties of generic PMMA using carboxyl and amine groups chemically functionalised MWCNTs.

Ormsby et al.\textsuperscript{6} reported significant improvements in mechanical properties of the PMMA bone cement as a result of a homogenous dispersion of the MWCNTs within the PMMA matrix aided by the negatively charged carboxyl groups and these improvements were less significant for nanocomposite incorporating amine functional groups. This was postulated to the difference in concentration level of functional groups present on the MWCNTs surfaces. The lower concentration of functional groups may result in a more heterogeneous dispersion of the MWCNTs within the cement matrix, therefore resulting in a less successful transfer of stress through the cement mantle.

In another study, Ormsby et al.\textsuperscript{8} used magnetically stirring, dry blending, and ultrasonic disintegration methods to incorporated different MWCNTs into the bone cement powder or monomer. They have stated that surface modification of MWCNTs with carboxyl groups did not result in significant improvements in the compressive or bending properties of PMMA Colacryl B866 bone cement on a consistent basis and MWCNTs were well-dispersed within cement matrix using an ultrasonic disintegrator method. They also concluded that the reductions in mechanical properties were ascribed to MWCNTs agglomerations occurring within the cement microstructure, and that the degree of these agglomerations was dependent on the method used to incorporate the MWCNTs into the bone cement. This result was consistent with our observations that surface modification of MWCNTs with hydroxyl or carboxyl groups had a slightly negative effect on the static biaxial properties of bone cement composites compared to unfunctionalised MWCNTs at same loadings level.

Singh et al.\textsuperscript{209} stated optimal homogeneity of MWCNTs in carbon-nanotubes reinforced PMMA/HA matrix using a freeze-granulation technique to synthesis a nanocomposite for biomedical applications. They established that the hardness and elastic modulus of MWCNTs-reinforced PMMA/HA nanocomposites increased with increasing concentration of nanotubes up to around 0.1 wt.% and further addition of MWCNTs to the matrix yielded a considerable decrease of these mechanical properties. The biaxial modulus and strength values achieved in the current study are in agreement with this result. This can be explained in terms of the nanotube–matrix contact homogeneity: a further increase of the amount of nanotubes after a concentration of 0.1% MWCNTs, a drop of homogeneity in the nanotubes–matrix contact occurs, giving rise to the formation of voids and internal cavities.
with the eventual reduction in the mechanical performance of the composite. These defects may act as nucleation sites for internal crack tips with a fast degradation of the structure integrity and eventual breakdown \[^{209}\].

Mechanical property such as strength is the first parameter to be assessed to understand the clinical potential and limitations of bone cement. Traditionally, the measurement of acrylic bone cement strength has been by compression testing and four-point bend testing and to a lesser degree three-point bending. Flexural strength is generally considered a meaningful and reliable method to assess the strength of brittle materials as they are much weaker in tension than compression \[^{176}\].

The 4-point and biaxial flexure tests develop lower levels of shear in the test section as compared to the 3-point flexure tests. The stress state in the 4-point and biaxial tests are, therefore, closer to pure bending. Furthermore, the biaxial tests are less sensitive to the edge effects than the 3- or 4-point flexural tests and are less sensitive to the surface imperfections resulting from specimen preparation. In addition, the biaxial test probes for the largest flaws oriented over a wider range of angles, while the 3- and 4-point bending tests are most sensitive to flaws nearly perpendicular to the beam axis of the specimen. It is difficult to separate edge effects from surface conditions. The biaxial flexure test is not dependent upon edge conditions because they are not directly loaded. Therefore, the biaxial test should minimise or reduce the reported variability in acrylic bone cement strength results \[^{182}\].

Our results revealed that the flexural strength of all composites evaluated fulfilled the minimum requirement specified in ISO 5833:02 (flexural strength >50 MPa) \[^{214}\]. Also, the flexural strength values obtained in ring-on-ring test were significantly higher than those obtained by 4-point bending test and lower than those obtained by (B3B) biaxial test, table (4-3) and figure (4-10).

This finding was in agreement with earlier studies conducted on glass-ionomer cements and alumina-ceramic materials. For example, Kanchanavasita \[^{231}\] found the biaxial strength (B3B) of resin-modified glass-ionomer cements to be three times greater (3:1) than their four-point bending strength. Shetty et al. \[^{232}\] also reported that the biaxial (ring-on-ring) strength of alumina was higher than its 4-point bending strength. The discrepancy is attributed to the difference in the effective area or volume of the material subjected to maximum tensile stress and associated with the different testing procedures. In the case of ring-on-ring biaxial test, a uniform line loading was applied as opposed to a point-like load in
the case of a ball loading and so the volume under stress was more representative of the four-point bend test, presenting a greater number of flaws. Therefore, both the ring-on-ring and 4-point specimen would have a greater volume of material under maximum stress compared with much lower effective area under maximum stress directly under the loading ball in the (B3B) test and correspondingly a lower strength \[174\].

In this study the biaxial strength of acrylic bone cement nanocomposites has been determined to depend on the contact area radius, figure (4-3). This supports the association of the biaxial strength value to the effective area or volume of the material under the tensile stress.

It has been established from finite element analysis that the stresses at the edge of the biaxial specimen are minimal and it may be argued that the edges do not play a role in strength determination for this reason alone. Therefore, when considering the different areas under stress, it is apparent that this reasoning comes part way in explaining the difference in biaxial and bending strength, thus, to a lesser degree this difference could be explained by stress gradients through the specimens \[174\].

As is the case for all flexural tests there are significant gradients in the tensile stress during loading. For the point loaded biaxial test the maximum tensile stress approaches a point location with a more gradual surface than through thickness gradients of the stress, thus, only a small volume of the material experiences the peak tensile stress. The tensile stress gradient within this area is significant and diminishes rapidly towards the support points \[174\].

The large standard deviation observed in the biaxial test methods, table (4-3) could be attributed to the complex stress state developed and the frictional force between the contact surfaces. In the present (B3B) loading configuration, the above effects were less significant as compared with the 4-point bend or ring-on-ring arrangements, which involved a larger contact area. Under the ball loading, which involves very small contact area, the material starts to yield when loaded beyond its elastic limit and maximum tensile stress occurs instantly at the centre of the specimen on the bottom support surface \[184\].

Whether measuring the tensile, flexural or compressive strength of a material the data obtained will only provide sufficient information for comparative purposes. Strength values become meaningful when the failure probability of the material is assessed \[183\]. The strength reliability and variability of materials should be studied as the failure stresses of brittle materials are statistically distributed as a function of the flaw size distribution in the material.
A commonly used statistical method to study the strength reliability and variability is Weibull analysis \cite{176}. The main parameters involved are the Weibull modulus and characteristic strength. Weibull modulus \((m)\) is used to describe the variation of the strength or asymmetrical strength distribution as a result of flaws and micro-cracks which may develop within the microstructure. The Weibull modulus can be used as a gauge of the data set’s variance; the higher value of \((m)\) indicates a narrow distribution of defects and thus greater structural reliability for a particular material. Conversely, the lower the value of \((m)\) indicates more flaws and defects in the material and hence decreased structural reliability. This provides the more detailed information required for the prediction of failure stress of brittle materials compared with quoting mean flexure strengths and associated standard deviations alone \cite{176}. The \((m)\) values are subject to specimen size, finish, and test environment because of the possible influences on subcritical flaw growth \cite{182}. Most ceramics are reported to have \((m)\) values in the range of 5–15, whereas metals, which produce ductile failures, have \((m)\) values in the range of 30–100 \cite{176}.

In this study, the Weibull moduli of specimens were in the range of 16.0–29.6 which is acceptable for bone cements, table (4-2) and figure (4-5). These Weibull values indicate that the flaws and stress distribution in these bone cement nanocomposites were similar. Despite the reported positive effects of MWCNTs addition, the data collected for the high concentration of MWCNTs (0.2 and 0.5 wt.%) showed less than ideal results. Unexpectedly, the Weibull modulus for SCOOSH1 cement samples sharply decreased when the MWCNTs were carboxyl groups surface modified. In another hand, when MWCNTs were surface modified with hydroxyl groups in SOH1 cement samples, Weibull modulus was the optimal among all composites. These irregularities are believed to be due to imperfectly disaggregating and dispersing large amounts of MWCNTs into the bone cement matrix. Agglomerations of MWCNTs can nucleate pores and other non-homogeneous regions in the resulting nanocomposite. While individual nanotube and perhaps even clumps of nanotubes can reinforce polymer matrices, clumps of such nanotubes also can have a detrimental role. An elevated number of MWCNTs agglomerations likely contributed to the premature failure MWCNT specimens at the highest stress amplitude.

Therefore, it is unclear whether the improvements in performance of these bone cement nanocomposites are a direct consequence of good MWCNTs dispersion within the bone cement matrix, providing mechanical reinforcement, or is due to a chemical interaction
between the MWCNTs and PMMA matrix. However, Eitan et al.\textsuperscript{[233]} confirmed that the efficiency of the load transfer from the matrix to the nanotubes is improved by surface modification of the MWCNTs.

The observed mechanical behaviour therefore represents the net of both the improvements made by well dispersed nanotubes and detractions made by clumped nanotubes. Thus, it is believed that the limitations of the mixing protocol were met with the addition of high MWCNTs loadings. Perhaps mixing for a longer period of time would further disaggregate such entanglements of MWCNTs, but the likelihood of irreversible damage to cement polymer matrix would become too great.

Weibull statistics are also essential for predicting the reliability of biaxial flexure strength data. In order to accurately assess the strength of a brittle material, the reproducibility of a flexural strength test must be optimised to achieve consistently reliable results\textsuperscript{[183]}. The failure strength corresponding to 1\% or 5\% probability of failure may be more clinically relevant when designing prostheses than the mean strength values\textsuperscript{[182]}. Therefore, perhaps the best way to compare the reliability of all bone cement nanocomposites is by using a survival graph. This line graph depicts the survival probabilities of bone cement at various biaxial flexural strength values; figure (4-8).

Scattering of flexure strength data is associated with $R^2$-value, and it should approximate a straight line on the Weibull plot where an $R^2$-value of 1 would be indicative of a perfect distribution of a single defect type. Multi-modal defects may distort the flexural strength data from a straight-line arrangement and thereby decrease the $R^2$-value, and as a result, the suitability of the Weibull analysis as a design parameter for these strength distributions\textsuperscript{[183]}. It can be seen that all six bone cement composites showed a high correlation with $R^2$-value being 0.90 or higher, suggesting decrease in scatter of the biaxial flexural strength data. It was proposed that cement composites with same $R^2$-values could show different failure mechanism at same stress levels depends on their uniform defect distribution for flexure strength survival probability distributions.

Upon failure, the biaxial specimen typically divided into two pieces and the crack originated from the centre of the specimen and traversed in a radial direction to one of the three supporting points. It is clear that fracture of brittle materials frequently originates at a surface defect or edge defect, and the strength is determined by surface conditions in conjunction with the internal microstructure/defect distribution rather than the internal microstructure.
alone. An explanation for this difference in strength can be attributed to edge effects [174]. The fracture origin observation for all bone cement composite samples showed failure with a threefold symmetry and the fracture initiated at or very near at the tensile surface plane underneath the loading ball; figure (4-11). These observations give clear evidence that the concept of the ball-on-three-balls loading geometry is appropriate.

Implant loosening is one of the most important causes of long-term failure of total hip replacements. Bone cement is used as grouting material to fix the prosthesis to the bone; it completely fills the space between the implant and the bone and does not chemically bond with the implant or the bone. The initial fixation of a cemented implant relies on the strength of the interface between the stem, bone cement, and adjacent bone. The curing process of bone cement is an exothermic reaction where cement undergoes volumetric changes that will generate transient stresses resulting in residual stresses once polymerisation is completed.

There are some discrepancies about the place where the loss of fixation initiates. Some clinical studies show that it begins at the bone–cement interface, but most of them conclude that the stem–cement interface is the most problematic. This is the reason why most of the previous works took into account the bone–cement interface as completely bonded and analysed the influence of bonding conditions of the stem–cement interface on the implant loosening [166, 234, 235].

The attachment of an implant material to bone relates to surface roughness and surface chemistry. There is a relatively low chemical bonding strength of so-called bioactive surfaces. Hydroxyapatite interfaces typically have an interfacial tensile strength of 0.15-1.5 MPa [236]. An attachment force similar to that of bioactive surfaces might also be reached through mechanical interlock with ordinary bone cement.

This study investigated the mode of failure and measured stem/bone cement/bone interfacial bond strengths to estimate the effect of MWCNTs chemical functionality and loadings in these bone cement nanocomposites. Interfacial bonding was evaluated by measuring the load required to detach the plate from bone cement surface in lap joint geometry by tensile force.

Single lap shear test results, table (4-5) and figure (4-17), showed that the bonding strength in both bonding systems is quite good and ranged between 342-470 MPa. The results show that MWCNTs improved the bonding strength between the aluminium sheet adherends and acrylic bone cement. Failure of all aluminium-cement joints occurred at the
cement/aluminium interface, showing interfacial mode of failure; figure (4-18), indicating relatively poor bonding strength between the acrylic bone cement and the aluminium sheets. The results suggest that surface irregularities and micro-interlock induced by MWCNTs on the bone cement surface enable an attachment that can resist tension between aluminium sheet and a cement surface. However, failure of a cohesive type occurred within the rigid polyurethane foam adherends; figure (4-19), indicating good bonding between the cement and the polyurethane foam. This could be attributed to the penetration of the cement mantle through the foam adherends pores. The bonding strength results did not explain much about the effects of the reinforcement of the cement composites as the failure occurred within the adherends, suggesting that the interfacial strength greater that the strength required failing the rigid polyurethane foam adherend.

The most important factor that determines the long-term implant stability is the bone resorption around the implant, which can be caused by different effects, such as, mechanical load alteration, inflammatory reaction associated with infection, inflammatory reaction stimulated by particles of wear debris, implant motion, high fluid pressures and others. Most of these effects are due to physical degradation of the cement and deterioration of the interface between cement and implant. Due to an absence of chemical adhesion, most studies assumed that there is no adhesion between PMMA bone cement and stem, but a geometrical adhesion of the cement to the surface does occur. The assumption of the interface as completely bonded or debonded is also unrealistic, because the disruption of the interface is progressive and its mechanical characteristics depend on the implant surface morphology. It is clear that the roughness of the implant can allow cement to adhere more strongly, thus, favouring bone cement function as an interpositional material assuring implant fixation without reliance on chemical adhesion, although rougher stems usually generate more particles. Besides their dependency on the stem surface finishing, all the mechanical properties of the interface value is related with the rest of the mechanical parameters of the interface \[166, 234\].

5.2 Thermal Analysis

Methacrylate monomers are highly reactive and release a considerable amount of heat during polymerisation. A quantitative understanding of the methacrylate polymerisation is necessary because the thermal history of the polymerisation has considerable influence on the final
properties of bone cement. A simple phenomenological model was used to describe the polymerisation reaction of cement. This model was integrated with an energy balance to predict temperature and degree of monomer conversion across the cement mantle.

Although a vast amount of literature is available on the polymerisation reaction of industrial PMMA, this is not directly applicable to bone cements. Because commercial bone cements consist of a complex mixture of radical initiator, modifiers, and inhibitors, it is very difficult to obtain the characteristics of the polymerisation reaction of acrylic bone cements without detailed and extensive chemical analysis. One of the problems lies in the fact that it is difficult to establish the amount of inhibitor in commercial resins, because it is incorporated during the manufacture of the pre-polymer, and is consumed during the storage of the resin. This means that the resin evolves, and the amount of inhibitor is variable over time. On the other hand, though the decomposition constants of the peroxides are tabulated, they have been obtained in conditions different from those in which the peroxide decomposes during the bone cement polymerisation \[^{189}\]. Due to the complexity involved in polymerising commercial acrylic bone cements, several simplifying assumptions were made to the fundamental reaction kinetic models to develop an empirical or phenomenological analysis to describe the kinetic behaviour, thus, the degree of monomer conversion.

Assuming the monomer reaches a maximum conversion value by providing an external heat source to the reaction. Therefore, reactions performed non-isothermally from 20-180°C at heating rate of 10°C/min were considered to be complete, resulting in a maximum heat released by the reaction \([\Delta H_{(tot)}]\) which is calculated by integrating the total area of the exotherm under the DSC curve. The value obtained for \([\Delta H_{(tot)}]\) represents the heat that would have been generated if the complete monomer conversion (i.e. \(x=1\)) had been achieved. Any heat released by the isothermal reaction at 37°C \([\Delta H_{(iso)}]\) measured below \([\Delta H_{(tot)}]\) value for a bone cement composite is attributed to incomplete monomer conversion and corresponds to a fractional monomer conversion of \(x\).

Isothermal DSC experiments showed that the developed heat, \([\Delta H_{(iso)}]\), is lower than \([\Delta H_{(tot)}]\), for all bone cement composites, thus indicating the presence of un-reacted monomer; tables (4-6) and (4-7). This was revealed by heating a sample immediately after an isothermal polymerisation at 37°C; figure (4-20).
By heating pre-polymerised sample to 150°C at 10°C/min, a residual reactivity exothermic peak corresponds to the reaction heat not released during the isothermal polymerisation is present, indicating that the cement material is not fully polymerised because vitrification prevented the polymerisation reaction from going to completion. This peak is shifted to a higher temperature than the test temperature because vitrification is unable instantaneously to arrest the reaction, as has been observed for thermosetting resins \[^{[222]}\]; figure (4-20b). This shift resulted in glass transition temperature, \((T_g)\), higher than the polymerisation temperature, as a result, the residual peak was overlapped with the \((T_g)\) of the cement powder particles which made it impossible to accurately estimate the residual heat of the remaining reaction. The glass transition temperature of the completely polymerised cement composite measured in a third scan at 10°C/min was \(\approx 114^\circ\text{C}\); figure (4-20c).

The polymerisation reaction started after the inhibitor was consumed and the rate rose up to the maximum due to the well-known gel effect. Finally, the reaction rate decayed rapidly to zero because of the transition of the polymer monomer system from a viscous paste to a glassy solid; figure (4-20a). At the start of the reaction, the reaction rate, \((k)\), was been described mechanistically by the usual kinetic rate equation; Eq.(3-18). The stage of kinetically controlled regime is observed up to the conversion at the point of maximum reaction rate. After the point of maximum reaction rate, in which the monomer conversion is \((x_{\text{max}})\), the reaction rate predicted is higher than that measured experimentally \[^{[189]}\]. This is attributed to a change from chemistry-controlled to diffusion-controlled kinetics in the vicinity of isothermal vitrification. As the polymer-monomer mixture approaches the glass transition point, small molecules become less mobile; hence non-entangled radicals should be subjected to diffusional effects. When a reaction is diffusion controlled, the diffusion of chemical reactants becomes a limiting step. Therefore, the empirical expression Eq.(3-19) has been used to describe the effect of diffusion control in the second part of the reaction peak on the reaction rate constant \((k^*)\).

Ormsby et al. \[^{[6, 8]}\] have reported that incorporation of MWCNTs to acrylic bone cement may reduce the polymerisation exotherm and the number of induced hot spots, therefore reducing the thermal necrosis of the surrounding tissue, adjacent to the cement mantle. Reducing the occurrence of such tissue damage may improve the mechanical integrity of the cement-bone interface, thereby promoting implant longevity. Also, they proposed that any effect on the reaction exotherm was dependant on MWCNTs loadings. The greatest reductions in peak
exothermic temperature were associated with the highest levels of MWCNTs loading. MWCNTs act as a heat sink within the PMMA bone cement and therefore assist in the dissipation of the heat generated during the polymerisation reaction. This behaviour is also a function of the extent of MWCNTs dispersion and distribution throughout the PMMA bone cement matrix, such that uniform dispersion of MWCNTs within the cement will dissipate the thermal energy throughout the cement matrix. This is further aided by the interconnectivity of MWCNTs entanglements and the very large surface area of MWCNTs.

Marrs et al.\(^4\) have stated that the thermal properties of MWCNTs-PMMA bone cement composites can be explained in terms of good dispersion of the MWCNTs within the cement microstructure. For thermally conductive composites, incorporated MWCNTs must form a percolated network of overlapping or touching MWCNTs to transport heat energy. Therefore, bone cements with poor mechanical properties due to relatively poor levels of MWCNTs dispersion within the PMMA matrix and consequent agglomerations, demonstrated the optimal thermal properties.

Ormsby et al.\(^8\) used this theory to explain why different incorporation techniques of MWCNTs into the cement matrix or different chemical functionality of MWCNTs provided differing thermal properties. They claimed that addition of unfunctionalised and amine functionalised MWCNTs provided more significant reductions in the polymerisation reaction when compared to carboxyl functionalised MWCNTs due to improved dispersion of the carboxyl functionalised MWCNTs within the cement matrix in comparison to the other composites.

Our results were consistent with these finding and showed that any effect on the reaction exotherm was dependant on MWCNTs loadings. The greatest reductions in peak exothermic temperature, and consequently un-reacted monomer, were associated with the highest levels of MWCNTs loadings. Conversely, incorporation of hydroxyl (-OH) and carboxyl (-COOH) functionalised MWCNTs increased the peak exothermic, but unexpectedly, carboxyl functionalised MWCNTs produce the maximum amount of un-reacted monomer.

The cement polymerisation reaction involves the conversion of liquid monomer (MMA) to solid polymer (PMMA). This is a free-radical polymerisation, induced and controlled by the availability of a Benzoyl Peroxide initiator (BPO) in the powder and an amine activator (\(N,N\)-dimethyl-paratoluidine) in the liquid. The conversion of monomer to polymer results in the creation of energy, and this energy is released as heat.
We propose that MWCNTs might take part in the reaction during the free radical polymerisation process of MWCNTs-acrylic bone cement nanocomposites whereby the growth of PMMA molecules will be obstructed and much more initiator (BPO) and amine activator will be consumed by the MWCNTs. In the polymerisation process MWCNTs can be initiated by BPO to open their π-bonds. By the opened π-bonds MWCNTs can link with the PMMA, produce a C–C bond between the MWCNTs and the PMMA, thus, a strong interface between the MWCNTs and the PMMA, which results in obstructing the growth of PMMA. Therefore, composites containing higher MWCNTs loadings, i.e. many more π-bonds, will consume much more initiator (BPO) and amine activator than MMA monomer. On the other hand, the chemical functionality of MWCNTs seems to alter the chemical reactivity of WMCNTs, thus the consumption of the activator. This theory explains the increased amount of un-reacted monomer with increase the MWCNTs loadings and why different chemical functionality of MWCNTs provided differing fractional monomer conversion. Also, we have suggested that by adding WMCNTs to the powder component and keeping the L/P ratio constant for all bone cement composites, there is a decrease in the nominal ratio of activator/pre-polymerised polymer powder/ WMCNTs in the mixture.

Ormsby et al.\cite{7} postulated that the presence of MWCNTs in the bone cement matrix not only altered the kinetics of the polymerisation reaction, but additionally played a role in dissipating heat energy. Incorporation of carboxyl and amine functionalised MWCNTs assisted in the dissipation of the heat produced during the exothermic polymerisation reaction of PMMA bone cement, thus, had a greater influence compared to the unfunctionalised MWCNTs.

Our results, however, have showed that the chemical interaction is the key factor for alerting the rate of polymerisation constant. That is, changing the unfunctionalised MWCNTs loadings in the cement matrix affects the polymerisation rate constants, but not as significantly as changing the MWCNTs chemical functionality for the same loading weight, this can be attributed to the larger functional group coverage, and thus, the more potential chemical interaction between the PMMA polymer and the MWCNTs.

Vallo et al.\cite{132} investigated the polymerisation of acrylic bone cements and quantified the amount of monomer present in the cured material using a developed empirical method based on the measurement of the rate at which heat is generated in an exothermic chemical reaction. It was reported that the rate and the degree of cure increased with an increase in the cure
temperature and the ultimate degree of polymerisation attainable by commercially available self-curing Subiton® bone cement cured isothermally at 35°C was ≈0.85. Furthermore, in later study, Vallo et al.\cite{189} reported that the maximum monomer conversion is conditioned by the cure temperature and the vitrification phenomenon and the amount of monomer remaining from the polymerisation is inversely proportional to the cement thickness. These findings were in agreement with experimental results reported by Linder et al.\cite{237} on measurements of monomer leakage from Simplex-P® bone cement by gas chromatography. The amount of leached-out monomer from samples having a same surface area but different thickness revealed that the weight percent of monomer present in samples of 1 mm thickness was about four times higher than that in samples of 5 mm thickness.

It has been reported that the un-reacted monomer acts as a plasticiser that influences the mechanical behaviour of the cement\cite{189}. Swenson et al.\cite{238} reported a 45% of reduction in fatigue strength of cement samples due to incomplete cure of the bone cement resin. On the other hand, De Wijn\cite{239} prepared incompletely cured samples by adding a polymerisation inhibitor to the liquid component of bone cement and reported a decrease of 50% in the bone cement compressive strength. Similarly, results reported in works conducted by Vallo et al.\cite{132, 163, 189, 240} showed that samples containing un-reacted monomer display lower yield stress and higher fracture toughness compared with samples free of un-reacted monomer.

In our mechanical results, the predicted increase in the monomer content that has arisen from high MWCNTs loadings seems to influence the mechanical behaviour of the cement, which is in accord with these findings. However, this theory did not interpret well the mechanical response to the amounts of residual monomer with composites containing functionalised MWCNTs, i.e. SCOOH1 bone cement composites showed higher mechanical properties and yet had a greater quantity of residual monomer compared to SOH1 bone cement composites which had lower quantity of residual monomer and yet showed lower mechanical properties.

However, investigating the polymerisation of acrylic bone cements to quantify the amount of monomer present in the cured bone cement composites using this simple method based on the comparison of the heat generated during isothermal and non-isothermal polymerisation failed to interpret the mechanical response to the amounts of residual monomer in the composites. DSC results imply degree of MAA monomer conversions ranging from 75-60%. If there was residual monomer of 40-25% in the cement mantle, the mechanical properties would reflect a
vastly reduced biaxial strength and modulus and extreme strain at break. This was not the case in our mechanical finding, and thus casts doubt on the validity of the DCS analysis of degree of monomer conversion.

The effect of temperature on biological tissue was first reported by Cornheim in 1873 [241], who observed tissue necrosis caused by supplying a temperature of 52°C for 6 or 7 minutes in rabbit ears. The effect of heat generation during bone cement polymerisation (>100°C) has been traditionally noted [7, 130, 241, 242] and it may result in a permanent cessation of blood flow, as well as causing bone tissue necrosis. The effect of temperature on bone tissue was first reported by Lundskog [242] in 1972. This author indicated that bone tissue heated at 50°C for 1 minute or 47°C for 5 minutes will not remain as functioning bone but will become resorbed and replaced with fat cells, and bone heated to 47°C for 1 minute causes a fat cell injury. This thermally-induced bone tissue necrosis shows no sign of repair after 100 days [7, 130]. Eriksson and Alberksson [243] found that the temperature threshold for impaired bone generation to be in the range of 44-47°C for 1 minute exposure. The higher the temperature the shorter the exposure duration before thermal bone necrosis occurs.

This implies that 44°C is the threshold temperature for the occurrence of morphologically evident bone tissue damage and proved that thermal necrosis is time-dependent.

Therefore, the competency of quantitative index value which represents the likelihood of the polymerising acrylic bone cement causing cellular damage with respect to the maximum polymerisation temperature and the exposure time is required. Hence, a cumulative thermal necrosis index (TNI) determined at two temperature levels calculated from the area under the temperature-versus-time plots related to the exothermic heat generated during polymerisation is much desired and allows for further characterisation of the exothermic reaction of the bone cements.

Henriques [224] demonstrated that burn injury could be treated as a rate process in which the progression of the injury is related both to the temperature and the duration of exposure, (TNI), and chose the threshold of full epidermal necrosis to assign TNI=1, but other data suggested that a value of TNI=0.53 can be taken to be the threshold for first-degree burn (erythema with no cell necrosis). Because of the method used to obtain the formula for TNI, values of TNI other than 0.53 and 1 have no physical meaning. Thus, an exposure resulting in TNI= 2 is not twice as severe as an exposure producing TNI=1.
However, Reed et al.\textsuperscript{[244]} proposed that TNI can be used to calculate a range of injury severities by using the depth of cell necrosis as the index of severity. A burn is more severe if cell necrosis has progressed to a deeper level. In relation to more commonly used descriptions of burn severity, TNI=0.53 corresponds to the threshold of first-degree burn and TNI=1 corresponds to a minimum-severity in which the depth of injury has extended fully through the epidermis. Therefore, estimating TNI for a range of cell depths to identify maximum depth at which TNI>1 is a useful technique.

The level of irreparable damage by bone cement caused by heat generation has been previously assessed using the cumulative thermal necrosis index, (TNI)\textsuperscript{[7, 130]}

Although the volume of cement used to measure the TNI of the cement as per ISO standard is relatively larger than that observed \textit{in vivo}; it is still relevant to use this data as an indicator of the probability of thermal tissue damage due to polymerisation. It should be noted this study, the temperature recorded for the polymerising bone cement is the temperature taken at centre of the sample and the TNI was assessed relatively to \textit{in vivo} temperature (37°C) in which the cement is contained.

It was suggested that the lower the value of reaction rate constant, the more likely it is for the cement to be favourable for the long-term anchoring of a total joint prosthesis\textsuperscript{[129]}. A slower rate of polymerisation extended the time taken for the bone cement to fully polymerise, which in turn reduced the $T_{\text{max}}$ and TNI values\textsuperscript{[130]}.

It can be observed from the results in figure (4-26) that all bone cement composites including the control had produced cumulative thermal necrosis damage of greater than one, TNI > 1 (ranging from 1.09 to 1.36). The TNI value decreased with increase MWCNTs concentration and the minimum values were for SOH1 and SCOOH1 bone cement nanocomposites. This reduced value can be attributed to the reduction in the peak exotherm during polymerisation, which could reduce the temperature increase or exposure time to high temperature experienced \textit{in vivo} therefore preventing the likelihood of the polymerising PMMA cement composites causing thermally-induced bone tissue necrosis.

The reduced rate of reaction due to the MWCNTs influencing the free radical polymerisation process in addition to the thermal conductivity properties of the MWCNTs allowed for these reductions in the peak exotherm during polymerisation. It was noted that the most pronounced influence was recorded for the chemically functionalised MWCNTs which had
the highest reaction rates, and thus, less exposure time. We propose that, considering the heat transfer rate from the maximum temperature ($T_{\text{max}}$) to the body’s temperature (37°C), the TNI value depends on the polymerisation reaction rate and to less degree on the maximum temperature ($T_{\text{max}}$), that is, the higher reaction rate results in less duration of exposure to the injury temperatures (>44°C), thus smaller peak exotherm areas. This theory explains why MWCNTs with chemical functionality provided differing TNI values at same loading weight.

Ormsby et al.\[7\] reported that incorporation of 0.1-1.0 wt% of MWCNTs into Colacryl B866 acrylic bone cement significantly altered the polymerisation reaction and cure kinetics of resultant bone cement; consequently, significant decreases in TNI values (ranging from 3% to 99%) were recorded. The most pronounced influence was recorded for the chemically functionalised MWCNTs. Also, it should be noted, the produced values of TNI were several orders of magnitude different to any other records. Dunne et al.\[130\] quantified the variations in TNI values (ranging from 0.00 to 1.36) as a function of mixing techniques of Palacos R® and CMW3® acrylic bone cements due to the alteration in the proportion of liquid monomer to the polymer powder.

It has been reported that the temperature at bone-cement interface is a function of the quantity of heat produced by the bone cement and the rate at which the heat is produced \[^{130}\]. The thermal properties of the bone, prosthesis and the cement also have significant affect on the temperature at the system interfaces as well as the initial conditions of the system materials, including initial and ambient temperature, and preparation technique of the cement. Since the quantity of energy is directly related to the quantity of cement, larger masses became hotter than smaller masses and some studies had shown that interface temperature during polymerisation of bone cement could reach as high as 100-120°C \[^{130}\]. At first, this would appear a significant issue, considering that protein denaturation occurs at 44°C, with the associated risk of thermal necrosis to bone and soft tissue \[^{131}\]. However, in the case of a conventional cemented femoral component (in THR), a number of factors may potentially combine to mitigate the thermal risk: the relatively large bulk of the metal hip prosthesis, the large surface area of the cement mantle, and the fluid flow around the femur, all acting as heat sinks, dissipating heat away from the cement \[^{131}\]. Kühn \[^{126}\] discussed the fact that, whilst the cement exotherm can achieve above 80°C in the laboratory (using the procedure in ISO 5833:02, for example), this only translates to a maximum of 40–50°C in the clinical setting (in a conventional cemented femoral stem, for example).
5.3 Biocompatibility Analysis

In the human physiological situation osteoblasts are responsible for bone formation and highly exposed to monomer and other cement components after release from bone cement composites in the cement cavity. Therefore, human osteoblast cells (HOB-c) were used to assess the in vitro biocompatibility.

The biological response of osteoblasts onto the surface of MWCNTs reinforced Simplex bone cements was not cytotoxic and exhibited good cell functionality related to cellular adhesion, growing, and viability. The results have also suggested that MWCNTs may possess some bioactive enhancing properties.

The cytotoxic effects of possible substances that could leach out of the acrylic bone cement composites were evaluated by quantifying the cellular viability using MTS-assay. The cytotoxicity assay was evaluated according to ISO 10993-5 "Biological evaluation of medical devices—Test for cytotoxicity: in vitro methods"[225], using direct and indirect contact. The blank reference was taken from wells with 5000 cells, also incubated with the MTS solution. The cell viability was calculated by the normalisation of optical density (OD) to the control.

Our results showed that all three main constituents of bone cement composite had small negative effect on the cell population when tested individually and the MWCNTs was the most toxic among these components; figure (4-27). On the other hand, when these constituents compounded, bone cement composites containing MWCNTs have showed higher cells population than of that of Simplex control bone cement and incorporation of 0.2 wt.% MWCNTs was optimal for cell viability; figure (4-28). Higher MWCNTs loading (0.5 wt.%) did not have a significant effect on the cells population compared to the Simplex control bone cement. Also, a negative correlation between extract concentration and cells population was found, indicating that leaching has a negative effect on cell viability.

These results support the assumption made earlier that MWCNTs take part in the polymerisation process of MWCNTs–acrylic bone cement nanocomposites whereby composites containing higher MWCNTs loadings will consume much more initiator (BPO) and amine activator than MMA monomer. Therefore, we suggest that the initiator, activator, and MWCNTs components of this type of acrylic bone cement composite are more toxic than the nonreactive monomer remaining. However, during the polymerisation reaction, defined
amount MWCNTs react with the initiator and/or activator resulting in new constituent and/or surface modified MWCNTs which improve the biological cellular response and promote cell viability.

This theory explains the reduction of the cell viability at higher MWCNTs. This could be attributed to the fact that the amount of the initiator and activator in the bone cement will react with and/or modify only certain quantity of the MWCNTs, thus, the leachable of these excess MWCNTs will impose its negative influence on the cytotoxic results of these composites depending on the dissolution rate of the matrix material in \textit{in vitro}.

All typical methods and techniques in the literature have been used to investigate the cytotoxicity of bone cement composites such as MTT assay, MTS assay, and fluorescence microscopy, are designed for the analysis of cell proliferation, viability and cytotoxicity, are single end-point qualitative measures of cell fitness. The established assays are labour intensive and comprise a number of manipulation steps that potentially can induce variation of the end-points. In addition there is a great tendency for compound interference because of the optics-based detection methods for most assays, such as absorbance, luminescence or fluorescence, which are vulnerable to distortions. Therefore, the competency of quantitative monitoring cell biological parameters in real-time in \textit{in vitro} cell culture is required. Hence, an automated assay that combines high reproducibility with respect to \textit{in vitro} cell proliferation and viability with easy manipulation is much desired \cite{245}.

Therefore, dynamic cell attachment and population at different densities were monitored in real-time using xCELLigence system to characterise and determine the growth curve of the HOB-c osteoblasts cell line over time; figure (4-29). The results demonstrate that the response seen in concentration of 750-3000 cells/well (≈ 2,800-15,300 cells/cm$^2$) experiments reflects cell cycle effects and can be applied as a conventional end-point in \textit{in vitro} assays. Concentration of 6000 cells/well (≈30,600 cells/cm$^2$) was not suitable for experimentation because of contact inhibition due to its high cell density.

All the factors that increase the number of attached cells on the electrodes, e.g., attachment from solution or cell viability leads to a higher cell index (CI) value. However cell death or toxicity induces cell-detachment, which will lead to a decreased CI value. Despite the same cell numbers, dimensional changes of the attached cells on the electrodes will lead to change the CI, e.g., an increase in cell adhesion or cell spread will lead to a higher CI value. Toxicity can induce cells to spread or cluster thereby leading to a larger cell surface-sensor contact,
which in turn can increase the CI value. On the other hand, toxic compounds can induce cells to round up and/or to detach leading to a decrease in CI \cite{245}.

xCELLigence assay was also used to examine the cytotoxic effects of MWCNTs on HOB-c osteoblasts. The osteoblast cells were challenged with extract solutions of Simplex control and SUNF5 composite which contained the highest loading weight of MWCNTs (0.5 wt.%); figure (4-30). The HOB-c cells were challenged with extracts after 3 h; despite the fact that they are still in the lag phase where they probably fully spread but were not actively proliferating. After feeding at 24 h, the steep increase of the CI of the HOB-c cells may be characterised by its similar adhesion and viability over time. The slow growth response at around 44 h could be attributed to the optimum saturation and confluence of the HOB-c osteoblast cells.

It has been reported that bone formation is mainly dependent upon the number, rather than the activity of osteoblastic cells, and cell number is largely dependent upon cell adherence and proliferation \cite{246}. Thus, the initial adhesion and cell recruitment on the material surface is of great importance to determine differentiation of cells in contact with a material.

For an implant material to be successful it must first promote cellular attachment. Therefore, the effects of MWCNTs chemical functionality and loadings on the initial adhesion of human osteoblasts cells onto the surfaces of Simplex control bone cement and its composites was assessed using direct contact MTS-assay after 24 h of culturing; figure (4-31). Although the numbers of attached cells onto the surface of all MWCNTs-bone cement composites were lower than the Simplex control sample, the cells number increased with increasing the MWCNTs weight loadings and the modified MWCNTs showed more attached cells compared to the unmodified MWCNTs.

The morphology of HOB-c osteoblast cells was investigated using SEM assessment to exam the influence of MWCNTs functionality and loadings on the adhesion and function of osteoblast cells on various bone cement composites, cells were cultured for 3 and 24 h; figure (4-32),

Our micrographs results demonstrate that both Simplex bone cement and its nanocomposites were able to support normal HOB-c osteoblast cell growth, with the well spread and flattened morphology of osteoblast on all samples, although cells were rapidly spreading on the MWCNTs–bone cement nanocomposite material surface rather than the Simplex control.
This result was consistent with previous study by Zhang et al.\textsuperscript{[247]} which reported no obvious changes in cell morphology in MG-63 osteoblast cells cultured in the presence of carboxyl acid groups (-COOH), polyvinyl alcohol (PVA), and biomimetic apatite surface modified MWCNTs. However, Lin et al.\textsuperscript{[248]} also claimed that rat bone marrow-derived mesenchymal stem cells (MSCs) cultured onto poly(lactic-co-glycolic acid) PLGA/carboxyl (-COOH) functionalised MWCNTs nanocomposites exhibited better adhesion over 21 days culture compared with PLGA and PLGA/unfunctionalised MWCNTs control groups.

SEM images highlight qualitatively the cell–surface interactions by depicting cell attachment, cell division (as suggested by the rounded cell spheres in the presence of flattened cells), and cell–cell interactions via their fibrous, which is a sign of cell vitality and effective binding between cell adhesion receptors and extracellular matrix molecules adsorbed on the material surface\textsuperscript{[228]}. Considering the osteoblasts cell viability, this result was in agreement with the confluence response observed with xCELLigence evaluation; figure (4-29).

For evaluation, a comparison of the experiment toxicology results values obtained by the commonly used MTS-based viability end-point assay with the values obtained previously by xCELLigence for Simplex control and SUNF5 extracts, which previously proved informative, revealed a close match between the xCELLigence and indirect contact MTS-assay data, demonstrating that the new xCELLigence system is a dynamic assay format for live cell cytotoxicity assessment.

Bacakova et al.\textsuperscript{[249]} used carbon nanoparticle layers for surface modification of bone implants to improve their integration with the surrounding bone tissue and postulated that these particles could create the nanostructure of the pore walls and enhance the ingrowth of bone tissue and its mineralisation. They also suggested that osteoblasts adhesion may be further enhanced by the derivatization of carbon nanoparticles with chemical functional groups or oligopeptidic ligands for cell adhesion receptors.

The activity of cells’ metabolism correlates with their viability and/or the cytotoxicity of the sample. Therefore, the influence of MWCNTs functionality and loadings on the cell viability was evaluated using live-dead confocal microscopy (LSCM) assessment. The results clearly evidenced that the incorporation of MWCNTs does not have any negative impact on cell viability and all bone cement composites had shown same value of $\approx 85\%$ viability.
In terms of cell function, rounded cellular morphology observed on the Simplex control culture while smaller rounded flattened morphology exhibited when MWCNTs were incorporated. Cell adherence enhanced by increasing MWCNTs loading. Cellular population seemed to increase with increasing MWCNTs loading; figure (4-33). These results were consistent with previous results observed with MTS-assay figures (4-28) and (4-30).

Although, no significant differences were seen for cell viability, figure (4-34) demonstrated that functionalisation of MWCNTs has negative effect on the value of live cells population and the cell morphology. This could be attributed to the fact that such treatments could also be used to remove any residual iron catalyst, which could negatively affect the biocompatibility of the composite \[4\]. This result was inconsistent with previous study by Zhang et al.[\textsuperscript{247}] which reported a reduction (≈20\%) of cell viability in MG-63 osteoblast cells cultured in the presence of carboxyl acid groups (-COOH) compared to polystyrene control. This difference could be attributed to the difference in the types of osteoblast cell lines, and the percentage of the functional groups contains (1.6\% OH groups and 1.2\% COOH groups), or the type control group, i.e. polystyrene.

Mammalian cells typically round up on 2D surfaces prior to mitosis \[228\]. LSCM live-dead images also highlight the absent of initial possible toxic response of the MWCNTs and rounded morphology at all samples surfaces.

Compared to conventional MTS-assay cell viability results, the live-dead laser scanning confocal microscopy (LSCM) viability assessment revealed identical cellular population trends in both methods, demonstrating that the live-dead LSCM viability assessment is an effective qualitative method to determine the cell cytotoxicity. Therefore, LSCM images should be interpreted in relation to the quantitative indirect contact MTS-assay data.

Many researches revealed an understanding of cell–materials interface relationships particularly related to protein adsorption \[248\]. Most mammalian cells are anchorage dependent and need a biocompatible, protein rich surface for attachment, differentiation and migration to form new tissue \[250\].

With acrylic bone cement, the surface presented to cells can be considered as a foreign chemical species with reactive sites \[195\]. The end groups of cement polymer chains may interact with reactive groups such as extracellular matrix (ECM) proteins or carbohydrate molecules in serum.
When a material is implanted in vivo, it is immediately covered with a thin layer of extracellular fluid, and the cells interact with the implant material through this layer. ECM proteins form the most important components of this surface layer for cellular attachment. It could be postulated that MWCNTs present correct scaffold for attachment of ECM adhesion proteins, compared to the plain PMMA Simplex matrix. In consequence, cell fibres probe the material surface to encourage integrin mediated cell adhesion to ECM components. Integrin proteins are transmembrane receptors that bind to specific ECM components and located within cell adhesion (focal contacts), and are thus involved in cellular adhesion in the response to material surfaces. Lin et al.\cite{248, 251} studied the serum protein adsorption for biodegradable poly(lactic-co-glycolic acid) (PLGA) filled with carboxyl-functionalised MWCNTs nanocomposite and reported that the total amount of protein adsorbed on the surface was higher for the nanocomposite compared to the unfilled polymer matrix and the presence of unfunctionalised pristine MWCNTs in the matrix resulted in higher protein binding compared to the carboxyl MWCNTs although the difference was not significant. Such a difference in protein adsorption capability was attributed to the surface chemistry, surface topography, and surface area of the material available for protein adsorption. Cai et al.\cite{252} also detected a little influence of nanometer scale roughness of titanium films on the amount or structure of absorbed albumin or fibrinogen proteins.

Following attachment, osteoblast cells begin a period of high proliferation. Implant materials that can support proliferation are more likely to promote good osteoblast differentiation and subsequent bone formation. Therefore, osteoblasts cell viability on the Simplex control bone cement and its composites was assessed as a function of the MWCNTs chemical functionality and loadings. Direct contact investigation was conducted on all bone cement composites and compared after 3 and 7 days of culturing using MTS-assay. The results were interpreted as early and late stages of cell viability; figure (4-35).

The results of early stage of cell viability (1-3 days) demonstrated that cell viability increased with increase the MWCNTs loadings in the bone cement composites. SUNF5 (0.5 wt.% MWCNTs) bone cement composite showed optimal cell viability and cellular growth rate, thus, is best support for enhancement osteoblast cell viability during early stage. These results correlate with live-dead LSCM assessment; figure (4-33), and MTS assay; figures (4-28) & (4-31). On the contrary, the results of late stage of cell viability (3-7 days) demonstrated that cell viability decreased with increase the MWCNTs loadings in the bone cement composites.
SUNF1 (0.1wt.% MWCNTs) bone cement composite presented the highest cell viability and cellular growth rate, thus, is best support for enhancement osteoblast cell during late stage of cell viability.

It can be concluded, that osteoblast cells responded well to the MWCNTs–Simplex bone cement composites and results in cell viability significantly higher than that on the Simplex control bone cement in this late cell viability stage which confirms the osteogenic effect of the MWCNTs component dispersed within the bone cement composite matrix.

MWCNTs chemical functionality significantly influences the growth rate rather than the cell population, this effect appear to be less significant in the early stage of cell viability; figure (4-35b). This could be attributed to the different chemical reactivity of the MWCNTs surfaces induce by these chemical groups, thus, possible different hydrophilicity of the cement surfaces. These results correlate with MTS assay; figure (4-31).

Incorporating of MWCNTs to the PMMA bone cement leads to higher surface roughness than plain bone cement due to the potential agglomeration of pristine MWCNTs, thus, increase the wettability of the nanocomposites in comparison with the pure polymer. The presence of hydroxyl and carboxyl groups on MWCNTs could improve the MWNTs dispersion in the PMMA matrix and the hydrophilicity of nanocomposites.

Since there were differences in the chemical composition, and any differences in the distribution of MWCNTs in the matrix and their interaction with the polymer matrix should relate to variance in their surface roughness. The rapid total confluence achieved in all cement composite surfaces is due to enhanced roughness and possible excellent bioactivity of MWCNTs. MWCNTs thought to behave as a bioactive booster possibly due to impurity and catalysts or even functional groups on its surface, precipitating apatite crystallisation on its surface, thus, promoting cell proliferation [253]. Apatite is a group of phosphate minerals, named for high concentrations of hydroxyl, fluorine, chlorine, and bromine ions in the crystal.

Zhang et al. [247] suggested that modifying CNTs with some functional groups could activate their bio-inert surface to attract calcium cations and thereby nucleate and initiate the crystallisation of apatite. White et al. [76] also suggested that CNTs possess some bioactive properties. However, there is a concern that catalyst particles may be released from the CNT interior, particularly in the case of CNTs damaged by functionalisation [76].
Although there has been a lot of debate about the biocompatibility of MWCNTs, our results have demonstrated that osteoblast cells adhered to and grew well on the surface of functionalised and unfunctionalised MWCNTs–Simplex-P® bone cement nanocomposites. The cytotoxicity results of all tested bone cement composites was acceptable for biomedical applications [225], therefore, those MWCNTs reinforced Simplex bone cement composites could be used as a scaffold for artificial bone growth due to their biocompatible properties.

George et al. [254] investigated the response of osteoblast cells to MWCNTs. They reported that these cells were well attached and survived on MWCNTs. Hahn et al. [255] also demonstrate that CNTs possess sufficient biocompatibility for use as biomaterials and the addition of CNTs to an HA coating is remarkably effective in improving the biological cellular response to the coating.

Zanello et al. [109] controlled cell growth on CNTs by functionalising the CNTs and reported a dramatic change in cell shapes in ROS (reactive oxygen species) 17/2.8 osteoblasts cultured on chemically modified MWCNTs with poly(m-aminobenzene sulphonic acid) homopolomer and poly(ethylene glycol) and the neutrally charged MWCNTs sustained osteoblast growth and bone formation.

Venkatesan et al. [256] reported that cell proliferation in scaffolds derived from chitosan grafted with functionalised MWCNTs in addition to HAp as bone graft substitutes was twice than in pure chitosan when checked in vitro using MG-63 cell line postulating a great potential applications in the field of bone tissue engineering.

Misra et al. [228] found that the addition of low concentration of MWCNTs (2 wt.%) improved MG-63 cell attachment and proliferation as opposed to higher MWCNTs concentrations (4–7 wt.%) in P(3HB)/bioactive glass composites.

Finally, it should be highlighted that the experimental results obtained here did not test the absolute biocompatibility of MWCNTs, but provided some preliminary evidence regarding the efficacy of using them for making composites in combination with acrylic bone cements.
6 Conclusions
6.1 Conclusions

The biaxial strength and stiffness were determined for a range of MWCNTs reinforced commercially available acrylic Simplex-P® bone cement nanocomposites using a ball-on-three-ball (B3B) biaxial flexural test. The most common approximation approaches found in the literature for the contact radius of the central loading ball were employed. The MWCNTs were incorporated into the commercial bone cement powder using clinically relevant method; ball milling blending technique. Finite element analysis was performed to verify the accuracy of the equivalent contact area. Different flexural strength tests were also conducted to compare the results determined. The influence of MWCNTs chemical functionality and loadings on the cellular function of an in vitro model of human osteoblast cells was studied in order to investigate the in vivo biomedical potential of these bone cement nanocomposites. The obtained results are as follows:

- The biaxial properties of acrylic cements can be determined accurately and simply using the ball-on-three-balls (B3B) biaxial flexure test and the concept of the (B3B) loading geometry is appropriate.
- The (B3B) testing geometry has some advantages compared to other testing assemblies, especially its test fixtures and specimen geometry simplicity, and is less sensitive to edge effects and surface imperfections.
- The approximation presented by Godfrey (3rd $r_0$) $^{[218]}$ is most representative of the area between the loading ball and the disc surface when used in conjunction with the equation presented by Kirstein and Wooley $^{[186]}$ to predict the biaxial strength of acrylic bone cements accurately.
- The difference in strength values among the (B3B) biaxial, ring-on-ring biaxial and four-point bending tests was explained on the basis of the stress gradient through the cement specimen sample rather than the relative areas under stress.
- The mechanical properties of acrylic bone cements can be improved by incorporating small loadings of MWCNTs, concentration of 0.1 wt.% unfunctionalised MWCNTs performs the best reinforcement for the PMMA/MWCNTs nanocomposites. Beyond this limit, further addition of MWCNTs to the PMMA Simplex-P® matrix yields a considerable decrease of the biaxial strength and modulus.


- Functionalisation of the MWCNTs surfaces with hydroxyl (-OH) and carboxyl (-COOH) groups did not enhance the dispersion of MWCNTs within the cement matrix, thus, did not improve the mechanical integrity. Therefore, it is unclear whether the improvements in performance of these cement nanocomposites are a direct consequence of good MWCNT dispersion within the PMMA matrix, providing mechanical reinforcement, or is due to a chemical interaction between the MWCNTs and PMMA matrix through surface chemical modification.

- Incorporation of MWCNTs reduces the exothermic polymerisation reaction for acrylic bone cement, which could potentially reduce the hyperthermia experienced in vivo. The most pronounced influence was recorded for the chemically functionalised MWCNTs.

- The biological response of osteoblasts onto the surface of MWCNTs reinforced Simplex-P bone cement was not cytotoxic and exhibited good cellular function. HOB-c osteoblast cells adhered to and grew well on the surface of functionalised and unfunctionalised MWCNTs reinforced PMMA Simplex-P® bone cement and were able to support normal osteoblast cell growth, with spread and flattened morphology of osteoblasts on all samples.

- The presence of MWCNTs in the Simplex-P® matrix has promoted adhesion and growth of HOB-c osteoblast cells at early stage of culturing suggesting that MWCNTs may possess some bioactive properties.

- Finally, on reviewing the mechanical tests data, it was thought that the fracture toughness testing is more informative and reliable test method than compression, biaxial and bending tests in evaluation the mechanical properties of orthopaedic bone cement materials.
6.2 Recommended Future Work

It is unclear whether the improvements of these bone cement nanocomposites are a direct consequence of good MWCNT dispersion within the PMMA matrix, or is due to a chemical interaction between the MWCNTs and PMMA matrix through surface chemical modification. Therefore, further study to investigate the effect of the type and density of different functional groups on MWCNTs surfaces have on the mechanical and thermal properties as well as the in vitro bioactive performance of these acrylic bone cement nanocomposites is recommended to address some of the limitations of this study is recommended.

Furthermore, the phenomenological method to measure the residual monomer content in cement samples using differential scanning calorimetry (DSC) was not reasonable, therefore, a suitable traditional technique such as gas chromatography (GC) should be used to establish the efficacy of the proposed method.
References
References


References


174


References


References

576578.
244. Reed, M. P. and Schneider, L. W., Skin burns from airbag exhaust gas: laboratory experiment and mathematical modeling. 1994, Transportation Research Institute: University of Michigan.

182
References


Appendix I

The composition of the powder and liquid components as well as initiator/activator ratios of most common bone cements

<table>
<thead>
<tr>
<th>Name</th>
<th>Responsible Manufacturer</th>
<th>Viscosity type</th>
<th>Powder sterilization</th>
<th>Market</th>
</tr>
</thead>
<tbody>
<tr>
<td>C-ment® 1</td>
<td>E. M. C. M. B.V.</td>
<td>high</td>
<td>beta irradiation</td>
<td>Central Europe, G</td>
</tr>
<tr>
<td>C-ment® 3</td>
<td>E. M. C. M. B.V.</td>
<td>low</td>
<td>beta irradiation</td>
<td>Central Europe, G</td>
</tr>
<tr>
<td>Cemex® Isoplastic (HV)</td>
<td>Tecres</td>
<td>high</td>
<td>ethylene oxide</td>
<td>South Europe, I</td>
</tr>
<tr>
<td>Cemex® RX (IV)</td>
<td>Tecres</td>
<td>medium</td>
<td>ethylene oxide</td>
<td>South Europe, I</td>
</tr>
<tr>
<td>Gerafix® LV</td>
<td>Ceraver Osteal</td>
<td>low</td>
<td>gamma irradiation</td>
<td>South Europe, F</td>
</tr>
<tr>
<td>CMW® 1 radiolucent</td>
<td>De Puy</td>
<td>high</td>
<td>gamma irradiation</td>
<td>worldwide</td>
</tr>
<tr>
<td>CMW® 1 radiopaque</td>
<td>De Puy</td>
<td>high</td>
<td>gamma irradiation</td>
<td>worldwide</td>
</tr>
<tr>
<td>CMW® 2</td>
<td>De Puy</td>
<td>high</td>
<td>gamma irradiation</td>
<td>worldwide</td>
</tr>
<tr>
<td>CMW® 3</td>
<td>DePuy</td>
<td>low</td>
<td>gamma irradiation</td>
<td>worldwide</td>
</tr>
<tr>
<td>Duracem™ 3</td>
<td>Sulzer</td>
<td>low</td>
<td>ethylene oxide</td>
<td>Central Europe, CH</td>
</tr>
<tr>
<td>Duros® H</td>
<td>Macmed Orthopedics</td>
<td>medium</td>
<td>gamma irradiation</td>
<td>South Africa</td>
</tr>
<tr>
<td>Endurance®</td>
<td>DePuy</td>
<td>low</td>
<td>gamma irradiation</td>
<td>USA</td>
</tr>
<tr>
<td>Osteobond®</td>
<td>Zimmer</td>
<td>low</td>
<td>gamma irradiation</td>
<td>USA</td>
</tr>
<tr>
<td>Osteopal®</td>
<td>Merck</td>
<td>low</td>
<td>ethylene oxide</td>
<td>worldwide</td>
</tr>
<tr>
<td>Osteopal® HA</td>
<td>Merck</td>
<td>high</td>
<td>ethylene oxide</td>
<td>F</td>
</tr>
<tr>
<td>Osteopal® VS</td>
<td>Merck</td>
<td>high</td>
<td>ethylene oxide</td>
<td>F</td>
</tr>
<tr>
<td>Palacos® LV/E Flow</td>
<td>Schering Plough</td>
<td>low</td>
<td>ethylene oxide</td>
<td>worldwide</td>
</tr>
<tr>
<td>Palacos® R</td>
<td>Merck/Schering Plough</td>
<td>high</td>
<td>ethylene oxide</td>
<td>worldwide</td>
</tr>
<tr>
<td>Palamed®</td>
<td>Merck</td>
<td>high</td>
<td>ethylene oxide</td>
<td>worldwide</td>
</tr>
<tr>
<td>Palavit® HV</td>
<td>Schering Plough</td>
<td>high</td>
<td>ethylene oxide</td>
<td>F, CH</td>
</tr>
<tr>
<td>Palavit® LV</td>
<td>Schering Plough</td>
<td>low</td>
<td>ethylene oxide</td>
<td>F, CH</td>
</tr>
<tr>
<td>Subiton</td>
<td>Prothoplast</td>
<td>high</td>
<td>ethylene oxide</td>
<td>Argentina</td>
</tr>
<tr>
<td>Surgical Simplex® P</td>
<td>Howmedica</td>
<td>medium</td>
<td>gamma irradiation</td>
<td>worldwide, USA</td>
</tr>
<tr>
<td>Zimmer® dough-type</td>
<td>Zimmer</td>
<td>low</td>
<td>gamma irradiation</td>
<td>USA</td>
</tr>
</tbody>
</table>

List of most common bone cement types.[a]
Composition of the powder components of most common bone cements.\textsuperscript{[a]}
Composition of the liquid components of most common bone cements.\textsuperscript{[a]}
Appendix I

In the chart below, the initiator/activator ratios of most common bone cements are depicted. DMAPE=2-[4-(dimethylamino)phenyl]ethanol.[a]


Note: there have been some minor changes in the composition of the powder and liquid components of some bone cements in the last decade, some of these changes are highlighted in section (2.3.3).
Appendix II

Osteoblasts cell line protocols according to the manufacturer’s instructions.

Protocol for Cryopreserved Cells.[a]

(1). Prepare the medium
Calculate the needed culture surface area according to the plating density. Fill the appropriate volume of PromoCell Growth Medium (at least 9 ml per vial of cells) in cell culture vessels. Place the vessels in an incubator (37°C, 5% CO₂) for 30 minutes.

(2). Thaw the cells
Remove the cryovial from the liquid nitrogen container and immediately place it on dry ice - even for short transportation. Submerge the vial into a water bath (37°C) and continuously agitate for 90 sec.

(3). Disinfect the vial and seed the cells
Thoroughly rinse the cryovial with 70% ethanol to avoid microbial contamination. Then, wipe the vial with a tissue. Open the vial under a laminar flow bench and resuspend the cells by carefully pipetting up and down. Transfer the cells to a cell culture vessel with the prewarmed medium from step 1.

(4). Incubate the cells
Place the vessel in an incubator (37°C, 5% CO₂) for cell attachment. Replace the medium after 16 – 24 hours. The cells should be subcultured, according to the subcultivation protocol, once they have reached 70 – 90% confluency.

Protocol for Proliferating Cells.[a]

(1). **Incubate the cells**
Unpack the culture vessel, do not open the lid, and immediately place it in an incubator (37°C, 5% CO₂) for 3 hours.

(2). **Replace the transport medium**
Carefully open the vessel, rinse the inner side of the lid with 70% ethanol, and let air dry. Aspirate the transport medium from the vessel. Add 10 ml of the appropriate PromoCell Cell Growth Medium.

(3). **Check and incubate the cells**
Check the cell density. Open the lid a half turn and place the vessel in an incubator (37°C, 5% CO₂). The cells should be subcultured, according to the subcultivation protocol, once they have reached 70 – 90% confluency.

Subcultivation Protocol[a]

(1). Prepare the reagents and wash the cells
Place the PromoCell DetachKit at room temperature for at least 30 minutes to adjust the temperature of the reagents. Carefully aspirate the medium from the culture vessel. Add 100 μl Hepes BSS Solution per cm² of vessel surface to wash the cells and agitate the vessel carefully for 15 sec.

(2). Detach the cells
Carefully aspirate the Hepes BSS from the culture vessel. Add 100 μl Trypsin/EDTA Solution per cm² of vessel surface. Note: We recommend detaching the cells at room temperature. Close the vessel and examine the cells under a microscope. When the cells start to detach, gently tap the side of the vessel to loosen the remaining cells.

(3). Neutralize the trypsin and harvest the cells
Add 100 μl Trypsin Neutralization Solution per cm² of vessel surface and gently agitate. Carefully aspirate the cell suspension and transfer it to a centrifugation tube. Spin down the cells for 3 minutes at 220 x g.

(4). Incubate the cells
Discard the supernatant (step 1), add 1 ml of the appropriate PromoCell Cell Growth Medium (step 2), and resuspend the cells by carefully pipetting up and down. Plate the cells according to the recommended seeding density in new cell culture vessels containing PromoCell Cell Growth Medium prewarmed to 37°C. Place the vessels in an incubator (37°C, 5% CO₂).