DOCTOR OF PHILOSOPHY

Development of a NanoHydroxyapatite-Collagen Composite Scaffold for Bone Tissue Engineering

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Award date: 2010

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Development of a NanoHydroxyapatite-Collagen Composite Scaffold for Bone Tissue Engineering

Gráinne Cunniffe
BA Hons

A thesis submitted to Queen’s University Belfast for the degree of

Doctor of Philosophy

School of Medicine, Dentistry and Biomedical Sciences

December 2009
DECLARATION

I declare that I am the sole author of this thesis and that the work presented here has not previously been submitted as an exercise for a degree or other qualification at any university. It consists entirely of my own work, except where references indicate otherwise.

Gráinne Cunniffe

QUB student number 17982065

December 2009
Abstract

Scaffolds for tissue engineering must meet certain requirements to make it suitable for use as a bone graft substitute, including a three-dimensional and highly porous structure, with an interconnected pore network to facilitate cell growth and transport of nutrients and metabolic waste products. In addition, the construct must be biocompatible and bioresorbable with controllable degradation and resorption rates to match cell/tissue growth in vitro and/or in vivo. Finally, a suitable surface chemistry for cell attachment, proliferation, and differentiation is required, along with sufficient mechanical properties to provide a support for the formation of new bone tissue.

Collagen based scaffolds are known to be highly biocompatible, with porosity, pore size and permeability suitable for bone tissue engineering. From a clinical perspective however, collagen scaffolds are limited by their mechanical properties, and therefore the primary objective of this thesis was to develop a technique to reinforce a highly porous collagen scaffold by the introduction of the bioactive ceramic, nano-hydroxyapatite (nHA). In Chapter 2 of the thesis, a technique to produce non-aggregating nano-sized particles (<100 nm) of HA was developed using a novel, dispersant-aided synthesis procedure.

In Chapter 3, the incorporation of the synthesised nHA into the collagen scaffolds was investigated. The optimised technique involved the addition of a nHA particle suspension (0 – 500 wt % nHA) to a collagen fibre slurry prior to freeze-drying, and
resulted in a highly porous (>99 %), resorbable composite material with significantly improved mechanical stiffness (18 fold increase) vs. a collagen only control. The homogeneous distribution of the osteoinductive nHA particles also influenced the behaviour of cells cultured on the composite constructs, and *in vitro* analysis (Chapter 4) demonstrated their excellent biocompatibility and capacity to support bone tissue formation. Superior cell attachment was achieved on the collagen-nHA constructs in comparison to the collagen control, in addition to promoting the earlier onset of mineralisation.

Recently in bone tissue engineering, the use of gene therapy has shown much promise for osteogenic applications, with therapeutic genes demonstrating enhanced bone regeneration and healing. However, the efficiency of current delivery systems must be improved to attain controlled and sustained localised gene delivery. The ability of the materials developed in this thesis to transfect cells with plasmid DNA was established in Chapter 5. The nHA particles served as a non-viral delivery method, and were combined with the collagen-nHA composite scaffolds to produce a gene activated matrix. These composite scaffolds facilitated sustained gene expression compared to the collagen only control scaffold, validating their potential for use in bone tissue regeneration.
# Table of Contents

Abstract ............................................................................................................................. ii  
Table of Contents ............................................................................................................ iv  
List of Tables .................................................................................................................... x  
List of Figures .................................................................................................................... x  
Acknowledgements ......................................................................................................... xix  
Nomenclature ................................................................................................................... xxii  
Courses attended, publications and conference presentations .................................. xxiv  

Chapter 1: Introduction and Literature Review .......................................................... 1  
1.1 Overview .................................................................................................................... 1  

Literature Review .............................................................................................................. 5  
1.2 Bone ........................................................................................................................... 5  
1.2.1 Bone Cells ............................................................................................................. 9  
1.2.2 Bone Remodelling and Fracture Repair ............................................................... 10  
1.3 Bone Grafting .......................................................................................................... 13  
1.3.1 Bone Graft Substitute Requirements ................................................................. 15  
1.4 Scaffold Biomaterials ............................................................................................... 20  
1.4.1 Synthetic polymers .............................................................................................. 21  
1.4.2 Natural polymers ................................................................................................ 22  
1.4.3 Ceramics .............................................................................................................. 23  
1.5 Biomaterials investigated in this thesis ................................................................... 25  
1.5.1 Collagen .............................................................................................................. 25  
1.5.2 Hydroxyapatite ................................................................................................... 28
1.5.3 Nano-sized HA (nHA) ................................................................. 30
1.5.4 Synthesis of nano-sized hydroxyapatite ............................................. 34
1.6 Polymer – Ceramic Composite Scaffolds .................................................. 37
  1.6.1 Composite scaffold fabrication ......................................................... 42
1.7 Gene Therapy ......................................................................................... 46
  1.7.1 Gene Expression .............................................................................. 46
  1.7.2 Plasmid DNA ................................................................................... 49
1.8 Methods for plasmid gene delivery .......................................................... 53
  1.8.1 Viral methods ................................................................................... 53
  1.8.2 Non-viral methods ......................................................................... 57
  1.8.3 Calcium Phosphates (CaP) in non-viral gene delivery ....................... 60
  1.8.4 Scaffolds for gene delivery ............................................................... 64

Aims of Thesis ............................................................................................. 67

Chapter 2: Synthesis and characterisation of nanophase hydroxyapatite (nHA) using a novel dispersant-aided precipitation method ......................................................... 68
  2.1 Introduction ......................................................................................... 68
  2.1.1 Aim ................................................................................................. 70
  2.2 Materials and Methods ................................................................. 70
    2.2.1 Materials ..................................................................................... 70
    2.2.2 Preparation of nHA particles ....................................................... 71
    2.2.3 Synthesis parameters ................................................................. 71
    2.2.4 Particle size determination ......................................................... 73
    2.2.5 Electron microscopy and atomic force microscopy ....................... 73
2.2.6 Physico-chemical characterisation ................................................................. 74
2.2.7 Statistical analysis .......................................................................................... 75
2.3 Results .................................................................................................................. 76
  2.3.1 Effect of synthesis conditions on average particle size ................................. 76
  2.3.2 Effect of surfactant/dispersant addition on average particle size ..................... 80
  2.3.3 Analysis of synthesised nHA particles ......................................................... 87
2.4 Discussion ........................................................................................................... 93
2.5 Conclusions ........................................................................................................ 98

Chapter 3: Development and characterisation of collagen nano-hydroxyapatite
composite scaffolds for bone tissue engineering .......................................................... 99
3.1 Introduction ......................................................................................................... 99
  3.1.1 Aim ............................................................................................................... 101
3.2 Materials and Methods ....................................................................................... 101
  3.2.1 Fabrication of Collagen Control Scaffolds ..................................................... 101
  3.2.2 Fabrication of collagen-nanohydroxyapatite (coll-nHA) composite scaffolds
      ......................................................................................................................... 102
  3.2.3 Mechanical Testing ....................................................................................... 103
  3.2.4 Weight Analysis ............................................................................................ 104
  3.2.5 Calcium Assay ............................................................................................. 104
  3.2.6 Scanning Electron Microscopy ...................................................................... 105
  3.2.7 Porosity ......................................................................................................... 105
  3.2.8 Fourier Transform Infra-Red spectroscopy .................................................... 106
  3.2.9 Energy Dispersive X-Ray analysis (EDX) ...................................................... 106
3.2.10 Degradation

3.2.11 Statistical Analysis

3.3 Results

3.4 Discussion

3.5 Conclusion

Chapter 4: Biological evaluation of Collagen - NanoHydroxyapatite (coll-nHA) composite scaffolds

4.1 Introduction

4.1.1 Aim

4.2 Materials and Methods

4.2.1 Scaffold fabrication

4.2.2 Cell culture and seeding

4.2.3 Hoechst DNA Assay

4.2.4 AlamarBlue™ analysis

4.2.5 Histological Assessment

4.2.6 Alkaline Phosphatase

4.2.7 Scanning Electron Microscopy (SEM)

4.2.8 Statistical analysis

4.3 Results

4.3.1 Cell number – DNA Hoechst assay

4.3.2 Metabolic activity

4.3.3 Cell distribution

4.3.4 Cell number
4.3.5 Histological Assessment ................................................................. 140
4.3.6 Scanning Electron Microscopy (SEM) .............................................. 147
4.3.7 Alkaline phosphatase ..................................................................... 149
4.4 Discussion ......................................................................................... 150
4.5 Conclusion ......................................................................................... 155

Chapter 5: Investigation of the potential of nHA particles to transfect in 2D and to sustain release in 3D using coll-nHA gene activated composite scaffolds .......... 156

5.1 Introduction ....................................................................................... 156
5.1.1 Aim ................................................................................................ 158
5.2 Materials and Methods ..................................................................... 158
  5.2.1 Plasmid propagation ...................................................................... 158
  5.2.2 CaP-pDNA complex synthesis .................................................... 159
  5.2.3 Binding analysis .......................................................................... 160
  5.2.4 CaP-pDNA complex analysis ...................................................... 160
  5.2.5 Lipid-pDNA complex preparation (positive control) ................. 161
  5.2.6 Cell culture .................................................................................. 161
  5.2.7 2D Transfection study ................................................................. 162
  5.2.8 3D Transfection study ................................................................. 163
  5.2.9 Luciferase Assay ......................................................................... 163
5.3 Results ................................................................................................ 164
  5.3.1 Analysis of the effect of Darvan on binding efficiency ................. 164
  5.3.2 Analysis of the effect of Darvan on average particle size .......... 164
  5.3.3 Zeta Potential (ZP) ................................................................. 165
5.3.4 2D Transfection study ................................................................. 166
5.3.5 Luciferase release study .............................................................. 168
5.3.6 Analysis of the effect of Darvan on 3D luciferase expression .......... 170
5.4 Discussion .................................................................................... 171
5.5 Conclusion ................................................................................... 178
Chapter 6 Discussion .......................................................................... 179
6.1 Introduction .................................................................................. 179
6.2 Future work: ................................................................................ 190
6.3 Conclusions .................................................................................. 191
References: ........................................................................................ 193
List of Tables

Table 1.1 Summary of a selection of collagen-HA scaffolds demonstrating *in vitro* and *in vivo* behaviour .....................................................41

Table 1.2 Commercial Collagen-HA scaffolds and their methods of fabrication ...45

Table 2.1 Summary of nHA synthesis parameter variations ..................................72

List of Figures

Figure 1.1 Schematic representation of the stages involved in tissue engineering. 1 – cell harvest, 2 – cell culture and expansion, 3 – seeding cells onto an appropriate scaffold with suitable growth factors and cytokines, 4 – incubation of the scaffold under suitable culture conditions and 5 – implanting the engineered construct into the defect site .................................................................2

Figure 1.2 The internal structure of a long bone .................................................6

Figure 1.3 Image showing a magnified view of the inner structure of bone ........6

Figure 1.4 Graph of bone formation markers expression over time showing that ALP (alkaline phosphatase) is expressed during matrix maturation .........................8

Figure 1.5 Schematic illustration of the different cell types present in bone ..........9

Figure 1.6 Schematic diagram showing the remodelling process in bone ............11

Figure 1.7 Diagram demonstrating the differences in appearance of trabecular bone in healthy and osteoporotic bone .........................................................................11

Figure 1.8 Illustration of the stages involved in regular fracture repair ..............13
Figure 1.9 Illustration of where bone can be harvested from within the iliac crest for use as an autograft.................................................................15

Figure 1.10 A selection of commercial products, demonstrating the range of shapes and sizes available.................................................................20

Figure 1.11 Illustration of the enhanced surface interaction of cells with nanocomposite constructs.................................................................33

Figure 1.12 A gene is a short segment of code within long strands of DNA which in turn make up the chromosomes...........................................47

Figure 1.13 Transcription and translation process for synthesising proteins from genetic code.................................................................48

Figure 1.14 Bacterial cell with extra-chromosomal plasmid DNA, showing its ability to integrate into host DNA and replicate................................49

Figure 1.15 Diagram representing the incorporation of a desired gene into plasmid DNA and transformation into bacterial cells for amplification........51

Figure 1.16: A diagram showing the stages involved in gene therapy using an adenovirus vector.................................................................56

Figure 1.17 Lipid-Mediated transfection in mammalian cells......................59

Figure 1.18 Demonstration of the size effect of the delivery vector on the method of internalisation, and hence transfection. (EPR - enhanced permeability and retention)........................................................................63

Figure 2.1 Scanning Electron Microscopy (SEM) images showing the aggregation of nanophase hydroxyapatite (nHA) particles precipitated in the non-optimised
synthesis at different magnifications (scale bars from top left; 100 µm, top right; 10 µm, bottom left and bottom right 1 µm)........................................................................................................77

Figure 2.2 Graph showing the relationship between average particle size and the order of addition of the calcium and phosphate precursors. Additionally, the effect of sonication is shown. (S) = sonication used (NS) = no sonication.............79

Figure 2.3 Graph showing the relationship between average particle size and the rate of addition of the calcium and phosphate precursors in the absence of sonication........................................................................................................79

Figure 2.4 Graph demonstrating the effect of pH on average particle size; a more variable behaviour and larger particles are formed above a reaction pH of 9.5..80

Figure 2.5 Graph showing the effect of the addition of poly(vinyl alcohol) (PVA) on average size of nanophase hydroxyapatite (nHA) particles.................................................81

Figure 2.6 Dynamic Light Scattering (DLS) results displaying the average particle sizes of nanophase hydroxyapatite (nHA) prepared with different concentrations (% v/v) of Darvan dispersant.................................................................82

Figure 2.7 Graph showing the comparative effects of the addition of 6 % w/v poly(vinyl alcohol) (PVA) and 0.1 % v/v Darvan on the average size of nHA particles.....................................................................................................................83

Figure 2.8 Graph showing the effect of the concentration of the initial reactants (calcium and phosphate precursors) on average particle size, when 0.1 % Darvan is present. Additionally, the effect of sonication (10 minutes) is also displayed. X: [Ca] = 0.001 M, 5X: [Ca] = 0.005 M, 10X: [Ca] = 0.01 M, (s) = sonication used, (ns) = no sonication.................................................................84
Figure 2.9 Graph showing average particle size as a function of sonication time. The particles were precipitated in the presence of 0.1 % v/v Darvan dispersing agent at an initial calcium concentration of 0.001 M and phosphate concentration of 0.0006 M.

Figure 2.10 Snapshots of particle size distribution measurements taken during NanoSight™ analysis for (a) nHA particles synthesised using 0.1 % Darvan and (b) the same synthesis conditions with no Darvan.

Figure 2.11 Graph showing the absolute values of zeta potential for suspensions synthesised in the presence of various quantities of dispersant, 0.1 % Darvan and 0.5 % Darvan.

Figure 2.12 Transmission Electron Microscopy (TEM) image showing the morphology of the nanophase hydroxyapatite (nHA) particles synthesised using 0.1 % Darvan dispersing agent.

Figure 2.13 Atomic Force Microscopy (AFM) image showing how the particles tend to re-aggregate slightly due to static forces following oven drying. (2-Dimensional and 3-Dimensional view of the same image).

Figure 2.14 X-Ray Diffraction (XRD) spectra of (top spectrum) the as prepared nanophase hydroxyapatite (nHA) with Darvan and (bottom spectrum) the same sample after calcination at 650°C. The vertical lines show the location of HA peaks based on the JCPDS standard, 72-1243.

Figure 2.15 The broader XRD spectrum (b) shows the presence of a small peak at 22.5° 2θ indicating the presence of ammonium calcium phosphate compared to the HA spectrum synthesised using the original "non-optimised" synthesis (a).
Figure 2.16 Fourier Transform Infra-Red (FTIR) spectra for nHA synthesised (a) with no Darvan, (b) with 0.1 % Darvan and (c) with 0.5 % Darvan present. * denotes the peaks due to Darvan in spectra (b) and (c).

Figure 3.1 (a) Young’s modulus of coll-nHA composite scaffold (powder method) vs. collagen only control, showing a significant increase in Young’s modulus following the addition of 50 wt % aggregated nHA particles (b) Scanning Electron Microscopy (SEM) image of the coll-nHA composite scaffold showing aggregated nHA particles assembled on the collagen fibres.

Figure 3.2 Young’s modulus of composite scaffolds produced using the suspension, *in situ* and immersion techniques (incorporating 10 wt % nHA using the suspension and *in situ* method, and the lowest concentration nHA solution for the immersion method; I-Low).

Figure 3.3 Young’s modulus of a range of composite scaffolds using the Suspension method (50 to 500 wt % nHA) and the immersion method (I-Low and I-High).

Figure 3.4 Graph of the quantity of nHA present in the range of scaffolds determined using weight analysis.

Figure 3.5 Graph of the relative quantities of nHA present in the various coll-nHA scaffolds, based on the content of calcium as determined using a calcium assay.

Figure 3.6 Low power Scanning Electron Microscope (SEM) images (x200 magnification) of a range of collagen and coll-nHA scaffolds, all displaying the porous nature of the constructs.
Figure 3.7 High power Scanning Electron Microscope (SEM) images (x1000 magnification) of a range of collagen and coll-nHA scaffolds, all displaying the detailed surface features of the constructs...........................................................115

Figure 3.8 Graph of scaffold percentage porosities, showing a slight decrease with nHA addition although all scaffolds retain porosity values above 98.9 %......................116

Figure 3.9 Fourier Transform Infra-Red (FTIR) spectra of coll-nHA composite scaffolds fabricated using the suspension and immersion methods (S–100 and I–High) compared to a collagen scaffold and commercial HA powder control spectra......................................................................................................................117

Figure 3.10 EDX spectra for (a) Collagen only control scaffold, (b) S–100 and (c) I–High showing the presence of nHA (Ca and P) in the composite scaffolds, but additionally a large presence of NaCl in the immersed scaffold I–High............118

Figure 3.11 Graph showing the increase in Young’s modulus achieved using an additional, chemical (EDAC/NHS) cross-linking treatment.............................................119

Figure 3.12 Graph showing the degradation of mechanical properties of composite scaffolds after incubation in PBS at 37°C for up to 6 weeks.........................120

Figure 3.13 Graph showing the decrease in weight of composite scaffolds as they degrade over time, up to 6 weeks.................................................................120

Figure 3.14 Micro-computed topography reconstructed image showing the distribution of nHA in the S-500 composite scaffold. The inset shows an image of a collagen only scaffold, demonstrating the inability to detect the collagen fibres using this technique.................................................................123
Figure 3.15 Representative Energy Dispersive X-Ray (EDX) map of a composite scaffold produced using the suspension method, showing the widespread distribution of calcium (green), phosphate (blue) and sodium (red) overlying a corresponding SEM image.

Figure 4.1 Graph showing cell number at 24 hours and 7 days on collagen, S-100 and S-500 scaffolds. The results show significantly more cells are present on the S-500 composite scaffold at each time point.

Figure 4.2 Graph showing metabolic activity on the collagen, S-100 and S-500 scaffolds after 24 hours and 7 days in culture.

Figure 4.3 Haematoxylin and eosin stained slices of the scaffolds. The images show the migration of cells from the surface of the constructs after 24 hours and 7 days in culture (arrows demonstrate cell infiltration). All images were taken at x4 and have the same scale bar (500 µm) as in the first image.

Figure 4.4 Graph showing cell number on collagen, S-100 and S-500 scaffold groups cultured up to 28 days in osteogenic media. The results show that there is no significant difference in cell number between the three scaffold groups.

Figure 4.5 Haematoxylin and eosin stained slices of the scaffolds. The images show the migration of cells from the surface of the constructs at days 0, 7, 14, and 28. All images were taken at x4 and have the same scale bar (500 µm) as in the first image.

Figure 4.6 Von Kossa stained slices of collagen, S-100 and S-500 constructs at days 0, 7, 14, 21 and 28. The brown/black staining shows the presence of phosphate. All
images were taken at x4 and have the same scale bar (500 μm) as in the first image.

Figure 4.7 Alizarin red stained slices, and the measured mineral content graphs of collagen, S–100 and S–500 constructs at days 0, 7, 14, 21 and 28 relative to collagen at day 0. The red staining in the images represents the presence of calcium, and all images were taken at x4 with the same scale bar (500 μm) as in the first image.

Figure 4.8 SEM images of seeded scaffolds at days 0, 7, 14, 21 and 28, showing the deposition of mineralised extracellular matrix (ECM) and presence of cells. The planar morphology and cytoplasmatic processes of the cells is visible at higher magnifications (days 21 and 28).

Figure 4.9 Graph showing the expression of alkaline phosphatase on each construct group, normalised per cell number group following 0, 7, 14, 21 and 28 days in culture.

Figure 4.10 SEM images of the S-500 scaffold at day 28, showing (a) the ability of the cells to bridge gaps (magnification = x1670) and (b) the attachment of cell processes to the scaffold surface (magnification = x16870).

Figure 5.1 Graph showing the percentage of pDNA binding to CaP particles with different amounts of Darvan dispersant present (no_D: 0 %, low_D: 0.016 %, med_D: 0.066 %, high_D: 0.1 %).

Figure 5.2 Graph showing the average particle size of CaP-pDNA complexes with different amounts of Darvan dispersant present.
Figure 5.3 Graph demonstrating the ZP of the CaP particles (no _D, low _D and high _D), and of their corresponding CaP-pDNA complexes, in comparison to the pDNA without CaP particles present (negative control). ................................................. 166

Figure 5.4 Fluorescent images demonstrating transfected MSCs expressing GFP using the no _D, low _D and lipid (positive control) methods. No transfection was observed in the pDNA in CaCl$_2$ (negative control) group ........................................ 167

Figure 5.5 Graph demonstrating luciferase expression from MSCs seeded on collagen, S-100 and S-500 constructs using the lipid (positive control) as the delivery agent (The $*$ symbol indicates when the culture media was changed) ........... 168

Figure 5.6 Graph demonstrating luciferase expression from MSCs seeded on collagen, S-100 and S-500 constructs using no _D as the delivery agent .................. 169

Figure 5.7 Graph demonstrating luciferase expression from MSCs seeded on collagen, S-100 and S-500 constructs using low _D as the delivery agent ............... 170

Figure 5.8 Graph demonstrating luciferase expression from cells transfected using no _D and low _D on the S-100 composite scaffold .......................... 171

Figure 5.9 The effect of Ca/P ratio is demonstrated by the GFP expression achieved using low _D and a suspension with 200 times the Ca/P ratio of low _D ....... 174

Figure 5.10 Fluorescence image demonstrating cell death (bright green cells) in lipid-treated MSCs ................................................................. 175

Figure 5.11 Lipid-mediated GFP expression from MSCs seeded on the S-500 scaffold (background cells dyed red with PKH26 and nuclei stained with DAPI) ...... 177
Acknowledgements

This is probably the hardest part of the thesis to write! Not only because it’s the only part that most people will actually read, but also because I’ve been very lucky with all the wonderful friends and colleagues I’ve been blessed with, so I have to try my best to include you all!

I would like to begin by thanking my dynamic duo of supervisors, Fergal O’Brien and Glenn Dickson, for all the guidance and patience you’ve shown over the last three years. I am very grateful for the opportunity you have given me, and belief you’ve shown through the ups and downs of PhD life. Also, this thesis would never have been finished without the never-ending support of Sonia Partap, and Garry Duffy, and I can not thank you enough for your help. I would also like to thank everyone who helped by reading sections, especially Tanya, Niall, Mike, Matt and Lauren. The support from all the colleagues I’ve worked with over the last few years has been immense, and I would really like to thank you all. In RCSI, I’ve really enjoyed the craic in the office, pub and many lunches out!! Thanks to Niamh, Tanya, Sonia, Matt, Orlaith, Oran, Ciara, Claire, Mike K, Laura, Lauren, Orla, Erica, Tara, Caroline, Amos, Elaine, Frank, Stephen, Maryanne, Amir, Ruth, Mike E, Sarah-Louise, Shona, Cornelia, Mike J, Christian, Peter, Johannes, John G, John O’B, Bernadette, Ross, Peter, Vinny, Amanda and Katie, and the extended tag rugby team and team Vinny running club! In Queen’s I would like to thank Mervyn, Susan, Grant, Sarah, Rosalyn, David and all the people who made me feel so welcome when I started. I’ve really enjoyed getting to know all the other bioengineering PhD students that I’ve met along the way, from Queen’s, Trinity, Jordanstown and UCD. Special thanks to Ken Stanton’s group in UCD for all their
assistance with the nHA work. For all the help with the various techniques used in this thesis, I would like to thank the QUB Chemistry and Pharmacy departments, the Physics and Engineering department in Trinity, and all the help from the Bone Research Group, the Pharmacy and Chemistry departments in RCSI. The Queen’s ladies football team should also get a mention, for all the craic on and off the pitch, made moving to Belfast a pleasure!

I would also like to thank all my friends from all aspects of life, who’ve always been there for me, Ciara, Elaine, Kathy, Mary, Joe, Fiona, Denise, Shane, Mary B, Cormac, Catherine, Sile, Siobhan, Sinead, Charly and all involved with the Brigid’s and Trinity football teams! I’m not sure if I should mention places such as Wagamamas, Butlers, Gerry’s, the Market Bar and Whelans, but they’ve kept me well fed and watered these last few years!

Niamh Walshe deserves a paragraph of her own! For all you’ve helped me with, and put up with over the last few years, I am, and always will be very grateful.

Finally, my wonderful family. My brother’s Gearóid and Dáire who make coming home so enjoyable, I’ve really appreciated all your support through the years, and probably never thanked you enough! I don’t know where to start with my parents, Anne Marie and Michael. Anything good that I have done or achieved has all been thanks to you. I have been so very lucky to have you both teaching me from day 1, and I hope some day to be able to re-pay you for all of your kindness and love.
Nomenclature

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
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<td>Bone Morphogenetic Protein</td>
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<td>Bovine Serum Albumin</td>
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<tr>
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<td>Bone Tissue Engineering</td>
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<tr>
<td>FDM</td>
<td>Fused Deposition Modelling</td>
</tr>
<tr>
<td>FGFs</td>
<td>Fibroblast Growth Factors</td>
</tr>
<tr>
<td>FTIR</td>
<td>Fourier Transform Infra-Red</td>
</tr>
<tr>
<td>g</td>
<td>acceleration due to gravity (m/s²)</td>
</tr>
<tr>
<td>GAG</td>
<td>Glycosaminoglycan</td>
</tr>
<tr>
<td>GAM</td>
<td>Gene Activated Matrix</td>
</tr>
<tr>
<td>GFP</td>
<td>Green Fluorescent Protein</td>
</tr>
<tr>
<td>H&amp;E</td>
<td>Haematoxylin and Eosin</td>
</tr>
<tr>
<td>HA</td>
<td>Hydroxyapatite</td>
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<tr>
<td>HIV</td>
<td>Human Immunodeficient Virus</td>
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<tr>
<td>IGFs</td>
<td>Insulin-like Growth Factor</td>
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<tr>
<td>K</td>
<td>Constant</td>
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<tr>
<td>LB</td>
<td>Lysogeny Broth</td>
</tr>
<tr>
<td>LMO2</td>
<td>LIM domain only 2</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
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</tr>
<tr>
<td>LSNTA</td>
<td>Light Scattering Nanoparticle Tracking Analysis</td>
</tr>
<tr>
<td>MC3T3-E1</td>
<td>Mouse Calvarial Osteoblastic Cell Line</td>
</tr>
<tr>
<td>MCS</td>
<td>Multiple Cloning Site</td>
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<td>MG-63</td>
<td>Human Osteosarcoma Cells</td>
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<td>mRNA</td>
<td>Messenger RNA</td>
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<tr>
<td>MSCs</td>
<td>Mesenchymal Stem Cell</td>
</tr>
<tr>
<td>μCT</td>
<td>Micro-Computed Topography</td>
</tr>
<tr>
<td>nHA</td>
<td>Nano-sized Hydroxyapatite</td>
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<tr>
<td>NHS</td>
<td>N-Hydroxysuccinimide</td>
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<td>NS</td>
<td>No Sonication</td>
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<tr>
<td>OTC</td>
<td>Ornithine Transcarbamylase</td>
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<td>p</td>
<td>Probability</td>
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<tr>
<td>PCL</td>
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<tr>
<td>PDGF</td>
<td>Platelet-Derived Growth Factor</td>
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<tr>
<td>PDLA</td>
<td>Poly(D-lactide)</td>
</tr>
<tr>
<td>PDLLA</td>
<td>Poly(D,L-lactide)</td>
</tr>
<tr>
<td>pDNA</td>
<td>Plasmid DNA</td>
</tr>
<tr>
<td>PE</td>
<td>Polyethylene</td>
</tr>
<tr>
<td>PEG</td>
<td>Poly(ethylene glycol)</td>
</tr>
<tr>
<td>PEI</td>
<td>Poly(ethylenimine)</td>
</tr>
<tr>
<td>PGA</td>
<td>Poly(glycolide)</td>
</tr>
<tr>
<td>PGE₂</td>
<td>Prostaglandin E₂</td>
</tr>
<tr>
<td>pGLuc</td>
<td>Plasmid containing Gaussia Luciferase</td>
</tr>
<tr>
<td>PMMA</td>
<td>Poly(methylmethacrylate)</td>
</tr>
<tr>
<td>PKH26</td>
<td>Fluorescent Dye</td>
</tr>
<tr>
<td>PLA</td>
<td>Polylactic Acid</td>
</tr>
<tr>
<td>PLGA</td>
<td>Poly(DL-lactic-co-glycolic-acid)</td>
</tr>
<tr>
<td>PLLA</td>
<td>Poly(L-lactic acid)</td>
</tr>
<tr>
<td>pMaxGFP</td>
<td>Plasmid containing Green Fluorescent Protein</td>
</tr>
<tr>
<td>PMMA</td>
<td>Poly(methylmethacrylate)</td>
</tr>
<tr>
<td>PVA</td>
<td>Poly(vinyl alcohol)</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic Acid</td>
</tr>
<tr>
<td>RP</td>
<td>Rapid Prototyping</td>
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<td>S</td>
<td>Sonication</td>
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<tr>
<td>SBF</td>
<td>Simulated Body Fluid</td>
</tr>
<tr>
<td>SCID-X1</td>
<td>Severe Combined Immunodeficiency</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium Dodecyl Sulphate</td>
</tr>
<tr>
<td>SEM</td>
<td>Scanning Electron Microscopy</td>
</tr>
<tr>
<td>siRNA</td>
<td>Small Interfering RNA</td>
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<tr>
<td>TCP</td>
<td>Tri-Calcium Phosphate</td>
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<td>TE</td>
<td>Tissue Engineering</td>
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<tr>
<td>TGF-β</td>
<td>Transforming Growth Factor β</td>
</tr>
<tr>
<td>TRAP</td>
<td>Tartrate Resistant Acid Phosphatase</td>
</tr>
<tr>
<td>tRNA</td>
<td>Transfer RNA</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular Endothelial Growth Factor</td>
</tr>
<tr>
<td>Symbol</td>
<td>Definition</td>
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<tr>
<td>v/v</td>
<td>volume per volume solution</td>
</tr>
<tr>
<td>w</td>
<td>weight</td>
</tr>
<tr>
<td>w/v</td>
<td>weight per volume solution</td>
</tr>
<tr>
<td>wt %</td>
<td>percentage by weight</td>
</tr>
<tr>
<td>XRD</td>
<td>X-Ray Diffraction</td>
</tr>
<tr>
<td>Xₘ</td>
<td>Crystallite Size</td>
</tr>
<tr>
<td>Zave</td>
<td>Average Diameter</td>
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<tr>
<td>ZP</td>
<td>Zeta Potential</td>
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<td>™</td>
<td>Trade Mark</td>
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<td>ρ</td>
<td>Density</td>
</tr>
<tr>
<td>®</td>
<td>Registered</td>
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<tr>
<td>βm</td>
<td>Full Width at Half Maximum</td>
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<tr>
<td>θ</td>
<td>diffraction angle</td>
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<tr>
<td>λ</td>
<td>wavelength</td>
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<tr>
<td>Pa</td>
<td>pascal</td>
</tr>
<tr>
<td>kPa</td>
<td>kilopascal (x10⁴)</td>
</tr>
<tr>
<td>MPa</td>
<td>megapascal (x10⁶)</td>
</tr>
<tr>
<td>GPa</td>
<td>gigapascal (x10⁹)</td>
</tr>
<tr>
<td>m</td>
<td>metre</td>
</tr>
<tr>
<td>μm</td>
<td>micrometre (x10⁻⁶)</td>
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<tr>
<td>mm</td>
<td>millimetre (x10⁻³)</td>
</tr>
<tr>
<td>cm</td>
<td>centimetre (x10⁻²)</td>
</tr>
<tr>
<td>μl</td>
<td>micro litre (x10⁻⁶)</td>
</tr>
<tr>
<td>ml</td>
<td>millilitre (x10⁻³)</td>
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<tr>
<td>M</td>
<td>molar</td>
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<tr>
<td>nM</td>
<td>nanomolar (x10⁻⁹)</td>
</tr>
<tr>
<td>μM</td>
<td>micromolar (x10⁻⁶)</td>
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<tr>
<td>mM</td>
<td>millimolar (x10⁻³)</td>
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<tr>
<td>N</td>
<td>Newton</td>
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<td>°</td>
<td>degree</td>
</tr>
<tr>
<td>°C</td>
<td>degree Celsius</td>
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<tr>
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<td>radians</td>
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<tr>
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<td>revolutions per minute</td>
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<tr>
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<td>millivolt (x10⁻³)</td>
</tr>
<tr>
<td>kV</td>
<td>kilovolt (x10³)</td>
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Courses attended, publications and conference presentations

Courses
Medical Sciences Course – anatomy and physiology course leading to awarding of Certificate in Medical Sciences: September 2006, Royal College of Surgeons in Ireland

Awards
- Shortlisted as a finalist for the Engineers Ireland Biomedical Research Medal, January 2010.
- Awarded 1st place prize for the “most innovative use of materials”, NIBES Spring Meeting, April 2009.
- Awarded 2nd place prize for best presentation at a NIBES Spring meeting, April 2008.

Publications

Conference presentations


N. Dunne, A. Boyd (Eds), Belfast, UK: P16)


xxvi


Chapter 1: Introduction and Literature Review

1.1 Overview

Tissue Engineering (TE) is an interdisciplinary field that applies the principles of engineering and life sciences towards the development of biological substitutes that can replace, restore, or improve tissue function [1]. A more recent classification described tissue engineering as "the creation (or formation) of new tissue for the therapeutic reconstruction of the human body, by the deliberate and controlled stimulation of selected target cells through a systematic combination of molecular and mechanical signals" [2].

Clinical success has been reported for a number of soft tissue applications, including bladder and skin [3-5], however difficulties exist in the attempt to engineer mechanically stronger, stiffer tissues such as bone. Bone is a dense connective tissue that consists of cells in an organic and inorganic matrix. The organic component of bone allows for a certain amount of elasticity and flexibility to our movements while the highly mineralised inorganic framework provides strength and stiffness that allows our bones to act as load bearing for our muscles and organs [6, 7]. Bone is a highly dynamic material, constantly undergoing a remodelling process involving the breakdown and removal of old bone and simultaneous deposition of new bone by bone cells. It is this process that enables the repair of micro-cracks and defects that can occur in the body. The majority of fractures can also be repaired through a fracture repair process, although there is a
threshold, or critical size, above which point this normal repair procedure cannot function. Spinal fusion, non-union fracture healing, reconstruction of bone defects resulting from trauma, tumours, infections, biochemical disorders or abnormal skeletal development; all require an intermediate step to augment healing and it is this need that bone tissue engineering (BTE) aims to address.

The basic strategy of BTE involves seeding bone cells onto a scaffold, culturing this cell/scaffold construct so that mineralisation occurs (through application of growth factors and induction of signalling mechanisms) before implanting it into a critical-sized defect in the body. A schematic view of this approach is shown in Figure 1.1.

![Figure 1.1 Schematic representation of the stages involved in tissue engineering. 1 – cell harvest, 2 – cell culture and expansion, 3 – seeding cells onto an appropriate scaffold with suitable growth factors and cytokines, 4 – incubation of the scaffold under suitable culture conditions and 5 – implanting the engineered construct into the defect site [8].](image)
The development of a suitable scaffold to act as a template for this process has been the focus of much investigation due to the many requirements it must satisfy. The role the scaffold plays is of vast importance for the success of the overall procedure as it represents a three-dimensional (3D) replica of the extracellular matrix (ECM) found within bone. The scaffold provides a physical support for the adhesion of cells and allows for their migration, proliferation and differentiation, leading to tissue formation, while acting as a local concentrated environment for signals such as growth factors and cytokines. The appropriate design of a tissue engineered scaffold to perform this function is thus vital, and this aspect of bone tissue engineering is addressed in this thesis.

Recent work in our laboratory has developed a series of novel collagen-based scaffolds with optimised properties to promote bone formation \textit{in vitro}. Collagen provides strength and structural stability to a number of tissues in the body including skin, blood vessels, tendon, cartilage and bone. With nano-sized hydroxyapatite, collagen is one of the two major components of bone. As such, it would appear to be an ideal candidate on which to culture cells to produce bone. The major disadvantage of collagen as a scaffold for orthopaedic tissue-engineering is that it has relatively poor mechanical properties. A number of recent projects in our laboratory have improved the mechanical properties of these scaffolds by developing collagen-hydroxyapatite (CHA) and collagen-calcium phosphate (CCP) scaffolds, and these scaffolds have shown potential in their ability to support bone tissue formation \textit{in vitro} and to heal bone defects \textit{in vivo} [9,
10]. However, some concerns remain over the use of micron-sized hydroxyapatite, as this can lead to brittle constructs with poor resorbability. Therefore attention has turned to the use of nano-sized hydroxyapatite (nHA) to overcome these drawbacks.

In this thesis, the combination of collagen and nHA particles is used to exploit the mechanical stiffness of the resorbable ceramic with the biocompatibility, biodegradability and pore architecture of collagen scaffolds. This composite scaffold would possess inherent properties suitable for use as an \textit{in vitro} template for both bone tissue formation and non-viral plasmid delivery for gene therapy purposes. Both of these topics are examined in this thesis. Gene therapy provides an opportunity to enhance the expression of tissue inductive factors [11]. Recent developments in the field of bone regenerative medicine have explored the transfection of cells \textit{in vitro} and \textit{in vivo} using gene delivery vectors contained within biodegradable scaffolds [12, 13]. Particles of nHA have long been explored for their ability to transfect cells, and thus their incorporation into a 3D collagen scaffold for a potential gene activated matrix is a logical forward step [14].

The focus of this thesis is the synthesis of non-aggregating nHA particles and the subsequent addition of nHA into collagen to form a novel collagen-nHA (coll-nHA) composite scaffold. The capacity of this composite scaffold to form bone tissue \textit{in vitro} is investigated, in addition to the potential use of the nHA particles
loaded on the coll-nHA scaffolds as a gene activated matrix for sustained cellular transfection.

Literature Review

1.2 Bone

Bone is an extraordinary dynamic and adaptable material which is in a continual state of remodelling. Bones function to move, support, and protect the various organs of the body, produce red and white blood cells and store minerals and growth factors. Bone is a nano-composite material, comprised of a mineral phase, collagen and cells. Two thirds of the mass of bone is a mineral which is made up of hydroxyapatite (HA, Ca₁₀(PO₄)₆(OH)₂) nanocrystals, and a small quantity of HA substituted with sodium, magnesium, carbon and fluoride. Collagen fibres account for approximately one third of the mass of bone, while about 2 % of the mass is made up by cells [15]. The mineral phase of bone is a hard, brittle material with strong compressive properties that is unable to endure large shear or tensile loads. On the contrary, the other main component of bone, the collagen fibres, display good bending flexibility and tensile properties but are weak in compression. Therefore, the combination of these two constituent parts provides a complex composite material in which ceramic phase nanoparticles reinforce a framework of collagen fibres, resulting in a strong, somewhat elastic material, resistant to impact loading.
Figure 1.2 The internal structure of a long bone [16].

Compact Bone & Spongy (Cancellous Bone)

Figure 1.3 Image showing a magnified view of the inner structure of bone [17].
In simple terms, there are two distinct regions in bone, an outer dense layer and a spongy internal structure, as shown in both Figure 1.2 and Figure 1.3. The hard outer layer is composed of compact bone tissue, or cortical bone. This accounts for 80% of the total bone mass of an adult skeleton. The inner trabecular bone accounts for the remaining 20%, although it has nearly ten times the surface area of the compact bone. Trabecular bone is an open interconnected porous network, also known as cancellous or spongy bone, through which blood vessels run. The marrow, located within the medullary cavity of long bones and interstices of cancellous bone, produces blood cells by a process called haematopoiesis. The ultimate compressive strength of human cancellous bone is reported as ranging from under 5 MPa to over 15 MPa, depending on age, density and site of test specimen [18].

The organic phase of bone is composed of 90% collagenous proteins (type I collagen 97% and type V collagen 3%) and of 10% non-collagenous proteins (osteocalcin 20%, osteonectin 20%, bone sialoproteins 12%, proteoglycans 10%, osteopontin, fibronectin, growth factors and bone morphogenetic proteins.) All these naturally occurring proteins are synthesised by osteoblasts (discussed in section 1.2.1) and most are involved in cellular adhesion. Fibronectin or vitronectin have been shown to be involved in osteoblast adhesion \textit{in vitro} [19].

Alkaline phosphatase is the bone formation marker analysed in this thesis. Alkaline phosphatase (ALP) is a glycoprotein enzyme present in bone and its activity is a
marker of the osteoblast lineage; it is upregulated during cell maturation, before the onset of mineralisation (Figure 1.4) [20].

![Graph of bone formation markers expression over time showing that ALP (alkaline phosphatase) is expressed during matrix maturation [20].]

Growth factors are proteins that function by stimulating cell proliferation and differentiation. Most growth factors regulate cell behaviour, acting as signalling molecules between cells. Growth factors involved in bone include transforming growth factor-β (TGF-β), insulin-like growth factors (IGFs), platelet-derived growth factor (PDGF), fibroblast growth factors (FGFs), and bone morphogenic proteins (BMPs). Bone formation is regulated by a complex sequence of events, beginning with the recruitment and proliferation of progenitor cells followed by cell differentiation, osteoid formation and eventually mineralisation [21]. The inorganic phase or mineral phase of bone is based on a nanocrystalline calcium phosphate material which is discussed in more detail later (section 1.5.3).
1.2.1 Bone Cells

Four types of cell are present in bone: osteoprogenitor, osteoblast, osteocyte and osteoclast cells (Figure 1.5).

![Figure 1.5 Schematic illustration of the different cell types present in bone][1]

Osteoprogenitor cells are immature progenitor cells derived from mesenchymal cells and differentiate into an osteoblast. They are also called preosteoblasts, as they are committed to the osteoblast lineage but not yet differentiated.

Osteoblasts are the immature mononuclear bone-forming cells that derive from osteoprogenitor cells. They are located on the surface of osteoid seams and produce proteins and organic components that form osteoid, which mineralises to become bone. Osteoid is primarily composed of Type I collagen. Osteoblasts also manufacture hormones, such as prostaglandins (PGE is anabolic to bone), to act on the bone itself. They produce alkaline phosphatase, an enzyme that has a role in the mineralisation of bone, as well as many matrix proteins.
Osteocytes are the most common bone cell. These are mature cells that occupy lacunae between layers of bone matrix. Osteocytes derive from osteoblasts that have migrated into and become confined and bounded by the newly produced bone matrix. Osteocytes have many processes (extended arms of cytoplasm) that reach out to meet osteoblasts and other osteocytes, connecting lacunae together. They have also been shown to act as mechano-sensory receptors—regulating the bone's response to stress and mechanical load.

Osteoclasts are the cells involved in bone remodelling responsible for removing bone via resorption. Osteoclasts are large, multinucleated cells located on bone surfaces in what are called Howship's lacunae or resorption pits. These lacunae, or resorption pits, are left behind after the breakdown of the bone surface. Because the osteoclasts are derived from a monocyte stem-cell lineage, they are equipped with phagocytic like mechanisms similar to circulating macrophages. Osteoclasts mature and/or migrate to discrete bone surfaces. Upon arrival, active enzymes, such as tartrate resistant acid phosphatase (TRAP), are secreted against the mineral substrate.

1.2.2 Bone Remodelling and Fracture Repair

A dynamic balance exists within bone, in which old bone is resorbed by osteoclasts while new bone is laid down by osteoblasts simultaneously (Figure 1.6). This continual process is known as bone remodelling, and is quite extensive - the entire human skeleton is replaced every ten years. The purpose of remodelling is to
regulate calcium homeostasis, repair micro-damaged bones (microcracks due to everyday stresses) and also to shape the skeleton during growth. An imbalance in this process can lead to osteoporotic bone and an increased risk of fracture (Figure 1.7).

**Bone Remodeling Cycle**

![Bone Remodeling Cycle Diagram](image)

**Figure 1.6** Schematic diagram showing the remodelling process in bone [22].

**Figure 1.7** Diagram demonstrating the differences in appearance of trabecular bone in healthy and osteoporotic bone [23].
Minor defects and micro-cracks can be repaired in the body based on this remodelling process. New material is produced rapidly in order to prevent fatigue causing major failure. Most fractures can also be healed by the body even after severe damage, provided that the blood supply and the cellular components of the endosteum and periosteum survive. This repair process is outlined in a number of steps illustrated in Figure 1.8. A large blood clot, or fracture hematoma is formed immediately following fracture, shutting off the injured vessels and leaving a fibrous network at the damaged site. Osteoprogenitor cells divide rapidly, and the new osteoblasts migrate to the fracture zone. An internal spongy bone callus combines with an external cartilage callus to temporarily stabilise the fracture. This callus is remodelled by osteoblast and osteoclast activity, firstly replacing the cartilage with spongy bone before the entire site is eventually reshaped over time, often leaving the original site stronger than prior to the fracture.

There is, however, a limit to the size of defect that the body can repair in this way. Above this critical size injury, the bridging necessary for repair can not occur and this is referred to as a critical size defect. These injuries require the use of a suitable bone graft to enable full healing.
Step 1
Immediately after the fracture, extensive bleeding occurs. Over a period of several hours, a large blood clot, or fracture haematoma develops.

Step 2
An internal callus forms as a network of spongy bone unites the inner edges, and an external callus of cartilage and bone stabilizes the outer edges.

Step 3
The cartilage of the external callus has been replaced by bone, and struts of spongy bone now unite the broken ends. Fragments of dead bone and the areas of bone closest to the break have been removed and replaced.

Step 4
A swelling initially marks the location of the fracture. Over time this region will be remodelled, and little evidence of the fracture will remain.

Figure 1.8 Illustration of the stages involved in regular fracture repair [15].

1.3 Bone Grafting

Approximately 5-10% of the 8 million bone fractures that occur in the US annually require the use of a bone graft to achieve full regeneration [24]. The selection of a material, suitable for use as a bone graft is complicated due to the complex nature of bone. A bone graft is defined as a material that when implanted in vivo will bring about a bone healing response by providing osteogenic, osteoconductive or osteoinductive activity to the implant site. The aim of the implanted material is to provide a template suitable for healing of the non-union fracture or defect, completely restoring function to the damaged site as quickly as possible. In clinical practice, bone grafts are used to replace diseased bone, fill bone voids after non-union fractures, tumour or cyst removal, dental implants, reconstructive surgery and in spinal fusion operations.
The use of an autograft is regarded currently as the gold standard for these procedures with an allograft being second choice, while both have associated drawbacks. Autologous bone (autograft) is bone removed from non-essential regions within the patients themselves, typically from the iliac crest (Figure 1.9), although other areas such as the distal femur, the greater trochanter and the proximal tibia have also been used [25, 26]. This harvested bone is then implanted back into the damaged site. This transplantation is expensive (requiring two major surgeries) and there is a limit to the quantity of available bone. Significant pain and morbidity at the donor site are further problems associated with this method along with the low survival rate of cells transplanted in an autograft which is another cause for concern.

Allografts and xenografts are also commonly transplanted bone graft materials. These derive from donor human or animal bone and are linked with the danger of infection, possible prion transmission or initiation of an immune response. Additionally, the use of coral has been investigated, although the structure differs greatly from bone, hence relating to difficulties with osteointegration [27]. Therefore, the need exists to develop an alternative bone graft substitute solution for the effective and reliable replacement of autografts and allografts.
1.3.1 Bone Graft Substitute Requirements

For a scaffold to be successful as a bone graft substitute or a template for tissue repair it must meet certain basic requirements. A vital issue is the biocompatibility of the material and the prevention of any detrimental immune response from the patient, requiring the substitute and any degradation products to be non-immunogenic, non-reactive in a negative manner and non-toxic. In addition, a biocompatible scaffold should also sustain cell function and phenotype. The choice of material for the scaffold plays an important role in the success or failure following implantation. The material should be bioinert at least, not provoking an immune response, but preferably the material should be bioactive, stimulating a desired positive reaction from the surrounding tissue. Therefore, a material that encourages osteogenesis would be beneficial. While biocompatible means not
eliciting an immune reaction, a graft material is required to be at least osteoconductive if not osteoinductive. Osteoconduction occurs when the bone graft material serves as a template for new bone growth that is perpetuated by the native bone, supporting the ingrowth of capillaries, tissue and cells into the scaffold. Osteoblasts from the tissue surrounding the defect utilize the scaffold material as a framework upon which to spread and generate new bone. Osteoinduction involves the stimulation of osteoprogenitor cells to differentiate into osteoblasts that then instigate new bone formation. A bone graft material that is osteoconductive and osteoinductive will not only serve as a scaffold for currently existing osteoblasts but will also trigger the recruitment of new osteoblasts, theoretically promoting faster integration of the graft. Generally, osteoconductive graft materials implanted into a defect area would not produce bone but osteoinductive materials would induce bone formation [29].

Another essential feature of scaffold performance is biodegradability. The scaffold must be designed to degrade into safe degradation by-products with a suitable degradation rate for the eventual repair or regeneration of the damaged tissue. The degradation process should be controllable so that the scaffold can maintain mechanical viability long enough to support the activity of the cells as they carry out the remodelling process, but additionally it is essential that the graft be resorbed over time to prevent further surgery for its removal. Ideally, resorbable graft materials act as a temporary support, being replaced gradually by osteoclasts removing the graft simultaneously whilst osteoblasts deposit new bone.
This aspect is closely linked with the inherent mechanical properties of the selected scaffold, which greatly influences the success or failure of the implanted material. To be useful clinically, the scaffold must facilitate in vitro handling and also remain viable within the defect site, although the use of external supports such as sutures and fixation devices are employed to bear mechanical loading during rehabilitation [30, 31]. External fixation devices are required, even following the implantation of cortical bone grafts [32]. Therefore, the belief that a scaffold for bone tissue engineering must have mechanical properties resembling that of native bone is negated, especially since other factors such as interconnected porosity and pore size are to be considered. Preferably a balance should be reached, between appropriate construct strength, and suitable pore architecture, rather than focusing on the development of a strong construct.

The mechanical properties of scaffolds have also been shown to influence cellular behaviour both in vitro and in vivo, by affecting processes ranging from cellular interaction with the substrate to guiding cell proliferation, differentiation and ultimately tissue formation and functionality of the implanted construct [33-39]. In a study by Engler et al. (2006), the differentiation of mesenchymal stem cells (MSCs) was shown to be dependant upon the mechanical properties of the various substrates upon which they were seeded [34]. Neurogenic, myogenic and osteogenic markers were found on substrates with Young’s moduli of 1, 11 and 34 kPa respectively. Cellular motion has also been shown to be guided by the rigidity of a substrate. Lo et al. (2000), demonstrated that fibroblasts could distinguish between stiff and compliant sides of a polyacrylamide gel covered in collagen [40].
Scaffolds for tissue engineering can be subject to cell-mediated contraction, leading to a reduction in scaffold volume and problems with porosity and permeability, hence affecting cellular proliferation [41]. Additionally, construct contraction in vivo would have detrimental effects on the interface between host tissue and implant. The degree of contraction was shown to be influenced by the intrinsic mechanical properties of the scaffolds [37, 42]. Therefore, the need for a scaffold with sufficient properties to withstand excessive cell-mediated contraction is essential for successful bone grafting.

It is imperative to achieve suitable mechanical properties without compromising pore architecture, necessary for graft success after implantation. Scaffolds for bone grafting require a high porosity to facilitate tissue in-growth and vascularisation [43-48]. Adequate interconnected porosity and suitable pore size dimensions are needed to enable cell migration to the centre of the construct for a homogeneous distribution, and also for the delivery of nutrients and removal of waste products from this region [49]. One view reported in the literature is that the pore diameter should be designed for specific cell types, with pore size balanced between being large enough to allow cell infiltration into the scaffold but small enough to offer sufficient ligand density for cellular attachment [47, 50-52]. A high surface area is hypothesised to enhance bone growth by increasing protein adsorption [53]. Osteoblast attachment on collagen scaffolds was found to be higher in scaffolds with smaller pore size, with optimal attachment achieved at 95 μm [47]. Large pores (>100 μm) are known to favour bone formation and vascularisation in vivo [45, 50, 54], whilst also increasing scaffold permeability, enabling nutrient
diffusion and waste removal [48, 55]. Porosity is inversely proportional to mechanical strength, so in order to maintain a high porosity some approaches need to be taken to reinforce the construct, especially when working with natural polymers such as collagen. The use of composite materials, where the natural polymer is strengthened by a stiffer material is one technique presented in this thesis which is combined with physical and chemical crosslinking methods developed in our laboratory.

A range of commercial products using ceramics, polymers and composites of the two are available for use as bone graft materials with some promising results (Figure 1.10). Despite many attempts, an ideal off-the-shelf product which delivers all the requirements of a bone graft has not yet been produced. The following section details the biomaterials employed for scaffold development, and how the correct choice of material, or combination of materials can result in different resultant constructs.
A selection of commercial products, demonstrating the range of shapes and sizes available [56].

1.4 Scaffold Biomaterials

A biomaterial can be defined as a material (natural or synthetic), intended to interface with biological systems to perform, augment or replace a natural function of the body. Recent discussion has suggested a renewed definition to include recent developments; “A biomaterial is a substance that has been engineered to take a form which alone or as part of a complex system is used to direct, by control of interactions with components of living systems, the course of any therapeutic or diagnostic procedure, in human or veterinary medicine” [2]. First-generation biomaterials were designed to purely restore function, remaining biologically inert, such as in early hip arthroplasties. Current investigations take a more proactive approach, often designing bioactive biomaterials to promote specific positive
biological responses from cells and host tissue. There are four distinct classifications of materials that have been used to fabricate scaffolds for use in tissue engineering: synthetic polymers, natural polymers, ceramics and composites, each having different characteristics that make them attractive as scaffold materials. In this thesis, the combination of a natural polymer, collagen, and a ceramic, nano-hydroxyapatite (nHA) is investigated, and both of these biomaterials are discussed in more detail in the following sections.

1.4.1 Synthetic polymers

Synthetic polymers are a highly versatile choice for tissue engineering, given the ability to easily tailor a wide range of mechanical properties and architectures. Polymers consist of chains made up from the combination of small unit monomers. Different chain lengths result in varying polymer stiffness and rigidity. In medical applications, polymers have been used to make catheters, prostheses, disposable instruments, artificial organs, blood vessels and scaffold materials. Many polymers in use in tissue engineering today have received FDA approval such as polyglycolide (PGA), polylactides (PLLA, PDLA), copolymers of both (PLGA), polycaprolactone (PCL) and polyethylene-glycol (PEG) [57, 58]. Poly(vinyl alcohol) (PVA) is an example of a synthetic polymer that can be combined with hydrogels, especially for use in skin regeneration. They provide a moist layer which has been found to promote wound healing while protecting the wound from infection. This material, while beneficial in skin tissue engineering, would not be suitable for use as a bone graft however due to insufficient mechanical strength and
poor biodegradability. The fundamental problem associated with the use of synthetic polymers is their degradation products, acidity and alcohol content [59-61]. In particular, scaffold acidity has been implicated in accelerating the degradation of the scaffold and causing a pronounced inflammatory response in the surrounding tissue leading to an inhibition of tissue formation [62]. Examples of this phenomenon were observed in the use of polyethylene (PE) in joint replacements and poly(methylmethacrylate) (PMMA) as cement to make titanium implants adhere to bone. Wear debris from PE induced an inflammatory response and the subsequent encapsulation around the implant by soft fibrous tissue led to implant loosening [63]. Similarly high levels of apoptosis (cell death) occurred following the use of PMMA leading to implant failure [64]. Mechanical activity between the implant and host bone will cause degradation of the synthetic polymer, and lead to an inflammatory reaction at this interface, preventing the formation of a strong bond at this vital position. In another study, lactic acid, the by-product of PLA degradation, was observed to cause an adverse cellular response, inducing the release of prostaglandin (PGE$_2$) which is a bone resorbing and inflammatory mediator [65].

1.4.2 Natural polymers

Naturally derived polymers such as hyaluronic acid, alginate, chitosan, silk fibrin, elastin, glycosaminoglycans and gelatine have received attention for use in biomaterial development and tissue engineering [66-74]. The success of natural polymers stems from their ability to provide innate biological information to cells
through the availability of natural surface ligands and peptides for adhesion as well as promoting chemotactic responses. Natural polymers are typically the constituents of natural extracellular matrix (ECM) and hence they have excellent biocompatibility and non-toxic degradation products [75]. A disadvantage of natural polymers is their poor mechanical properties, which are not sufficient for high strength applications unless the polymer is chemically treated or reinforced by a stiffer filler material. In particular, research into the natural polymer collagen has proved promising and will be discussed in detail below.

1.4.3 Ceramics

Ceramics are inorganic materials that are generally crystalline in structure. Their characteristics include hardness, high compressive strength and high melting points. Ceramics also tend to be chemically inert and thermodynamically stable. Drawbacks of ceramic scaffolds for tissue engineering include brittleness, poor tensile properties and some difficulties with fabrication. The properties that make ceramics attractive for use in scaffolds (particularly in hard tissue applications) are their bioactivity and their high compressive strength. Common ceramic materials used in tissue engineering are calcium phosphates, calcium sulphates and bioactive glasses. The calcium phosphate (CaP) family of minerals contains calcium ions, orthophosphates, meta-phosphates and occasionally hydrogen. The most common forms are tri-calcium phosphate (TCP) and hydroxyapatite (HA) [76-82]. Calcium phosphates (CaP) are the most ubiquitous family of bioceramics, well known for their use in biological applications. There are several forms of CaP structures
which typically form a family of compounds called apatites. This term describes a family of compounds having a similar hexagonal structure. The inorganic component of bone and teeth is very similar to the most common CaP, hydroxyapatite (HA) $\text{Ca}_10(\text{PO}_4)_6(\text{OH})_2$ and as such has led to much investigation of HA for use in bone tissue engineering. Other CaP phases include brushite ($\text{CaHPO}_4\cdot2\text{H}_2\text{O}$) and tricalcium phosphate (TCP; $\text{Ca}_3(\text{PO}_4)_2$). Brushite has a Ca/P ratio of 1 and can be synthesised at a pH of 4 to 6. However, it has poor stability and is generally used as a precursor for HA synthesis. TCP has a Ca/P ratio of 1.5 and can be found in two allotropic forms, $\beta$-TCP and $\alpha$-TCP. $\beta$-TCP is generally converted to $\alpha$-TCP following sintering at high temperatures. TCP is generally more resorbable than HA. Giannoudis et al. (2005) found that porous TCP is removed from the implant site as bone grows whilst HA is less resorbable [83]. Both brushite and TCP are calcium deficient as they have a Ca/P ratio less than the stoichiometric value of HA, 1.67 [84]. HA is discussed in greater detail in a following section.

Calcium and phosphate based biomaterials such as calcium phosphate ceramics and calcium phosphate silica glasses (bioactive glasses) exhibit excellent bone-bonding properties [85]. Calcium sulphate materials are fully resorbable and mostly used as osteoconductive bone void fillers such as for the filling of cysts, bone cavities, segmental bone defects and the filling of bone-graft harvest sites [31]. Bioactive glasses are another subset of ceramics, typically composed of silica, calcium oxide, disodium oxide and pyrophosphate and when implanted they form
strong bonds with collagen, fibrin and growth factors. Bioglasses are also considered bio-mimetic as they stimulate the formation and precipitation of calcium phosphate from solution and therefore enhance native ECM formation [85, 86]. The resorbability of bioactive glasses can be modified by combination with crystalline HA to persist for longer periods following implantation. However, they are extremely brittle and are not suitable for use in load bearing conditions in vivo [87]. Ceramics such as coral (calcium carbonate ceramic) may be harvested from nature and have an interconnected porous structure similar to trabecular bone. However, coral is highly brittle, lacking tensile strength similar to synthetic ceramics.

1.5 Biomaterials investigated in this thesis

1.5.1 Collagen

Collagen is a major component of the ECM and is the most common structural protein found in the body. It is a key constituent of tendons, skin, blood vessels, ligaments, cartilage and bone. Over twenty genetically different types of collagen molecules exist [88], and are located in tissues that are subjected to mechanical forces within the body [89]. Collagen type I is the most abundant collagen protein present in the human body and is found in many tissues such as skin, ligaments, tendons and bone. For this reason, along with its biocompatibility, biodegradability and hydrophilic nature, the use of collagen type I as a bone graft substitute has
been of much interest to researchers, including investigations within our laboratories.

Collagen type I is a heterotrimer, consisting of triple helical fibrils made of polypeptide chains with carboxyl groups, interconnected by covalent and hydrogen bonding \[90, 91\]. The triple helical structure is important to the nature of collagen as it protects the collagen from being broken down by proteases and is important for cell adhesion and the assembly of the ECM \[92\]. The degradation rate of collagen can be controlled by varying the degree of crosslinking (i.e. covalent bonds) present in and between collagen molecules \[93\]. Intra-molecular bonds are covalent bonds that form between the three helices within the collagen molecule, while bonds between individual molecules also develop and these are called inter-molecular bonds. In this thesis both physical and chemical crosslinking methods were employed to improve the mechanical properties of collagen scaffolds. Dehydrothermal (DHT) crosslinking is a physical method which involves heating the collagen scaffold in a vacuum. This method has the added advantage of sterilising the scaffold although care must be taken not to overheat the collagen as this causes denaturation to occur. EDAC crosslinking is a chemical method which involves immersing the collagen scaffold in a solution of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide and N-hydroxysuccinimide. It has been found that a combination of DHT at 105°C for 24 hours with subsequent EDAC treatment is suitable for improving the mechanical stability of collagen scaffolds \[94\].
The favourable characteristics of collagen has inspired recent research to focus on its use in tissue engineering applications, with investigations of collagen synthesised into gels, foams, beads and sheets in an attempt to manufacture an appropriate 3D scaffold for cell culture [95]. Cells have been shown to attach, proliferate and form ECM on porous collagen scaffolds, confirming collagen as a promising scaffold material [96, 97]. The intrinsic biological properties of collagen allow it to be easily absorbed into the body. It also exhibits a high affinity for water, this fluid binding capacity allows extra proteins such as fibrin and glycosaminoglycans to become attached to it, therefore, improving the physical and biological properties [98]. In relation to bone tissue engineering, collagen type I has been shown to positively affect the expression of the bone cell phenotype and is also fundamental to the development and expression of the osteoblast phenotype and formation of a mineralised tissue [99].

Scaffolds combining natural polymers collagen and glycosaminoglycans (CG scaffolds) have been used successfully in skin grafting [100], and work has been conducted in our laboratory in adapting this scaffold for bone tissue engineering, with results demonstrating their ability to support both chondrogenesis and osteogenesis [101]. Another study showed that varying the collagen or glycosaminoglycans (GAG) content of a scaffold affected its osteogenic performance using MC3T3-E1 cells in vitro without affecting the structural properties of the scaffolds [102]. A drawback associated with using CG scaffolds is their poor mechanical properties, and attention in our lab has focused on enhancing
their mechanical properties [94, 102]. Improvements have been obtained with methods such as crosslinking, although the scaffolds still lack stiffness signifying a limitation in the usability of CG scaffolds in load bearing situations in vivo.

A lyophilisation (freeze-drying) process is used to fabricate the CG scaffolds produced in our laboratory, and will also serve as the basis for the scaffolds presented in this thesis [47, 49, 94]. This method has many advantages, including the low temperature fabrication and the ability to yield reproducible highly porous scaffolds. In this process, blended, acetic-acid based collagen slurry (viscous suspension) is frozen at a constant cooling rate and subsequently the ice crystals that form are sublimated. This gives a controllable, interconnected, homogeneous pore structure. The pore size of the resultant scaffold is influenced by the size of the ice crystals that form, which are dependent upon the cooling rate and the final freezing temperature. The pore size affects the attachment of cells to the scaffold so control of this factor is vital in bone tissue engineering [47].

1.5.2 Hydroxyapatite

Hydroxyapatite is one of the main materials used for bone regeneration since it is biocompatible, osteoinductive and analogous to the mineral in bone [103]. HA can occur as non-stoichiometric apatite in the body, with the Ca/P ratio ranging from 1.62-1.64 in teeth, and up to 1.71 in bone [104]. It occurs as nanocrystals of 50 nm x 5 nm x 5 nm [105]. The compressive strength of sintered HA is 509 MPa [106] but in the body, resorbable HA is present in its amorphous form. HA monoliths
formed at 38°C in a dissolution-precipitation reaction had a Young’s modulus of 6 GPa and a compressive strength of 174 MPa [107].

Early in vivo studies investigating the implant potential of dense HA blocks found that although the HA was biocompatible, the constructs were not resorbed over long periods, in some cases with cells only infiltrating regions where cracks had formed [108, 109]. Therefore, while these implants encouraged good binding to host bone and provided structural stability to the defect, the implant could not be remodelled and remained brittle and in danger of re-fracture. Porous HA blocks were more successful, with cubes of 70% porosity used by Inoue et al. (1993) to fill bone cyst removal defects. By 12 weeks, new bone had been observed around and on the cubes however resorption was still an issue, with the majority of the cubes still remaining after 2 years [110]. Porous ceramic constructs of HA and TCP seeded with MSCs were capable of healing critical sized bone defects, which had been unable to heal via resident cells or by the addition of an osteoconductive device alone [111, 112]. Zhang et al. (2006) also found that despite a porosity of over 90%, seeding HA-TCP composite constructs with cells was necessary for mineralisation and vascularisation to occur [113]. Osteointegration of the scaffold depends on cellular adhesion and interaction with the construct, and a high porosity is required to improve the osteoconductive abilities of the scaffold [114]. However, despite using porous constructs, the lack of resorption and hence the prevention of remodelling of HA scaffolds remain a limiting factor. Quarto et al. (2001) found that porous HA scaffolds implanted into human tibiae were still present after 7
years, despite good integration of the scaffolds with host bone and this corresponded with similar findings [115-117].

Resorption of HA mineral occurs in bone due to the amorphous nano-sized nature of the ceramic and its increased surface area. Bauer and Muschler (2000) reported an increase in resorption with decreasing HA particle size, stating that particles below a 2 μm threshold can resorb while larger particles do not [26]. Nevertheless, implants made with HA of particle size of 0.25-1 mm have also been reported as resorbable, although particles of HA still remained after 6 years [118]. In addition, resorbability is influenced by crystallinity; the more amorphous the ceramic is, the quicker it will degrade [119] [120]. Therefore, despite the bioactivity and osteoconductive properties of HA, scaffolds fabricated using micron sized HA particles are brittle when highly porous and display poor resorbability. Evidence has shown that these issues may be addressed by reducing particle size and crystallinity of the HA particles, sparking interest in the development and use of nano-sized HA (nHA) [121].

1.5.3 Nano-sized HA (nHA)

Many motivating factors serve as the rationale behind using nHA. Firstly, 70 % of the bone matrix is nanocrystalline HA, and so its use in the development of an orthopaedic replacement scaffold is logical. In addition, compared to conventional ceramic formulations, nanophase HA properties such as surface grain size and wettability could control protein interactions (e.g. adsorption, configuration and
bioactivity); therefore, modulating subsequent enhanced osteoblast adhesion and long-term functionality. Nanometer grain size topography and surface wettability are properties that not only promote increased selective vitronectin adsorption (a hydrophilic protein that mediates osteoblast adhesion) but also enhances osteoblast functions [122]. A review paper by Tran et al. (2009) stated that for orthopaedic-related research, the superiority of nanomaterials has been established compared to conventional counterparts, with recent results implying that nanomaterials may improve osteointegration, which is crucial for long-term implant efficacy [123]. The bioactive and osteoconductive properties of nHA are of much benefit to a wide range of biomedical applications including producing bone tissue engineered constructs, coating medical implants and as a carrier for plasmid DNA in gene delivery [124].

Many investigations of nanophase materials to date have illustrated their potential for bone repair [121]. Lawson and Czernuszka (1998) have shown that smaller plate-like particles of the order of 200 nm×20 nm×5 nm achieved enhanced osteoblastic adhesion and proliferation compared to HA particles of an order of magnitude larger [125]. Webster et al. (2000, 2009) examined a number of different parameters looking at osteoblast and osteoclast responses, and the influence of nanophase materials compared to their larger, conventional form. Their findings correspond with other studies, showing that increased osteoblast adhesion and proliferation is observed on nanograined ceramics, while osteoclasts also demonstrated modified behaviour [126-128]. Tran et al. (2009) report
enhanced *in vitro* and *in vivo* osteoblast response across a wide range of nanomaterials, highlighting the role of nanoscale roughness and an increase in surface energy [123]. Another possible source of these improved biological responses may be the increase in protein adsorption due to surface nano features, with Tran *et al.* (2009) documenting an increase in fibronectin and vitronectin adsorption on nano-structures, proteins known to induce osteoblast adhesion [123].

The exploitation of the enhanced properties of nHA in a 3D environment has also been the subject of much interest recently. Compared to particles with sizes in the micron range, nano-particles have a large surface area, and consequently, a nano-composite may exhibit improved mechanical properties arising from interactions at interfaces whilst simultaneously giving a more homogenous material due to less agglomeration providing cells with increased access to the bioactive particles throughout the scaffold. Kothapalli *et al.* (2005) hypothesised that by using nano-sized particles a greater interaction is obtained between the matrix and scaffold which leads to less de-bonding when a stress is applied and consequently the modulus and strength would be improved [129].

A schematic representation of the effect of using nanostructured composites compared to conventional constructs is given in Figure 1.11. Bone cell functions can be enhanced by interacting with nanophase ceramics and nanostructured polymers collectively when compared to individual components [130-133].
Heo et al. (2009) and Wei et al. (2004), have shown that in a comparison between the use of nHA and micron sized HA in composite scaffolds, the nHA scaffolds have improved mechanical properties and are more biocompatible [135, 136]. They reported increased levels of attachment and proliferation of MG-63 cells on the nHA composite and this correlates with other findings. Shi et al. (2009) also observed a size effect of nHA particles on the proliferation and apoptosis of MG-63 cells, reporting that the smaller particles promoted cell growth and also inhibited cell apoptosis [127]. In vivo studies have also demonstrated increased new bone formation on metals coated with nHA compared to conventional apatite [137]. Meirelles et al. (2008) demonstrated significantly increased bone formation after 4 weeks on implants coated with nHA compared to uncoated controls.
indicating that early bone formation is influenced by the size of the hydroxyapatite [138].

Conventional HA scaffolds are known to be brittle despite having good compressive strength [139, 140], however, Ahn et al. (2001) found that the use of nano-sized HA improved compressive strength as well as bending strength and fracture toughness [141]. Small sintered particles of less than 1 μm have been cautioned against in bone implants due to their increased inflammation response and cell toxicity in vitro [142] and according to a review by Tran et al. (2009) further research into the toxicity of nanoparticles needs to be conducted to ensure clinical applications can exploit the benefits of nanotechnology [123].

1.5.4 Synthesis of nano-sized hydroxyapatite

A drawback associated with the use of nano-sized HA particles lies in the complexity of their fabrication [143]. Difficulties arise during the synthesis of nHA, the resultant particles are in the micron range with a wide particle size distribution, low surface areas and widespread agglomerate formation [144-147]. There is an inability to effectively control flocculation of particles as they are created in aqueous solutions and often an extensive ball-milling step is required to separate the agglomerated particles [143, 148-151]. The term nanomaterial is somewhat vague, and in a recent leading opinion paper by the editor of Biomaterials, David Williams (2009), questions also arise regarding the suitability of its use [2]. Is it the dimension of a crystal within the material, or of a grain
boundary, a domain, or a molecule, or is it a parameter of a surface feature of the sample that qualifies a material to be called "nano"? In many nHA papers, the particles are in fact aggregates of nanocrystals, many microns in dimension. While these materials may still offer advantages, many of the attractive properties of nanoparticles such as high surface area and resorbability may be compromised.

Several methods are reported for the production of HA, including wet chemical precipitation, sol-gel processing, mechanochemical synthesis, solid-state reactions, chemical vapour deposition, hydrothermal synthesis, combustion method, solvothermal and reverse micro-emulsion techniques [148-165].

Of these methods, wet precipitation is the most common due to its simplicity and low cost. The stability of the calcium phosphates are affected not only by small compositional changes, but also by variations in pH and the reaction conditions such as the solvent, temperature, pressure, nature of precursors and the complexing agents used for controlling the reaction kinetics [84]. Various HA phases can be formed such as calcium-deficient hydroxyapatite, oxy-hydroxyapatite and carbonate substituted hydroxyapatite depending on the type of environment employed in the synthesis steps. Biological apatites differ chemically from pure HA in that they often include cations such as Mg$^{2+}$, Na$^+$, K$^+$, Cl$^-$ and F$^-$ which can be introduced into the HA lattice by substitution of one or more Ca$^{2+}$ [84].
The most widely used aqueous chemical precipitation routes can be represented by the following chemical reactions [84]:

\[
10 \text{Ca(OH)}_2 + 6\text{H}_3\text{PO}_4 \rightarrow \text{Ca}_{10}\text{(PO}_4)_6\text{(OH)}_2 + 18 \text{H}_2\text{O} \tag{1}
\]

\[
10 \text{Ca(NO}_3)_2 + 6(\text{NH}_4)_2\text{HPO}_4 + 2\text{H}_2\text{O} \rightarrow \text{Ca}_{10}\text{(PO}_4)_6\text{(OH)}_2 + 12\text{NH}_4\text{NO}_3 + 8\text{HNO}_3 \tag{2}
\]

Methods based on reaction (1) represent more of a dissolution–precipitation reaction involving a solid–liquid reaction of phosphoric acid dissolving Ca(OH)_2 to form hydroxyapatite. The latter reaction represents a more typical precipitation reaction comprising the reaction of a weakly acidic solution of a calcium salt with a basic phosphate generating hydroxyapatite. Both approaches require stringent control and maintenance of pH above 10 to initiate the formation of stoichiometric and stable HA. The reactions are limited by the acidic nature of H_3PO_4 and the formation of toxic by-products [84]. Kumta et al. (2005) developed a precipitation synthesis route using CaCl_2, Na_3PO_4 and NaOH as initial reactants to overcome these issues. They produced nHA particles that were safer and more efficient for drug delivery and more biocompatible for cells [84]. The synthesis method described in this thesis uses these same starting precursors but with different reaction conditions.

The difficulty with controlling aggregation during precipitation reactions has led to investigations into the use of surfactants, such as cetyltrimethylammonium bromide (CTAB) and sodium dodecyl sulphate (SDS) with moderate success, although they do not overcome the necessity for the time consuming ageing and heat treatment stages [127, 150, 166, 167]. Instead, poly(vinyl alcohol) (PVA) was
chosen as a surfactant due to its biocompatibility and established use in biomedical applications [168], whilst, Darvan 821A® is a dispersing agent which has been reported in the literature to disperse commercial HA particles in aqueous solutions. Bhattacharjee et al. (2006) compared the effectiveness of three anionic polyelectrolytes, Darvan 821A, Darvan 811 and Darvan C for the dispersion of HA powder in a slurry and found that the smaller dispersants, Darvan 821A and Darvan 811 worked better at preventing agglomeration [169]. While Darvan 821A, an ammonium based polyacrylic acid, has been used for dispersing HA powder for this purpose, it has not been previously used during the synthesis process.

1.6 Polymer – Ceramic Composite Scaffolds

As discussed in the last section, the addition of nHA to a 3D scaffold can improve the success of the scaffold as an implant for bone defect repair. The combination of materials to make a composite scaffold is a logical and popular approach to developing bone graft substitutes, as the advantages of each constituent part are merged, yielding a construct with more potential than either individual part alone [170-174]. Composite materials are heterogeneous materials made up of two or more phases, with the purpose of optimising the performance of the material, choosing complementary materials to achieve a good balance of material properties. A reinforcing material is usually combined with a matrix phase with the aim of lending mechanical support to the surrounding matrix, while maintaining the biological performance and structure of the matrix. This occurs naturally in bone, where nanocrystalline hydroxyapatite reinforces the organic matrix
containing collagen and non-collagenous proteins. The ceramic phase supplements
the flexible collagen fibres, adding stiffness and compressive strength.

Many studies have been conducted, combining polymers both natural and
synthetic, with bioceramics and generally finding that mechanical and biological
performances were enhanced in the composites compared to controls [175-180].
The reinforcing materials used in these studies were generally particulate in nature,
with sizes ranging from nano- to millimeters. Juhasz et al. (2004) reported an
increase in mechanical properties with increasing bioactive filler content, and
importantly he also noted a reduction in mechanical strength as the filler particle
size increased, implying that the use of higher content and smaller particles is
optimal for composite fabrication [181]. Composites of PLA with nanophase
alumina, titania, and HA particles added, showed significantly greater bending
moduli than that of composites with conventional coarser-grained ceramics [182].
The degradation rate of collagen can be altered by the introduction of a bioceramic
and for example, a study by Wu et al. (2004) demonstrated that collagen-HA gel
beads showed improved resistance to degradation by collagenase compared to
collagen only control [183]. Clearly, both the choice and quantity of filler will
affect the degradation rate, and these parameters will also impact on the composite
pore architecture. Maquet et al. (2004) found that the pore structure changed,
becoming more irregular with a poorer distribution of particles throughout the
matrix as the percentage of particles was increased in bioglass-PDLLA scaffolds
[184]. Anisotropic pores were also obtained in HA and gelatin scaffolds as the
percentage of HA in the scaffolds was increased [185].

The mechanical strength of PLLA and PLGA foams have been shown to be significantly improved on addition of HA and a similar outcome was experienced following addition of bioactive glass particles to both PDLLA and PLGA [184, 186]. Wang et al. (2007) reported that nHA-polyamide scaffolds displayed good in vitro biocompatibility and in vivo analysis demonstrated the osteoconductivity of the constructs, while MSC-seeded constructs enhanced new bone formation [187].

Composites of carbonated HA, collagen and PLGA in a three-layered membrane showed a more positive response to osteoblast culture than scaffolds of PLGA alone [59] and the same effect was observed when HA particles were added to a gelatin scaffold with enhanced osteoblastic activity as a result [185]. Wei and Ma (2004) demonstrated that enhanced osteoblast adhesion is achieved due to the increased protein adsorption capacity of scaffolds following nHA particle addition [136].

Collagen-HA composite scaffolds have been shown to support the osteogenic differentiation of human bone MSCs both in vitro and in vivo [72]. Similarly, the presence of a mineral phase in collagen scaffolds was shown by Bernhardt et al. (2008) to encourage the osteogenic differentiation of human bone marrow stromal cells over a four week period [188]. Xie et al. (2004) reported an enhancement of osteoblast differentiation using both collagen and HA independently, with an
acceleration of osteogenesis obtained when the two materials were combined [189]. Additionally, a composite matrix when embedded with human-like osteoblast cells, showed better osteoconductive properties compared to conventional HA, producing calcification matrix similar to bone tissue [96]. Du et al. (1999) developed collagen-nHA composite scaffolds which promoted the deposition of a new bone matrix and also demonstrated that the porous collagen-nHA scaffold provided a microenvironment resembling the in vivo situation, with osteoblasts within the construct acquiring a three-dimensional polygonal shape [176]. Kikuchi et al. (2001) implanted collagen-HA scaffolds into critical-sized defects in beagle tibiae, finding that the composite was incorporated into the remodelling process, with resorption by osteoclastic cells and evidence of new bone observed after 12 weeks [173]. Using a nHA-collagen scaffold, Pek et al. (2008) demonstrated the excellent bioactivity and osteoconductivity of the nanocomposite construct, and the successful in vivo healing of defects in rat femur and pig tibia models [190].

A paper by Wahl et al. (2006) reviewed a number of collagen-HA scaffolds, and summarised their in vitro and in vivo behaviour in a table shown below (Table 1.1) [142]. This summary illustrates the vast potential of collagen-HA scaffolds, but also the variety of results that can be obtained by altering for example, the method of fabrication, the porosity of the scaffolds and the particle size of the HA filler.
Table 1.1 Summary of a selection of collagen-HA scaffolds demonstrating \textit{in vitro} and \textit{in vivo} behaviour [142].

<table>
<thead>
<tr>
<th>Composites</th>
<th>Method</th>
<th>Cell culturing or implantation</th>
<th>Period (new tissue formed)</th>
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</thead>
<tbody>
<tr>
<td><strong>In vitro experiments</strong></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>HA-Collagen (Du et al., 1999)</td>
<td>\textit{In vitro} mineralisation</td>
<td>Bone extract wrapped around composite</td>
<td>21 days (mineralised bone)</td>
</tr>
<tr>
<td></td>
<td>Composite porous sheets</td>
<td>Osteogenic cells</td>
<td></td>
</tr>
<tr>
<td>HA-Collagen (Clarke et al., 1993)</td>
<td>Remineralisation of decalcified bone</td>
<td>Human derived bone cell culture</td>
<td>32 days (connective tissue and mineralised bone)</td>
</tr>
<tr>
<td>HA-Collagen (Wu et al., 2004)</td>
<td>Water-in-Oil emulsion Composite gel beads</td>
<td>Osteoblasts from rat calvaria</td>
<td>21 days (mineralised bone)</td>
</tr>
<tr>
<td></td>
<td>Porous composite</td>
<td>Chondrocytes in closed chamber</td>
<td>49 days (cartilage)</td>
</tr>
<tr>
<td>HA-Collagen-Elastin (Rovira et al., 1996)</td>
<td>Direct mix, air dried Low porosity</td>
<td>Osteoblasts from trabecular bone</td>
<td>15 days (mineralised bone)</td>
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<tr>
<td><strong>In vivo experiments</strong></td>
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</tr>
<tr>
<td>C0,Apatite-Collagen (Okazaki et al., 1990)</td>
<td>Direct mix, centrifuged, air dried Low porosity</td>
<td>Implantation in rat periosteum crania</td>
<td>21 days (mineralised bone)</td>
</tr>
<tr>
<td>PCCA-TCP-Collagen (Du et al., 2000)</td>
<td>\textit{In vitro} mineralisation</td>
<td>Composite porous sheets</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Implantation in rat thigh muscle</td>
<td>7 days (connective tissue and capillary vessels)</td>
</tr>
<tr>
<td></td>
<td>\textit{In vitro} mineralisation</td>
<td>Dense cylinders with internal tunnels</td>
<td>54 days (mineralised bone)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Implantation in beagle tibia</td>
<td></td>
</tr>
<tr>
<td></td>
<td>\textit{In vitro} mineralisation</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Implantation in beagle cervical spine</td>
<td>14 days (start of callus formation)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Implantation in rat periosteum crania</td>
<td>14-28 days (mineralised bone)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Implantation in rat femur</td>
<td>14 days (mineralised bone)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Implantation in rat infraorbital bone</td>
<td>60 days (mineralised bone)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Implantation in rabbit skin</td>
<td>98 days (mineralised bone)</td>
</tr>
</tbody>
</table>

A number of issues remain that must be resolved before a model scaffold for bone tissue engineering can be developed. The particle size, content and resorbability of the bioceramic filler within the composite scaffolds must be optimised. In addition, the correct balance between retaining a high porosity while improving the mechanical properties of the scaffold needs to be achieved. These complex issues are being addressed currently in the endeavour to create ideal bone graft substitutes.
1.6.1 Composite scaffold fabrication

There are a number of scaffold fabrication methods available, for the development of particular materials, and the production of specific 3D structures. Sintering is usually conducted to fabricate ceramic scaffolds, using a binder that is removed during heating or hydrogen peroxide which causes the ceramic to effervesce yielding a foam-like formation. Rapid prototyping (RP) techniques can be used to form polymer scaffolds from CAD (computer-aided design) models. 3D printing (3-DP) and fused deposition modelling (FDM) are based on this method, and use a layer-by-layer approach to build the construct although a lack of precision and constraints regarding materials that can be used remain as limitations [171]. Injection moulding can be used for both polymer and composite scaffolds although there are some limits to the pore sizes that can be obtained (approximately 200 – 500 μm) [191]. Electrospinning is another method used to fabricate scaffolds and works by pumping a slurry through a syringe in an electric field, causing the slurry to solidify as a fine (usually nanoscale) fibre. A wide range of scaffolds with varying porosities can be manufactured using this method [192].

Lyophilisation (freeze-drying) is a highly effective method for producing porous polymer scaffolds. The polymer material is dispersed in a solvent, which is removed via sublimation to yield an interconnected porous sheet of scaffold. The nature of the pores within this sheet can be controlled by the variables of the procedure such as the freezing rate, undercooling temperature, solvent and polymer used and their relative content within the dispersion. Crystals of the solvent
nucleate and grow during the freezing step, forcing the polymer to the grain boundaries. The pressurised drying phase causes the solvent to vaporise and the polymer remains in place generating the fibrous network [47]. Annealing can also be used in order to give a suitable grain (and hence pore) structure [193]. This method has been used successfully to produce composite scaffolds [186]. The effect the quantity and size of the reinforcing particles will have on the freeze-drying process and resultant scaffold structure must be considered [193]. For example, irregular pores were obtained in gelatin-HA composites as the number of HA particles in the polymer solution increased [185]. This was due to the change of the crystallization front leading to irregular and impeded crystal growth, and so care must be taken in this regard.

Additional methods for producing composite scaffolds include vacuum infiltration, enzymatic mineralisation and water-in-oil emulsion system. Pompe et al. (2003) used a vacuum to get collagen fibres to infiltrate a porous HA scaffold before freeze-drying the composite to attain micro-pores [194]. In the enzymatic mineralisation method, alkaline phosphatase was loaded onto a collagen sheet before coming into contact with calcium and phosphate solutions [195]. The sample was then coated again with a collagen suspension, air dried and cross-linked with UV irradiation. Repeating this cycle resulted in multilayered composite sheets of calcium/phosphate and collagen with a sheet thickness of 7 μm [195]. The water-in-oil method is particularly used for making injectable bone fillers as microspheres or gel beads are formed.
In a review of methods for fabricating collagen-HA composite scaffolds, Wahl et al. (2006) viewed freeze drying and critical point drying as superior to these other methods as they have the fewest residual solvent problems, also the easy removal of ice crystals compared to other porogens used in conventional polymeric-porogen leaching methods eliminates any dimensional restrictions [142].

Alternative methods exist for incorporating a ceramic phase into polymer scaffolds such as immersing the polymer scaffold in SBF solution, or in separate solutions containing calcium and phosphate ions [176, 196]. These methods firstly require the fabrication of the polymeric scaffold. Al-Munajjed et al. (2009) fabricated collagen-calcium phosphate (CCP) constructs, using a number of immersion techniques and found that these CCP scaffold induced significant bone formation in vivo [10, 197]. Lawson and Czernuszka (1998) fixed a collagen film in between calcium and phosphate solutions, allowing the calcium and phosphate ions to diffuse into the fibrils, although a gradient effect is observed using this procedure [198]. The biomimetic approach of soaking collagen scaffolds in SBF causes mineral to become deposited on the scaffold surface [199].

Having met clinical regulatory standards, some commercial collagen-HA scaffolds have become available for hard tissue repair. A review by Wahl (2006) summarises some of these composites and the manufacturing method used in their production (Table 1.2) [142]. The fabrication methods used in this thesis include adapted lyophilisation (freeze-drying) and immersion procedures.
### Table 1.2 Commercial collagen-HA scaffolds and their methods of fabrication [142]

<table>
<thead>
<tr>
<th>Composite</th>
<th>Name (Company)</th>
<th>Manufacturing</th>
</tr>
</thead>
<tbody>
<tr>
<td>Synthetic HA</td>
<td>Collapat II (BioMet, Inc)</td>
<td>Direct Mix</td>
</tr>
<tr>
<td>Bovine Collagen type I (calf skin)</td>
<td></td>
<td>Freeze dried</td>
</tr>
<tr>
<td>Synthetic HA</td>
<td>Healos (Depuy Spine, Inc)</td>
<td>HA-coated collagen matrices</td>
</tr>
<tr>
<td>Bovine Collagen type I Bone marrow aspirate</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Biphasic HA/TCP</td>
<td>Collagraft (Neucoll, Inc; Zimmer, Inc)</td>
<td>Composite Paste (direct mix of equal mass proportions) with a 4:1 volume ratio of autologous bone marrow aspirated from the ilium. Available in a lyophilised Composite Strip</td>
</tr>
<tr>
<td>Bovine Collagen type I Autologous bone marrow</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Synthetic HA</td>
<td>Biosite (Vebas, s.r.l)</td>
<td>Freeze Dried</td>
</tr>
<tr>
<td>Equine Collagen type I Chondroitin-6-Sulphate (CS)</td>
<td></td>
<td>HA (88wt%), Col (9.5wt%), CS (2.5wt%)</td>
</tr>
</tbody>
</table>

The goal of tissue engineering is to synthesize substitutes that mimic the natural ECM to help guide the growth of new functional tissue \textit{in vitro} or \textit{in vivo}. The combination of a 3D scaffold and a gene delivery vector has recently been investigated to achieve this objective. In particular, a gene activated matrix (GAM) system developed by Bonadio \textit{et al.} (1999), consisting of plasmid gene delivery vectors contained within a 3D polymer scaffold, showed the potential of this approach, obtaining the induction of stable and reproducible bone formation when implanted \textit{in vivo} [13]. Particles of nHA also have the ability to transfect cells, and the combination of this aptitude within a 3D scaffold is examined in this thesis.
1.7 Gene Therapy

Gene therapy has the potential to treat a wide variety of diseases, both genetic and acquired, by replacing, altering, or supplementing a gene that is either absent or abnormal, causing the disease [200]. Gene therapy can be a valuable tool to avoid the limitations associated with the alternative approach of local growth factor delivery, including short half-life, large dose requirement, high cost, need for repeated applications, and poor distribution [201]. Actually getting the replacement gene into the correct location in the body is the challenging and complex aspect. Transfection, or the introduction of nucleic acids (DNA or RNA) into living cells is a process that allows the selective introduction of genetic material for protein synthesis, or alternatively the selective inhibition of protein synthesis (gene silencing). There are two forms of transfection; transient transfection, where the DNA does not integrate into the host genome, and stable transfection, where the DNA is integrated with the host genome, and subsequently replicated in the next generation.

1.7.1 Gene Expression

A gene is a short sequence of DNA residing within long strands of DNA that make up the chromosomes which contains genetic information that can act as a template for synthesis of a specific protein (Figure 1.12) [202]. Within a gene, the sequence of bases along a DNA strand defines a messenger RNA (mRNA) sequence, which then defines a sequence for one or more specific proteins. The gene contains both a
“coding” sequence, detailing for example the protein to be synthesized, and a “non-coding” region that determines when the gene is expressed [15, 200].

Figure 1.12 A gene is a short segment of code within long strands of DNA which in turn make up the chromosomes [202].

When a gene is active, the coding and non-coding sequences are copied in a process called transcription, which then directs the synthesis of proteins via the genetic code. The genetic code consists of three-letter codons, formed from a combination of three nucleotides (e.g. ACT, CAG, TTT). The relationship between the nucleotide sequences of genes and the amino-acid sequences of proteins is determined by the process of gene expression.
Figure 1.13 Transcription and translation process for synthesising proteins from genetic code [203].

In the transcription step, the codons of a gene are copied into messenger RNA (mRNA) by RNA polymerase. This RNA copy leaves the nucleus through pores in the nuclear membrane and mature mRNA is then decoded by a ribosome in the translation stage (Figure 1.13). The ribosome uses the mRNA sequence as a template for synthesising a new protein, by base-pairing the messenger RNA to transfer RNA (tRNA). The genetic code is read three nucleotides at a time and each tRNA molecule has three unpaired bases known as the anticodon which is complementary to the codon it reads. The tRNA binds to its complementary codon
in an mRNA strand and the ribosome assembles amino acids into a new polypeptide chain for the synthesis of the new protein [15, 200].

1.7.2 Plasmid DNA

Although most DNA is chromosomal DNA, there also exists another form known as plasmid DNA which is found in bacteria. Plasmid DNA (pDNA) is usually circular and double-stranded (Figure 1.14) and its size can vary from 1 to over 1,000 kilobase pairs (kbp). Research into this type of DNA is of great interest due to the ability to encode a gene into its composition which can then be replicated and amplified before incorporating it into cells to induce the expression of the encoded gene. Plasmids used in genetic engineering are called vectors. Bacteria can be used to yield large amounts of proteins, as bacteria containing the specific plasmid can be grown up containing the pDNA. This method is used as a cheap and easy way of mass-producing a gene or the protein it codes for example insulin.

Figure 1.14 Bacterial cell with extra-chromosomal plasmid DNA, showing its ability to integrate into host DNA and replicate.
In bone tissue engineering, it would be advantageous to have the expression of particular genes upregulated within cells as proteins that encourage bone formation and mineralisation could be synthesised. An example of this would be the synthesis of osteoinductive cytokines which are molecules that promote the formation of bone such as bone morphogenic proteins (BMP) which are members of the transforming growth factor β (TCF-β) family [204, 205]. Over 20 types of BMPs have been identified in humans, the most promising recombinant BMPs for bone tissue engineering include bone morphogenic protein 2, 4 and 7 (BMP-2, BMP-4 and BMP-7) showing significantly improved bone formation in both in vitro and in vivo studies [206-214].

BMP-2 and BMP-7 have received FDA approval for use in clinical studies [215]. However, a review by Gautschi et al. (2007) highlighted the contrast between the promising results found in animal pre-clinical studies to the unconvincing results achieved in recent clinical studies, citing a number of possible reasons for this disparity [216]. The presence of underlying conditions in the patient, a history of prior operations attempting to heal a non-union fracture and previous soft tissue damage in the patients were amongst the explanations given in the review. It has also been noted that BMPs need to be present at the fracture site for a prolonged period of time to be effective and improvements with this regard are being investigated. Other proteins of interest include TGF-β1 which plays an important role in bone remodelling, and vascular endothelial growth factor (VEGF) for vascularisation. Simple diffusion and protein denaturisation can dilute protein
concentration significantly before inducing any effects if they are just added \textit{in vivo}. Due to the rapid turnover of proteins in the body, protein therapy is an inadequate form of therapy and gene therapy is required to provide a prolonged supply of the proteins producing them in a sustained and controllable manner [217]. The gene of interest is inserted into the stable pDNA molecule and replicated within bacteria cells as shown in Figure 1.15.

\textbf{Plasmid Insertion}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{plasmid_insertion.png}
\caption{Diagram representing the incorporation of a desired gene into plasmid DNA and transformation into bacterial cells for amplification [218].}
\end{figure}
The gene to be replicated is inserted into a plasmid that also contains an antibiotic resistant gene and a multiple cloning site (MCS), which is a short region containing several commonly used restriction sites allowing the easy insertion of DNA fragments at this location. Next, the plasmids are inserted into bacteria by a process called transformation, usually using a heat shock treatment. Then, the bacteria are cultured on a plate containing a specific antibiotic and hence only bacteria which have taken up the plasmid survive due to the antibiotic resistant gene in the plasmid. In this way the antibiotics act as a filter to select only the transformed bacteria and these bacteria can be grown in large amounts, harvested and lysed to isolate the plasmid. The plasmid can then be used to transfect cells. The plasmid enters the cell and the DNA is either integrated into the chromosomal DNA (stable transfection), or into the nuclease in an extrachromosomal location (transient transfection). Both methods lead to the expression of the desired gene.

Successful transfection requires the addition of a suitable carrier as naked pDNA cannot internalize (enter the cell) on its own. This is due to the molecular size and negative surface charge of pDNA. For example, a tail-vein injection of naked pDNA into mice did not result in gene expression in major organs because of its rapid degradation by nucleases in the blood [219].

The pDNA molecule is a polyanion of phosphate group-repeated chain and has an expanded molecular structure due to the negatively charged intramolecular repulsion forces present at the physiological pH. Similarly, the cellular membrane
is also negatively charged and therefore the pDNA cannot interact with the cells due to the electrostatic repulsive forces, and as a consequence the pDNA will not be internalized and transfection will not occur. To enhance the internalization of pDNA into cells, a pDNA-carrier complex is required, which should have an appropriate molecular size and surface charge, suitable for the interaction with the cell membrane and leading to internalization [220]. The pDNA-carrier complex is internalized via the endocytosis pathway and carried into the endosomal compartment, followed by subsequent lysosomal degradation. Therefore, the carrier should be designed to allow the effective escape of the pDNA-carrier complex from the endosome into the cytoplasm. Specifically, vectors (carriers) are required which allow for efficient transfection, controllable expression period and cell or spatial specificity, while avoiding safety concerns. Furthermore, these vectors should be mass-producible and relatively inexpensive if they are to have clinical impact [221]. The plasmid DNA can be delivered into the cells via two main approaches, viral and non-viral delivery systems.

1.8 Methods for plasmid gene delivery

1.8.1 Viral methods

The use of viral methods for genetic transfer dates back to 1952 with the investigation of salmonella for this purpose [222]. Viral methods are used as they have the inherent ability to invade cells and deliver its genetic material into the
cytoplasm and later the nucleus. The two main types of viruses used for this purpose are retroviruses and adenoviruses.

Retroviruses are easily produced and are highly efficient carriers for gene delivery into dividing cells, but do not infect non-dividing cells [217, 223]. The genetic material in retroviruses is in the form of RNA molecules unlike the genetic material of their hosts which has a DNA formation. When a retrovirus infects a host cell, it will introduce its RNA along with some enzymes, namely reverse transcriptase and integrase into the cell. This RNA molecule from the retrovirus must produce a DNA copy from its RNA molecule before it can be integrated into the genetic material of the host cell. The process of producing a DNA copy from an RNA molecule is termed reverse transcription and is carried out by the reverse transcriptase enzyme. Following production of the DNA copy, it must then be incorporated into the genome of the host cell. This process is done by another enzyme carried in the virus called integrase. However, as the insertion site of the gene into the DNA chain is arbitrary and occurs by chance, there is the danger of insertional mutagenesis or the insertion of the new genetic material in the middle of an original gene. For example, patients suffering from human severe combined immunodeficiency (SCID-X1) disease were treated by retroviral vector enabled gene therapy which had shown some promising results. However, many patients later developed leukaemia-like syndrome due to the insertion of the retrovirus gene near the LIM domain only 2 (LMO2) oncogene, subsequently activating its expression [224-227]. Lentiviral vectors are a type of retrovirus that can infect both
dividing and non-dividing cells because their preintegration complex (virus "shell") can get through the intact membrane of the nucleus of the target cell. However insertional mutagenesis is still a concern associated with this approach. Human immunodeficient virus (HIV) is an example of an effective lentivirus, and safety fears exist regarding its use and the potential for self-replication of the virus, leading to HIV infection.

Adenoviruses infect both dividing and non-dividing cells (Figure 1.16). The adenovirus vectors are efficient and can achieve high gene expression [223]. They carry their genetic material in the form of double-stranded DNA and when these viruses infect a host cell the DNA molecule is introduced into the cell, but not incorporated into the host cell's genetic material (transient transfection). The DNA molecule is transcribed just like any other gene. The only difference is that these extra genes are not replicated when the cell undergoes cell division, so the descendants of that cell will not have the extra gene. As a result, treatment with the adenovirus will require re-administration in a growing cell population, although the absence of integration into the host cell's genome should prevent the type of cancer seen in the SCID-X1 trials [217]. However, the gene expression is not always stable using this method leading to an unpredictable end therapeutic effect, in addition, the adenovirus genes can enter the transfected cells along with the therapeutic genes causing immunogenicity problems. A patient suffering from ornithine transcarbamylase (OTC) deficiency took part in a clinical trial in 1999 involving the use of adenovirus to deliver the OCT gene, instead the viral vector
entered his circulatory system, triggering an extensive inflammatory response that ultimately led to his death [223].

Figure 1.16 A diagram showing the stages involved in gene therapy using an adenovirus vector [228].

In summary, the use of viral vectors seems to satisfy at least the initial requirements, as most viruses can deliver nucleic acids showing high transduction efficiency. However, the success of viral vectors is limited due to their potential immunogenicity and oncogenicity as well as their unsuitability for mass production [229-235]. Thus, despite relatively lower transfection efficiency, non-viral vectors have become increasingly more attractive [236, 237].
1.8.2 Non-viral methods

Although non-viral methods do not yet have high transfection efficiencies similar to viral methods, their use does present particular advantages such as their simple large scale production and low host immunogenicity. Therefore, investigation into non-viral methods and techniques for improving their efficiency are being enthusiastically explored. There are several non-viral transfection techniques and they can be separated into physical and chemicals techniques. Commonly used physical methods include electroporation and the gene gun technique. Chemical methods used are generally based on compounds which form a complex with the pDNA, such as dendrimers, lipids and calcium phosphates.

Physical stimuli have been shown to enhance the transfer of pDNA into cells by escorting the DNA to the cell membrane proximity and/or by temporarily disrupting the cell membrane [237]. Based on this approach, several physical methods have been established to augment gene expression in vivo by using: electroporation (electric pulses applied to increase cell membrane permeability) [238-243], hydrodynamic pressure (vascular delivery of genes using pressure) [244-246], gene gun (DNA coated gold particles shot at high speed) [247, 248], magnetofection (guiding paramagnetic particle carriers using strong magnetic fields) [249], microinjection (localised injection of pDNA into a single cell) [250] and ultrasound (used to burst microbubbles containing naked pDNA that were infused intravenously, at the desired location) [251-253].
Chemical techniques modify the DNA to increase internalisation into cells. Examples of non-degradable, non-viral systems are mainly based on cationic polymers, dendrimers and cationic lipids and their electrostatic interaction with pDNA. Polymers including poly(ethylenimine), poly(2-dimethyl-aminoethyl methacrylate) and poly-L-lysine form complexes with the pDNA molecule which are typically called polyplexes (polymer-DNA ion complex) that can be introduced into target cells [2, 254]. The negatively charged DNA binds to the polycation and the complex is taken up by the cell via endocytosis. Another class of polymer is the highly branched organic compound, dendrimers. A dendrimer is a spherical shaped macromolecule, and its surface can be functionalised in many ways controlling the resulting properties, although their production can be slow and expensive. Cationic dendrimers, i.e. with a positive surface charge can form a temporary association with nucleic acids, allowing the complex to be internalised. A difference between the action of polyplexes and lipid-pDNA complexes (lipoplexes) is that some polyplexes cannot release the pDNA into the cytoplasm, therefore, requiring co-transfection with endosome-lytic agents. Also, a recent study by Cohen et al. (2009) showed that once the plasmid was integrated into the nucleus, greater expression efficiency was obtained using lipoplexes compared to the use of polyplexes [255].
Lipids self-assemble with the DNA to form complexes (lipoplexes) that are suitable for cellular uptake [236, 257, 258]. Liposomes, *i.e.* small, membrane-bound bodies that have a similar structure to cells can fuse with the cell membrane, releasing the DNA into the cell (Figure 1.17). For eukaryotic cells, cationic lipid based transfection is more efficient due to the stable nature of the lipoplex. Cationic lipids, due to their positive charge can also be used to condense negatively charged DNA molecules so as to facilitate the encapsulation of DNA into liposomes. Although cationic liposomes have been widely used as an alternative for gene delivery vectors, a dose dependent toxic effect of cationic lipids was also observed which could limit their therapeutic use. The size of the lipoplex is another factor for consideration, reports in the literature vary, with some indicating larger particles are preferable when endocytosis is the dominant method of uptake due to the increased levels of membrane contact, whilst others report that higher rates of transfection are achieved using smaller lipoplexes [259]. The combination of
liposomal bubbles and ultrasound technology may prove a more efficient method of targeted plasmid delivery [260].

Lipofectamine or Lipofectamine 2000 is a commercial transfection reagent used to introduce small interfering RNA (siRNA) or pDNA into *in vitro* cell cultures by lipofection [255]. Lipofectamine treatment alters the cellular plasma membrane, allowing nucleic acids to cross into the cytoplasm and has been shown to be very effective compared to other lipoplex and polyplex methods, although the dependency of all lipids on serum-free media is a drawback [261]. This issue in addition to some toxicity concerns with lipoplex transfection is avoided in studies using CaP as the transfection agent.

**1.8.3 Calcium Phosphates (CaP) in non-viral gene delivery**

Therapy with genes encoded for osteoinductive factors could potentially provide long-term bone formation activity and healing. Calcium phosphate (CaP) is the most widely studied inorganic non-viral delivery vector for gene transfer, inspired in part by its well established biocompatibility and biodegradability [262]. The advantage of using the CaP approach is its ability to transfect a wide variety of mammalian cells *in vitro*, while avoiding problems such as immune responses and the high levels of toxicity associated with other techniques [262-265]. Despite this, a disadvantage associated with CaP transfection has been reports of poor reproducibility [266-268] since its discovery over 40 years ago [14]. However, recent significant developments and adaptations to this original approach have
been reported in a number of papers demonstrating \textit{in vivo} CaP transfection in various models [269].

The original CaP transfection method was established by Graham and van der Eb in 1973 [14]. Preparation of the carrier involved mixing DNA and calcium chloride (CaCl\(_2\)), with the subsequent addition of HEPES, a phosphate buffered saline solution, which resulted in nano- and micron-sized precipitate formation. The good binding observed between the DNA molecule and calcium phosphate particles may have resulted from the strong affinity of CaP towards the phosphate groups in nucleic acids. Particles in this dispersion were reported to be taken up by the cells, and the transfection efficiency of this standard CaP method was determined by a number of parameters including the type of cell, pH value, concentrations of CaCl\(_2\) and DNA, temperature and the time between precipitation and transfection. Drawbacks with this original method include a poor transfection efficiency and reproducibility.

Pedraza \textit{et al.} (2008) reported that optimisation of parameters involved in an adapted CaP method can lead to potent transfection, achieving results comparable to commercial vectors without the need for transfection enhancement methods such as the use of specialised media, cell synchronization, glycerol shock and chloroquine treatment that have been used to improve CaP transfection previously [262]. The study also highlighted the necessity for the presence of CaCl\(_2\), as a combination of calcium and chloride ions efficiently enhanced green fluorescent
protein (GFP) expression suggesting that these ions play a specific role during vector synthesis and transfection.

The particle size is also reported to have a major role in transfection efficiency [270]. While one report claimed that the effect of the nanoparticles was to concentrate DNA on the cell surface by sedimentation [266] thus favouring larger particles, the majority of studies maintain that small particles are the most effective [124, 262, 271, 272]. This may be due to the mechanism by which nano-sized particles are usually taken up by cells (endocytosis) which is limited to a size range of approximately 20–200 nm in diameter, above this size phagocytosis is thought to dominate (Figure 1.18) [273]. The chemistry behind CaP formation is important as the nucleation and crystal growth is prone to agglomeration leading to the synthesis of microcrystals, which would affect the particles ability to transfect cells [274].
1) Nano-sized gene carriers

In vitro
- DNA
- Ligand
- (DNA-carrier complex)

(a) Enhancement of gene internalization (cell-specific gene internalization)
(b) Efficient intracellular trafficking (endosomal escape, transport to the nucleus)

In vivo
- (c) Blood circulation for long time
- (d) Gene delivery using EPR effect and gene targeting delivery

Figure 1.18 Demonstration of the size effect of the delivery vector on the method of internalization, and hence transfection. (EPR - enhanced permeability and retention) [237].

Liu et al. (2003) highlighted some concerns with the biocompatibility of CaP nanoparticles, indicating an apoptotic response of CaP nanoparticles in the concentration range of 50-200 mg/L, albeit there is a question regarding the size of the CaP particles used in the study [254, 275]. The adverse reaction by cells may be due to the increase in concentration of intracellular calcium. Neumann et al. (2009) reported that the use of nano-sized particles of CaP avoided the issue of intracellular calcium concentration which is observed when using larger particles from the original CaP transfection method [231].
Successful transfections have been reported using a number of modifications to the original CaP approach. Olton et al. (2007) found that employing a very high Ca:P ratio of between 100:1 and 300:1 yielded CaP nanoparticles suitable as a delivery vector [124]. DNA-loaded calcium phosphate nanoparticles functionalized with bovine serum albumin (BSA) also produced functional transfection [276]. The transfection efficiency of calcium phosphate nanoparticles can be increased by applying additional layers of calcium phosphate and DNA for DNA encapsulation, thus protecting it from intracellular enzymatic degradation [277].

Recent in vivo studies have shown that calcium phosphate has been used to effectively transfect bone tissue [278-281]. Endo et al. (2006) showed that implanting a scaffold consisting of bovine atelocollagen and expression plasmid vector encoding for human BMP-2, with CaP, achieved bridging of a rat tibial bone defect at 4 weeks, and the strength of the bone was comparable to that of an intact tibia at 6 weeks [278]. This illustrates the potential of scaffolds loaded with a suitable gene delivery vector for transfection both in vitro and in vivo and this topic is discussed below.

1.8.4 Scaffolds for gene delivery

The sustained release of pDNA is required to enhance long term therapeutic gene expression and defect healing. This can be done by the use of a biodegradable scaffold acting as a reservoir that protects the condensed DNA resulting in a slower transfection rate, with a longer expression time of the transgene. The 3D
environment may also enhance cellular transfection as it has been observed that some cell types respond differently and are transfected more efficiently in 3D than in 2D [221, 282]. It is possible that primary cells are more sensitive to matrix interactions and thus, the 3D environment acted as a more powerful adjuvant for these cells. However, the relatively higher transfection levels might simply be a function of cellular invasiveness in the scaffold architecture. Hosseinkhani et al. (2006) observed an increase in alkaline phosphatase activity and osteocalcin content of transfected MSC cells cultured in 3D vs. 2D conditions [235].

Many different types of constructs have been studied to date. Such scaffolds include poly(lactic-co-glycolic acid) (PLGA) scaffolds, type I/III collagen scaffolds, PLGA-HA scaffolds as well as the DNA hydrogel [283-286]. They are biocompatible, biodegradable, inexpensive to fabricate and easily moulded into desired shapes and sizes. A porous PLGA scaffold containing poly(ethylenimine) (PEI) condensed pDNA has been shown to lead onto a more mineralised bone tissue within a rat cranial defect than scaffold alone or uncondensed plasmid controls [287]. In addition, Huang et al. (2005) have also shown that sustained high levels of gene expression were achieved for up to 15 weeks when this scaffold was implanted into rat subcutaneous tissue [286]. Krebs et al. (2009) reported similar success in a study investigating the effectiveness of CaP-pDNA complexes delivered from an alginate hydrogel. Bony tissue formation in a subcutaneous mouse model was observed after two and half weeks due to the sustained release of the pDNA encoding BMP-2 [269].
Holladay et al. (2009) studied the effectiveness of a 3D collagen scaffold incorporating DNA molecules complexed with dendrimers for sustained pDNA release [221]. Using this complex with collagen scaffolds, prolonged transfection period was observed with relatively high levels of reported gene expression. A collagen-based 3D scaffold mentioned briefly in a previous section (section 1.6.1) showed similar potential. Bonadio et al. (1999) developed a gene activated matrix (GAM) system for in vivo plasmid delivery, and when implanted in a canine bone defect model led to sustained expression for up to 6 weeks, with retention and expression of pDNA evident for up to 6 weeks, leading to significant new bone formation [13]. GAMs are biomaterials that can be used to deliver DNA to cells, based on the principle that a plasmid DNA is incorporated into the matrix, and when the matrix is degraded, the DNA is slowly released and taken-up by the cells surrounding the matrix. A GAM was also used to deliver plasmid DNA into a segmental defect in a rat femur by soaking lyophilized bovine tracheal collagen in BMP-4 and implanting this into the defect. Although non-treated defects remained unbridged, defects implanted with pBMP-4 GAMs regenerated in 9 weeks [288].

Looking beyond bone tissue engineering, achieving controlled, localized gene expression within an engineered scaffold will have broad potential to be applied to the regeneration of many tissue types, with great promise for clinical therapies [229]. Collagen-based matrices may serve as an ideal template for these wide-ranging applications [289], and as such are the basis of the study conducted in this thesis.
Aims of Thesis

The primary objective of this research work was to develop a tissue-engineered scaffold with inherent structural and material properties suitable for use as a bone graft substitute. The two major constituents of bone; collagen type 1 and nano-hydroxyapatite, are combined to yield a porous, resorbable and osteoconductive composite scaffold.

The specific aims of this thesis were:

- To develop a protocol for the quick and reproducible synthesis of non-aggregated nHA (Chapter 2)

- To incorporate the nHA into a range of highly porous, resorbable composite scaffolds, applying characterisation techniques to determine the scaffolds with most potential for use as a bone graft (Chapter 3)

- To investigate the \textit{in vitro} behaviour of these scaffolds (optimised from Chapter 3) using short-term analysis to look at initial cell attachment and proliferation, and a long term study to examine osteogenesis, matrix deposition and mineralisation (Chapter 4)

- To investigate the potential of nHA developed in Chapter 2 as a non-viral plasmid gene delivery vector, both in 2D culture and in a 3D gene activated matrix, utilising techniques developed in Chapter 3 (Chapter 5).
Chapter 2: Synthesis and characterisation of nanophase hydroxyapatite (nHA) using a novel dispersant-aided precipitation method

2.1 Introduction

Bioceramics have been extensively researched for use in engineered bone grafts. One of the most commonly used is hydroxyapatite (Ca_{10}(PO_4)_6(OH)_2; HA) which displays excellent bioactivity and biocompatibility [110, 290-293]. Currently it is used in numerous clinical applications; in the form of powders, granules, coatings, dense and porous blocks, as a non-viral carrier for plasmid DNA gene delivery, in photodynamic therapy as well as in biocomposites [124, 138, 262, 284, 294-300]. However, as micron-sized HA exhibits poor bioresorbability and brittle characteristics [59, 301-306], the study of nanophase HA (nHA) has become more imperative [121, 122, 126, 127, 129, 135, 174, 307].

Investigations of nanophase materials to date have illustrated their potential for bone repair. For example, increased osteoblast adhesion on nano grained materials in comparison to conventional (micron grained) materials has been reported [127, 128]. Osteoblast proliferation in vitro and long-term functions have also been shown to be enhanced on ceramics with grain or fibre sizes less than 100 nm [126]. In addition to osteoblast responses, increases in osteoclast-like cell function has
also been documented on nanophase ceramics [137], while in vivo studies have demonstrated increased new bone formation on metals coated with nHA compared to conventional hydroxyapatite [122, 152-156].

Various methods are reported for the synthesis of HA, including wet chemical precipitation, sol-gel processing, mechanochemical synthesis, solid-state reactions, chemical vapour deposition, hydrothermal synthesis, combustion method, solvothermal and reverse micro-emulsion techniques [154, 156-165]. Of these methods, wet precipitation is the most common, mainly due to its simplicity and low cost, which also makes it suitable for industrial production. However, difficulties arise during the synthesis of nano-sized HA [143]; the resultant particles are often formed in the micron size-range with a wide particle size distribution, low surface areas and widespread agglomeration [144-146]. There is an inability to effectively control flocculation of particles as they are created in aqueous solutions and often an extensive ball-milling step is required to separate the agglomerated particles [148-151]. Alternatively, the use of surfactants in precipitation reactions has been investigated with moderate success, albeit they do not overcome the necessity for time consuming ageing and heat treatment stages [127, 167, 308]. Surfactants and dispersing agents can prevent widespread agglomeration from occurring by controlling the nucleation and crystal growth process. In this thesis, poly(vinyl alcohol) (PVA) was chosen as a surfactant due to its biocompatibility and established use in biomedical applications [168, 309-311]. Darvan 821A® was also used as a dispersing agent. The use of the ammonium-
based polyacrylic acid, Darvan 821A, has been reported in literature for the dispersion of commercial HA particles in aqueous solutions [169, 312, 313], however, its use during the synthesis phase of HA particles has not previously been explored.

2.1.1 Aim

The aim of this study was to develop and optimise a novel aqueous, low-temperature rapid precipitation reaction to create non-aggregating particles of nHA (< 100 nm). The goals were to determine the role of reaction pH, the rate of reactant mixing, use of sonication, order of addition and concentration of the primary reactants on the formation of nHA. In addition, the effect of using a surfactant (PVA) and dispersing agent (Darvan 821A®) on the final product was also examined.

2.2 Materials and Methods

2.2.1 Materials

The chemicals used in this study were as follows: calcium chloride dihydrate (analytical reagent grade 100 %, Fisher Scientific, Pittsburgh), sodium phosphate tribasic dodecahydrate (min 98 % Sigma, Dorset), sodium hydroxide anhydrous (Sigma, Dorset). Darvan 821A® was provided by R.T Vanderbilt (Norwalk). Poly(vinyl alcohol) (Sigma, Dorset) was used in solution as a surfactant. A Ca/P
ratio of 1.67 was maintained throughout the study by adding reactants in the appropriate stoichiometric ratio to ensure the formation of hydroxyapatite.

2.2.2 Preparation of nHA particles

The initial procedure used for the preparation of nHA particles was based on work by Kumta et al. [124]; aqueous solutions of CaCl$_2$.2H$_2$O (0.41 M) and Na$_3$PO$_4$.12H$_2$O (0.25 M) were prepared using double distilled H$_2$O. NaOH (0.0003 M) was added to the phosphate precursor to control the pH at 10.5. These were mixed by dropping the calcium precursor into the phosphate precursor, at a rate of 18,000 ml/hr. The precipitation reaction occurred immediately under stirring, according to Equation 1. The solution was then centrifuged for 99 minutes at 4000 rpm and washed (with water) to remove the NaCl by-product. The precipitate was resuspended using sonication to yield nHA suspension, or freeze-dried to generate a fine powder. These particles will be referred to as our controls herein; all other variants are compared to these samples.

Equation (1)

\[10\text{CaCl}_2.2\text{H}_2\text{O} + 6\text{Na}_3\text{PO}_4.12\text{H}_2\text{O} + 2\text{NaOH} \rightarrow \text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2 + 20\text{NaCl} + 92\text{H}_2\text{O}\]

2.2.3 Synthesis parameters

The following parameters were varied from this original synthesis protocol in order to optimise the production of nHA, while maintaining a stoichiometric Ca/P ratio of 1.67 (Table 2.1). The concentration of the initial reactants (Ca: 0.414 to 0.001 M, PO$_4$: 0.248 to 0.0006 M), the order (Ca into PO$_4$ and vice versa) and the rate of their addition (5, 10, 300 and 18,000 ml/hr) were altered to establish what effect they had on nHA particle formation. In addition, the pH of the reaction was varied
from 8 to 12.5 by adding different quantities of NaOH to establish the effect of pH on the particle size distribution. The use of different quantities of PVA (1, 3 and 6 % w/v) and Darvan (0.1 to 1 % v/v) was also investigated by adding the respective solutions to the phosphate solution, prior to conducting the precipitation reaction. Each factor was varied while keeping all others constant to investigate their role independently. The effect of sonication was also examined in conjunction with the other factors.

Table 2.1 Summary of nHA synthesis parameter variations

<table>
<thead>
<tr>
<th>Synthesis Parameter</th>
<th>Variations</th>
</tr>
</thead>
<tbody>
<tr>
<td>(i) Synthesis Conditions</td>
<td></td>
</tr>
<tr>
<td>Concentration of Calcium Precursor (M)</td>
<td>Varied from 0.001 M to 0.414 M</td>
</tr>
<tr>
<td>Concentration of Phosphate Precursor (M)*</td>
<td>Varied from 0.0006 M to 0.248 M</td>
</tr>
<tr>
<td>Order of addition</td>
<td>Calcium added to Phosphate and <em>vice versa</em></td>
</tr>
<tr>
<td>Rate of addition (ml/hr)</td>
<td>5 ml/hr, 10 ml/hr, 300 ml/hr and 18000 ml/hr</td>
</tr>
<tr>
<td>Reaction pH</td>
<td>Varied from 8 to 12.5</td>
</tr>
<tr>
<td>(ii) Surfactant/Dispersant addition</td>
<td></td>
</tr>
<tr>
<td>PVA (% w/v)</td>
<td>1, 3 and 6</td>
</tr>
<tr>
<td>Darvan (% v/v)</td>
<td>Varied from 0.1 to 1</td>
</tr>
<tr>
<td>Technique used in combination with other variants</td>
<td></td>
</tr>
<tr>
<td>Sonication (minutes)</td>
<td>Used (2 minutes) in combination with order of addition, PVA addition and Darvan addition studies. Also investigated its use on the optimised synthesis; Varied from 2 minutes to 14 minutes</td>
</tr>
</tbody>
</table>
2.2.4 Particle size determination

Particle size distributions were measured using dynamic light scattering (DLS) (ZetaSizer 3000 HS, Malvern instruments, UK). Measurements \((n \geq 3)\) were carried out under monochromatic, coherent He-Ne laser light of fixed wavelength \((633\, \text{nm})\) at room temperature with each size determination yielding an average particle size expressed as the mean diameter \(Z_{\text{ave}}\) together with a plot of the size distribution.

Light scattering nanoparticle tracking analysis (LSNTA) was employed to verify particle size distributions for nHA synthesised using the optimised parameters, with and without Darvan (0.1%). LSNTA allows the visualisation of particles through a light microscope, using a laser light source to illuminate the particles as they move under Brownian motion. The attached NanoSight™ image analysis software (NanoSight LM 20, Wiltshire, UK) tracked the real-time motion of these particles, and thus calculated their size based on their movement and the Stokes-Einstein relationship, providing a particle size distribution and mean diameter, similar to DLS.

2.2.5 Electron microscopy and atomic force microscopy

Analysis of particle morphology was performed using Transmission Electron Microscopy (TEM; JEM 100CXII, JEOL, Japan) by placing a drop of nHA suspension onto pioloform-coated copper grids and allowing it to dry. For Scanning Electron Microscopy (SEM), aggregated nHA samples were adhered to
rapid setting epoxy resin on an aluminium specimen stub and then sputter coated with gold using a Polaron Sputter Coater, to a thickness of 10 nm. Imaging was carried out using a JEOL 840 SEM, operated at 15 kV. In addition, nHA suspension was placed onto a silicon disc and dried in a vacuum oven at 60°C for 60 minutes in preparation for Atomic Force Microscopy (AFM) imaging. This was carried out using a Nanoscope 3A Multimode AFM, operated in tapping mode using silicon nitride tips over an area of 25 mm².

2.2.6 Physico-chemical characterisation

nFJA precipitates, from suspensions synthesised using optimised parameters, both with and without Darvan (0.1 %), were freeze dried to yield a fine powder (Advantage EL, Vir-Tis Co., Gardiner, NY). The crystal phase of the product was determined using a Bruker Advance D8 X-Ray diffractometer (XRD). Cu-Kα radiation (λ = 0.1542 nm) was used and all samples were run for 2 hours in the 2θ range from 20–60° at a scan speed of 4 sec/step using an increment step size of 0.02°. A comparative heat-treated sample (optimised nHA with 0.1 % Darvan) was calcined at 650°C for 4 hours to further crystallise the sample. Analysis of spectra and crystallinity calculations were performed using DIFFRACplus EVA software. Peak broadening occurs and can be used to estimate the crystallite size using Scherrer’s equation [314]

\[ X_s = \frac{K \lambda}{\beta_m \times \cos \theta} \]  

(2)

Where \( X_s \) is the crystallite size in nm, \( K \) is a constant, taken for HA as 0.89, \( \lambda \) the wavelength of monochromatic X-Ray beam (0.154 nm); \( \beta_m \) the full width at half
maximum for the diffraction peak under consideration (rad) and $\theta$ is the diffraction angle (°).

Fourier Transform Infrared microscopy (FTIR) analysis was carried out on nHA powders prepared using optimised parameters, both with and without Darvan (0.1% and 0.5%) using a Spectrum One FTIR (Perkin Elmer, UK). Freeze-dried powders were mixed with potassium bromide using a mortar and pestle, before being pressed into a transparent sample. Spectra were collected between wavenumbers 4000 and 400 cm$^{-1}$.

Zeta Potential (ZP) readings ($n = 6$) were conducted on freshly prepared suspensions using a ZetaSizer 3000 HS (Malvern instruments, UK). The ZP value of a sample serves as an indication of the stability of the suspension.

2.2.7 Statistical analysis

All data was analysed for significance ($p \leq 0.05$) using one-way analysis of variance (ANOVA) with SPSS v15. Post hoc tests to determine significant differences between group means were performed using the conservative Tukey test.
2.3 Results

2.3.1 Effect of synthesis conditions on average particle size

*Initial control reaction*

Initial hydroxyapatite particles produced using the technique described in section 2.2.2 [84], *i.e.* using the control procedure with a Ca/P ratio of 1.67, were shown using dynamic light scattering (DLS) to be approximately 24 \( \mu \text{m} \) in diameter with large deviations while in suspension. Scanning Electron Microscopy (SEM) of dried samples (after grinding using a mortar and pestle) revealed that large aggregates had formed during synthesis as shown in Figure 2.1. These images, taken at different magnifications of the same sample, show a representative view of the powders obtained before optimisation of the synthesis procedure. Conventional SEM was unable to image optimised non-aggregating nHA powders due to the resolution of the technique.
Figure 2.1 Scanning Electron Microscopy (SEM) images showing the aggregation of nanophase hydroxyapatite (nHA) particles precipitated in the non-optimised synthesis at different magnifications (scale bars from top left; 100 μm, top right; 10 μm, bottom left and bottom right 1 μm).

Varying synthesis conditions

The concentration of both the calcium and phosphate precursors was the first parameter to be varied from the initial protocol. The average particle size of synthesised nHA was found to decrease significantly with decreasing concentrations. The initial calcium solution concentration of 0.414 M yielded particles that were immediately visible on precipitation. This indicated that they were many microns in size. The calcium concentration was sequentially diluted to 0.001 M, at which stage the particles were no longer visible during the synthesis.
phase. However, the yield of nHA decreased corresponding with decreasing concentration, and so a lower limit was set at this 0.001 M value. Therefore, reducing the concentration of the reactants corresponded to a reduction in aggregate size; however, altering the concentration of the precursors alone was not sufficient to prevent aggregation.

Changing the order of addition of the reactants so that the phosphate solution was added to the calcium solution resulted in a significant decrease in particle size; 2879.7 ± 1305 nm vs. 1105.9 ± 224 nm (Figure 2.2). Sonication was found to aid average particle size reduction especially when the phosphate solution is added into the calcium precursor (144.1 ± 50 nm), although it was not sufficient to prevent aggregation, either independently or combined with low concentration and optimal order of addition. The rate of reactant addition was not found to have a significant (p ≤ 0.05) effect on the size of the resulting precipitate (Figure 2.3), and so the fastest rate (18000 ml/hr) was selected for further experiments.
Figure 2.2 Graph showing the relationship between average particle size and the order of addition of the calcium and phosphate precursors. Additionally, the effect of sonication is shown. (S) = sonication used (NS) = no sonication.

Figure 2.3 Graph showing the relationship between average particle size and the rate of addition of the calcium and phosphate precursors in the absence of sonication.

The pH of the reaction was controlled by the addition of sodium hydroxide (NaOH), and it was established that the particle size was lowest and most consistent at a pH between 8 and 9.25. As seen in Figure 2.4, the particle size rises sharply above pH 9.5, with variable results obtained.
Therefore, in summary, the above results indicate that the optimised conditions involve the rapid addition of the phosphate precursor (0.0006 M) into the calcium precursor (0.001 M) at a pH between 8 and 9.5, using sonication to resuspend particles following the washing stage. This thus serves as the control synthesis during the investigation of surfactant and dispersant addition.

2.3.2 Effect of surfactant/dispersant addition on average particle size

*Surfactant (PVA) addition*

The addition of increasing amounts of the surfactant, poly(vinyl alcohol) (PVA), to the control synthesis reaction (optimised conditions) led to a significant reduction in average particle size, (1 %: 2926 ± 3268 nm, 3 %: 1686 ± 2377, 6 %: 366 ± 196...
nm) as seen in Figure 2.5. However, large deviations can be seen due to irregular particle size ranges within samples. Preparing the 6 % w/v sample of PVA proved troublesome as it had a ‘bubbly’ consistency and although it resulted in a 26 fold reduction in aggregate size, it also led to a significant decline in the yield. Sonication was not effective when working with PVA and actually increased the particle size compared to non-sonicated samples.

![Graph showing the effect of the addition of poly(vinyl alcohol) (PVA) on average size of nanophase hydroxyapatite (nHA) particles.](image)

**Figure 2.5** Graph showing the effect of the addition of poly(vinyl alcohol) (PVA) on average size of nanophase hydroxyapatite (nHA) particles.

**Dispersant (Darvan) addition**

Although the surfactant PVA was shown to be capable of reducing the average particle size with respect to the control synthesis, regular nano-sized nHA particles were not obtained. Subsequently, the use of the dispersing agent, Darvan 821A® was investigated. Nano-particles of HA were synthesised by using the optimal reaction conditions (0.0006 M phosphate solution added to 0.001 M calcium solution at 18000 ml/hr) with the dispersing agent, Darvan 821A®. This optimised
synthesis yielded uniform, nano-sized particles in suspension rather than the large random sized agglomerates observed previously. The required amount of dispersant to prevent aggregation was found to be in the range of 0.1 % to 0.5 % v/v. A combination of reduction in particle size above 0.1 % v/v and a significant decrease in particle yield above 0.5 % v/v led to this determination (Figure 2.6). Therefore, for subsequent experiments a value of 0.1 % v/v Darvan was chosen. Darvan influences the reaction pH, increasing it to over 11, although this does not have an impact on particle size, as was observed previously in the synthesis without the use of a dispersing agent, in which larger irregular particles were observed at a pH of over 9.5.

![Figure 2.6 Dynamic Light Scattering (DLS) results displaying the average particle sizes of nanophase hydroxyapatite (nHA) prepared with different concentrations (% v/v) of Darvan dispersant.](image)

Figure 2.6 Dynamic Light Scattering (DLS) results displaying the average particle sizes of nanophase hydroxyapatite (nHA) prepared with different concentrations (% v/v) of Darvan dispersant.
In a comparative study between the use of “optimal” PVA (6 % w/v) surfactant and 0.1 % v/v Darvan dispersant, a smaller average particle size (360 ± 194.7 nm vs. 140 ± 10.7 nm respectively) with a much tighter standard deviation was observed when Darvan 821A® was used (Figure 2.7).

![Graph showing the comparative effects of the addition of 6 % w/v poly(vinyl alcohol) (PVA) and 0.1 % v/v Darvan on the average size of nHA particles.](image)

Figure 2.7 Graph showing the comparative effects of the addition of 6 % w/v poly(vinyl alcohol) (PVA) and 0.1 % v/v Darvan on the average size of nHA particles.

Sonication was required to fully disperse the nanoparticles for this optimised synthesis (0.0006 M phosphate solution and 0.1 % v/v Darvan, added to 0.001 M calcium solution). Control reactions with no dispersant, followed by sonication, yielded average particle sizes in the micron range (5.556 ± 5.454 μm) whereas, the presence of a dispersing agent prevented particle agglomeration and reduced the average particle size to 91.4 ± 0.7 nm following sonication. A concentration effect is evident in Figure 2.8 where the higher concentrations (calcium precursor concentration: 5X - 0.005 M, 10X - 0.01 M, i.e. 5 and 10 times the initial concentration used) yield micron sized aggregates with no sonication, but the sizes
could be reduced significantly to within the nano range after sonication (10 minutes). In addition, increasing the time of sonication was shown to reduce particle size further up to 12 minutes, at which stage no further significant reduction was obtained (Figure 2.9). Therefore, the use of sonication enables the synthesis of a higher yield of consistent nHA particles using the higher concentration of initial reactants. Consequently, the final optimised synthesis protocol developed in this study is the rapid (18000 ml/hr) addition of phosphate solution (0.0006 M) to calcium solution (0.001 M) in the presence of 0.1 % Darvan, although the concentrations may be increased to 0.006 and 0.01 M, followed by 10 minutes sonication, if required for large-scale production.

Figure 2.8 Graph showing the effect of the concentration of the initial reactants (calcium and phosphate precursors) on average particle size, when 0.1 % Darvan is present. Additionally, the effect of sonication (10 minutes) is also displayed. X: [Ca] = 0.001 M, 5X: [Ca] = 0.005 M, 10X: [Ca] = 0.01 M, (s) = sonication used, (ns) = no sonication.
Figure 2.9 Graph showing average particle size as a function of sonication time. The particles were precipitated in the presence of 0.1 % v/v Darvan dispersing agent at an initial calcium concentration of 0.001 M and phosphate concentration of 0.0006 M.

An additional size analysis technique was utilised to verify the dispersing effect of Darvan on the nHA particles formed. The light scattering nanoparticle tracking analysis (LSNTA) technique and Nanosight™ image analysis provided a particle size distribution with an average value of 184.4 ± 34.4 nm (n = 10) for unsonicated nHA suspension with 0.1 % Darvan (sample a). In contrast to this, the average value for samples prepared using the same conditions, but with no Darvan present, was 898 ± 305.5 nm (sample b, n = 2). It should be noted that the NanoSight™ technology has a working range of 10 nm-1000 nm and the sample (b) nHA suspension prepared using no Darvan, is too close to the upper limit of this range to be considered an accurate reading. The result should be taken only as a comparison to the non-aggregated sample (a) prepared with 0.1 % Darvan. The low n number for the second sample is due to aggregation occurring during measurement, leading to a lack of completed tracks, and thus prevented measurements of the required
quality being obtained. Figure 2.10 shows a screen print view of the analysis running during measurement, with evident differences visible between sample (a) (0.1 % Darvan) and sample (b) (no Darvan). A small, homogenous range is visible for the dispersed sample, while in contrast to this, the measurements being collected for sample b show a wide spread of values, with some outcomes well above the nanometer range.

Figure 2.10 Snapshots of particle size distribution measurements taken during NanoSight™ analysis for (a) nHA particles synthesised using 0.1 % Darvan and (b) the same synthesis conditions with no Darvan.
All subsequent analysis is conducted on particles synthesised using the optimised protocol (the rapid (18000 ml/hr) addition of phosphate solution (0.0006 M) to calcium solution (0.001 M) in the presence of 0.1 % Darvan, with 10 minutes sonication following the washing stage), unless otherwise stated.

2.3.3 Analysis of synthesised nHA particles

Zeta Potential

Zeta Potential (ZP) measurements were taken for nHA suspensions prepared using the optimised synthesis conditions, with varying quantities of the dispersing agent. Results show that the presence of the dispersing agent serves to increase the ZP value, to -46.9 ± 0.7 mV for 0.1 % Darvan and -40.2 ± 0.3 mV for 0.5 % Darvan, hence increasing the stability of particles (Figure 2.11). Aggregation is known to occur without a dispersing agent, even with all other parameters optimised, and this corresponds with a lower ZP value of -27.1 ± 1.4 mV falling within the range known to cause agglomerate formation (+30 mV to -30 mV). Increasing the amount of the dispersant from 0.1 % to 0.5 % decreased the ZP value, thus slightly decreasing the stability of the synthesised particles, which may be responsible for the decrease in particle yield which was observed visually in the study of quantity of Darvan vs. particle size (Figure 2.6).
Figure 2.11 Graph showing the absolute values of zeta potential for suspensions synthesised in the presence of various quantities of dispersant, 0.1 % Darvan and 0.5 % Darvan.

Microscopical investigation of optimised particles

Transmission Electron Microscopy (TEM) showed that the nHA particles generated using the optimised conditions in the presence of Darvan 821A® dispersing agent were non-aggregating rod shaped crystals approximately 30 nm by 10 nm (Figure 2.12). This is less than the corresponding DLS findings which gave an average size of 82.4 ± 11.1 nm for the imaged sample.
Figure 2.12 Transmission Electron Microscopy (TEM) image showing the morphology of the nanophase hydroxyapatite (nHA) particles synthesised using 0.1 % Darvan dispersing agent.

Individual nHA particles were also viewed using the high resolution of Atomic Force Microscopy (AFM). The samples were oven dried before imaging, causing some aggregation to occur, which is likely due to static forces arising during drying. The heights of individual particles appeared to be under 50 nm and particle aggregations were found to have approximately similar height dimensions (Figure 2.13).
Figure 2.13 Atomic Force Microscopy (AFM) image showing how the particles tend to re-aggregate slightly due to static forces following oven drying. (2-Dimensional and 3-Dimensional view of the same image).

Material characterisation

X-Ray Diffraction (XRD) confirmed the dried nHA particles to be hydroxyapatite without the presence of any other calcium phosphate phases (JCPDS 72-1243C) [315, 316]. The crystallinity of the non-heat treated sample was determined to be 72.9 % and, as shown in Figure 2.14, the crystallinity can be significantly increased on calcination of the sample at 650°C for 4 hours. When nHA made without Darvan is compared to nHA made with Darvan there is a close correspondence in their spectra, but with broader peaks observed in the dispersant-aided nHA sample (Figure 2.15); this may be due to smaller particle size, and lack of long range order of the nano crystals in this sample. The crystallinity of the nHA prepared without Darvan is slightly greater than that with Darvan; 74.3 % vs. 72.9 %. There is also a small peak at 22.5° 20 which is attributed to ammonium calcium phosphate
(JCPDS 21-0697). This is known to decompose to HA over time at room temperature and is not evident after calcination in Figure 2.14 [317].

**Figure 2.14** X-Ray Diffraction (XRD) spectra of the as prepared nanophase hydroxyapatite (nHA) with Darvan (bottom spectrum) and the same sample after calcination at 650°C (top spectrum). The red vertical lines show the location of HA peaks based on the JCPDS standard, 72-1243C.

Taking the (002) peak from these XRD spectra, at approximately 25.9° 2θ, the crystallite size of the nHA samples were determined using the Scherrer’s equation [301]. The crystallite size of nHA synthesised using the optimised procedure and 0.1% Darvan was found to be 26.8 nm compared to a crystallite size of 50.4 nm when the synthesis was conducted without the dispersing agent.
Figure 2.15 The broader XRD spectrum (b) shows the presence of a small peak at 22.5° 2θ indicating the presence of ammonium calcium phosphate compared to the HA spectrum synthesised using the original "non-optimised" synthesis (a).

Fourier Transform Infra-Red (FTIR) spectra of nHA synthesised both with and without the presence of Darvan reveal characteristic peaks for carbonated hydroxyapatite (Figure 2.16). Peaks due to phosphate vibrational modes are visible throughout the spectra; v4 at 562 and 603 cm\(^{-1}\), the broad band for v3 from 1090 to 980 cm\(^{-1}\). The phosphate v1 band at 1031 cm\(^{-1}\) appears to be masked by the v3 band. The v2 band at 469 cm\(^{-1}\) is less evident in the spectrum for nHA with 0.5 % Darvan present (C) [318]. There are also peaks associated with Darvan present, which are representative of the R-COO- stretch and may be shifted from 1573 to 1557 due to an intermolecular bridge R-COO-Ca complex formation (indicated by * in Figure 2.16) [313, 319]. The relative lack of resolution of the P-O absorption bands in the 0.5 % Darvan nHA sample may indicate that the nHA in this sample has a more amorphous nature than the other spectra [320].
2.4 Discussion

The rationale behind developing a quick, low temperature method for synthesising nHA is to enable convenient production of the particles for use in bone tissue engineering applications, specifically as a filler material in a composite scaffold or a delivery vehicle for plasmid DNA. The aqueous suspension is an appropriate form for these functions, although the suspension can also be freeze-dried to yield a fine nanophase powder if desired. The use of nano-sized HA has led to improved mechanical properties, resorbability and biocompatibility when compared to using conventional micron-sized particles [117, 126, 135, 136, 141, 321]. Tran et al.
(2009) report enhanced in vitro and in vivo osteoblast responses across a wide range of nanomaterials, including nHA, highlighting the role of nanoscale roughness and an increase in surface energy [123]. However, fine-tuning synthesis methods in order to construct nano-sized, non-aggregating nHA particles has proven difficult, often employing the use of surfactants in conjunction with time consuming heating and aging steps in precipitation reactions [322]. Results of the current study indicate that controlling reaction parameters such as the concentration of initial reactants, reaction pH and the use of sonication has important implications for the final product in terms of aggregation and particle size. Reducing the calcium and phosphate precursor concentrations correlated to a decrease in particle size although a lower limit is set due to the reduction in yield. Therefore, a balance is required between achieving nano-sized particles and obtaining a sufficient yield. This trend showing a reduction of particle size with reducing concentration agreed with other studies and a range of calcium concentrations, from 0.001 M (X) to 0.01 M (10X), was selected for experiments in the rest of this investigation [124]. The original synthesis method [84] specified the addition of the calcium into the phosphate precursor, however, reversing this, and combining the use of sonication led to a large reduction in aggregation and thus a decrease in resultant particle size. This may be due to the relative atomic sizes of the calcium and phosphate ions. Sonication has long been used to disperse solids in a suspension, but to work it must overcome the attractive forces between molecules. In this work, it was found that the forces between newly forming nHA
particles were too strong and sonication alone was not sufficient to overpower these.

Contrary to previous findings, in this simple chemical precipitation reaction the rate of addition of the precursor solutions did not play any significant role on the particle size formed when using optimal concentration and order of addition conditions (0.0006 M phosphate into 0.001 M calcium) [84]. This may be due to the optimisation of the protocol for working with these particular starting materials and we speculate that the rate of introducing phosphate ions into a calcium ion solution is not important if the concentration is sufficient to keep the nucleation sites separated, thus preventing widespread aggregation from occurring.

Without the addition of a surfactant or dispersing agent, the synthesis required strict control over reaction pH, maintaining it between 8 and 9.5 in order to minimise particle size. It was observed that conducting the reaction above pH 9.5 formed large and polydisperse agglomerates, while reactions below pH 7.5 are known to produce other forms of calcium phosphate such as brushite (CaHPO₄·2H₂O) and tri-calcium phosphate (Ca₃(PO₄)₂). These results indicate that unaided (i.e. no surfactant or dispersant) precipitation reactions, synthesising nHA, should be executed within the range pH 8-9.5.

The factor with the largest effect on aggregation and particle size was established to be the use of a dispersing agent. The surfactant, poly(vinyl alcohol) (PVA),
reduced the particle size range to less than 400 nm but required a large volume (6 % w/v) to do this and a large variability was obtained. In contrast to this, using the dispersing agent, Darvan 821A, led to uniformly sized particles of under 100 nm using 0.1 % v/v. The effect the Darvan had on reducing the range of particle sizes produced was also made evident using the LSNTA method. Darvan 821A is an ammonium based dispersing agent with polyacrylic acid as the active agent. Its molecules are negatively charged along their length and they attach to particles causing them to repel each other resulting in a disperse nanophase precipitate suspension. Other forms of Darvan and polyacrylic acid have been used in the dispersion of ceramics in aqueous media, particularly in high solid loading [169, 312, 313, 323, 324].

Volumes of the dispersant below 0.1 % resulted in no reduction in particle size, while volumes greater than 0.5 % significantly reduced the particle yield, and this may be due to the change in Zeta Potential (ZP) at this higher dispersant concentration, indicating the reduction of stability in the suspension. The ZP of a suspension is the potential difference between the suspension medium and the layer of fluid surrounding the dispersed particle and provides an indication of the tendency of a suspension to remain stable or to agglomerate. ZP essentially is a measure of the repulsion between similarly charged particles in suspension. For sufficiently small particles, a large ZP value (positive or negative) will denote stability; however, a low ZP will imply that attraction exceeds repulsion between particles, causing the dispersion to aggregate.
Examining the particles under TEM revealed rod shaped crystals approximately 30 nm in length, although this is less than 90 nm, which the DLS results indicated. AFM showed small crystals beginning to clump together during the oven drying, and these individual crystals were also under 30 nm in dimension. XRD and FTIR analysis demonstrated the presence of phase pure nHA. The presence of the organic component of Darvan is visible in the FTIR spectra of particles synthesised using the dispersing agent. XRD also shows an effect of the Darvan, although it is shown to be removed following heat-treatment if required. The broadness of the XRD peaks in the spectrum of nHA powder synthesised with Darvan is due to the small particle size leading to a lack of long range order of crystals (similar to biological HA) within the sample, which is contrary to the well defined peaks in the heat-treated sample. The decreased crystallinity of nHA has been shown to have a positive effect on the adhesion of osteoblasts [325]. The spectrum also reveals the crystallite size of nHA, synthesised using Darvan, via the Scherrer’s equation to be 26.8 nm, in comparison to 50.4 nm when Darvan is not utilized. This agrees closely with the TEM and AFM imaging analysis which shows individual crystals of approximately these dimensions. The discrepancy between these results and the DLS analysis may be due to the specific technique used to determine average particle sizes using DLS. According to the manufacturer’s website the average particle size measured is in fact the hydrodynamic diameter, which is based on how a particle diffuses within a fluid, taking surface structure, concentration and ions present in the media into account during measurement
[326]. The website also states that the size may be larger than corresponding measurements using electron microscopy.

2.5 Conclusions

Results from this research have shown that the described novel low temperature synthesis technique produced non-aggregating nanoparticles of HA. The optimised precipitation reaction involved the rapid (18000 ml/hr) addition of phosphate solution (0.0006 M) to calcium solution (0.001 M) in the presence of 0.1 % Darvan, with 10 minutes sonication employed to resuspend the particles following the washing stage. Time-consuming heating and ageing treatments to produce the nHA crystals were avoided using this procedure and the resultant nHA particles are producible in a form for their potential use in a myriad of biomedical applications. The introduction of Darvan 821A® dispersing agent was the principal factor behind the prevention of agglomerate formation. Further significant factors which were found to reduce particle size included maintaining the reaction pH between 8 and 9.5, decreasing the initial concentrations of reactants, using sonication, and changing the original order of reactant addition by adding the phosphate precursor to the calcium precursor.
Chapter 3: Development and characterisation of collagen nano-hydroxyapatite composite scaffolds for bone tissue engineering

3.1 Introduction

The ideal scaffold for tissue engineering acts as a template, supporting cell growth and differentiation and ultimately the deposition of regenerated tissue [1, 48]. In bone tissue engineering, the scaffold should be biocompatible with osteoconductive and osteoinductive properties and should allow for cells to attach, proliferate and form extracellular matrix (ECM). It requires an open and interconnected pore structure (with a porosity >90 %) that allows nutrients to diffuse into the scaffold \textit{in vitro} and accommodates vascularisation to occur \textit{in vivo} [47, 171, 327]. The scaffold should also degrade at a suitable rate to match the rate of regenerative tissue formation.

This chapter focuses on the development of a novel composite scaffold for bone regeneration using the two major constituents of bone; collagen type 1 and hydroxyapatite (HA; \(\text{Ca}_{10}p\text{O}_{4}\text{H}_{6}\)) in the form of nanoparticles (<100 nm). Composite materials are heterogeneous materials made up of two or more phases which allow the advantageous properties of each phase to be combined, resulting in a superior material compared to that achieved by each individual component.
Composites usually comprise of a matrix material and a reinforcing material. The reinforcing material provides mechanical support to the surrounding matrix which can be of a fibrous or particulate nature. Collagen (a fibrous matrix material) is used extensively as a scaffold biomaterial due to its biocompatible and biodegradable properties [102, 215, 221, 285, 328]. However, from an orthopedic perspective, collagen scaffolds are limited by their poor mechanical characteristics and for this reason many studies, including other research in our laboratory, have combined collagen with calcium phosphates and hydroxyapatite in a particulate form (reinforcing material) to improve scaffold mechanical properties [10, 120, 142, 197, 329]. However, the majority of these composite scaffolds are produced using micron-sized particles and hence some concerns exist regarding poor resorbability and brittleness of the constructs [135, 136].

Many reported advantages of using nano-sized particles of HA have been discussed in Chapter 2, including the attainment of enhanced mechanical properties, cellular responses and resorbability [126, 136, 141]. Therefore, the incorporation of nanophase HA into collagen scaffolds is hypothesised to provide a reinforced, biocompatible composite scaffold suitable for bone tissue engineering. To achieve this a well established lyophilisation technique which provides highly porous collagen scaffolds with a controlled pore structure in a reproducible manner was appropriately modified [49].
3.1.1 Aim

The objective of this study was to produce a range of collagen-nHA (coll-nHA) biocomposite scaffolds by developing novel methods of incorporating nano-sized HA particles (developed as described in Chapter 2) into the highly porous collagen scaffolds based on the lyophilisation technique [49, 94]. A variety of techniques will be utilized to characterise and identify the optimal construct.

3.2 Materials and Methods

3.2.1 Fabrication of Collagen Control Scaffolds

Collagen control scaffolds were produced by freeze-drying a collagen slurry (0.5 % w/v) containing type 1 bovine collagen (Integra Life Sciences, Plainsboro, NJ) in 0.05 M glacial acetic acid. Blending was carried out at 15,000 rpm using an overhead blender (Ultra Turrax T18, IKA Works Inc., Wilmington, NC), in a cooled reaction vessel maintained at 4°C via a circulation cooling system (WKL 230, Lauda, Germany). The resultant slurry was degassed under a vacuum, before freeze-drying in a stainless steel pan by cooling to -40°C at 0.9°C/min, followed by a sublimation step for 17 hours at 0°C and 2.67 mbar (Advantage EL, Vir-Tis Co., Gardiner NY) [94]. This low cooling rate and controlled process provides a homogeneous distribution of ice crystal nucleation throughout the slurry, leading to a homogeneous pore structure [49].
3.2.2 Fabrication of collagen-nanohydroxyapatite (coll-nHA) composite scaffolds

The coll-nHA composites were synthesised using four different methods to combine the nHA particles (synthesised in Chapter 2) with collagen scaffolds. These methods are hereafter described as (1) powder, (2) *in situ*, (3) suspension and (4) immersion.

**Method 1 - Powder** involved the addition of oven-dried aggregated nHA powder (using the original non-optimised nHA synthesis procedure outlined in section 2.2.2) to the collagen slurry before freeze-drying. 1.8 g of the aggregated nHA powder (50 wt % with respect to the collagen fibre component) was suspended in 40 ml of 0.5 M acetic acid and pumped into the blending collagen fibres at a rate of 10 ml/hr before freeze-drying as described above.

**Method 2 - In situ** involved conducting the optimised nHA synthesis (section 2.2.3) reaction within the collagen slurry, by adding the calcium precursor (0.001 M CaCl₂), followed by the phosphate precursor (0.0006 M PO₄ solution containing 0.1 % Darvan and 0.0003 M NaOH). A syringe was used to enable addition of the solutions beneath the surface of the blending slurry, and the mixture was left blending for one hour before the freeze-drying stage.

**Method 3 - Suspension** involved the addition of nHA particles, suspended in water, to the collagen slurry during the blending stage, followed by lyophilisation. Different concentrations of aqueous based nHA suspensions were added to produce a range of coll-nHA scaffolds with different quantities of nHA present (10 % - 500
%, relative to the weight of collagen used), for example adding 3.6 g nHA yielded a 100 wt % scaffold, \((S-100)\) and 18 g nHA yielded a 500 wt % scaffold \((S-500)\).

**Method 4 - Immersion** involved the immersion of collagen scaffold discs (9.5 mm x 4 mm) in an aqueous based nHA suspension for 4 days before freeze-drying. The concentrations of the calcium and phosphate solutions used were varied to generate two scaffold variants; calcium concentration 0.001 M \((I-Low)\) and 0.01 M \((I-High)\). The Ca/P ratio for all suspensions was 1.67.

All scaffolds were cross-linked using a dehydrothermal (DHT) treatment (0.05 bar at 105°C for 24 hours [94]), and stored in aluminium foil in an airtight container until use. An additional, chemical cross-linking method is used to further improve the mechanical properties of the scaffolds, prior to degradation analysis (3.2.10). The scaffolds were initially hydrated in phosphate buffered saline (PBS, Sigma-Aldrich) prior to being immersed in a solution of EDAC/NHS for two hours; 14 mM N-(3-Dimethylaminopropyl)-N’-ethylcarbodiimide hydrochloride and 5.5 mM N-Hydroxysuccinimide (EDAC/NHS) in distilled water. Residual EDAC was removed by washing the scaffolds (two 30 min washes) in PBS using an orbital shaker.

### 3.2.3 Mechanical Testing

Uniaxial compression testing was conducted to evaluate the effect of nHA incorporation on the Young’s modulus of the composite scaffolds. All testing was carried out using a mechanical testing machine \((Z050, \text{Zwick/Roell, Germany})\) fitted with a 5 N load cell. Samples \((n \geq 8)\) were pre-hydrated in phosphate buffered saline (PBS; Sigma-Aldrich, Dublin) prior to, and during testing. A pre-
load of 5 % strain was applied before testing was performed at a strain rate of 10 %
per minute. The resultant load/displacement curves obtained from the Testxpert
software (Zwick/Roell, Germany) were analysed using Microsoft Excel and the
compressive modulus was calculated from the slope of the stress-strain curve,
between 2 - 5 % strain.

3.2.4 Weight Analysis

The quantity of nHA present in the coll-nHA scaffolds (n ≥ 6) was determined by
mass calculations of the composite scaffolds in comparison to collagen only
controls. Measurements were taken using a Mettler Toledo MX5 weighing balance
(accuracy = 0.001 mg; Mason Technology, Dublin) and the weight of nHA present
was given by the following equation

\[ W_{\text{nHA}} = W_{\text{coll-nHA}} - W_{\text{coll}} \]

where \( W_{\text{nHA}} \) is the average weight of nHA in mg, \( W_{\text{coll-nHA}} \) is the average weight of
the composite scaffolds and \( W_{\text{coll}} \) is the average weight of the collagen only
scaffold.

3.2.5 Calcium Assay

A calcium assay was used to determine the amount of calcium salt present in all
scaffolds, hence giving a further indication of the nHA content in the composites.
The concentration of calcium in the scaffold was measured using a Stanbio Total
Calcium Liquicolor kit (Stanbio Laboratory, Texas, USA). 200 µl of the working
reagent (Equal measure of the colour and base reagent from the kit) was added to
each scaffold (n ≥ 6) and the mixture was centrifuged at 15,000 rpm for 5 minutes.
10 µl of the surfactant was diluted in a further 200 µl of the working reagent and transferred into wells of a 96 well plate. The absorbance of the colour change, produced by the Ca-ortho-cresolphthalein complexone (OCPC) complex, was then measured at 572 nm using a Victor³V, 1420 multilabel counter (Perkin Elmer, Waltham, Massachusetts, USA) and compared to a standard curve of dilutions of a calcium standard provided in the kit.

3.2.6 Scanning Electron Microscopy

Scanning Electron Microscopy (SEM) was used to examine the microstructural topography of the scaffolds. Scaffold samples were cut using a punch and fixed to an adhesive carbon stub. Imaging was carried out using a TableTop SEM (TM-1000, Hitachi High-Technologies Corp., Japan) operated at 15 kV.

3.2.7 Porosity

The porosity of the scaffolds (n > 8) was calculated using the following equations:

\[
\text{Porosity} = \left( \frac{\rho_{\text{sample}}}{\rho_{\text{material}}} \right) \times 100
\]

where \(\rho_{\text{sample}}\) is the density of the sample and \(\rho_{\text{material}}\) is the density of the material from which the scaffold is made. For the composite scaffolds, \(\rho_{\text{material}}\) is worked out based on the densities of collagen and nHA as follows:

\[
\rho_{\text{material}} = \frac{m_{\text{collagen}} + m_{\text{nHA}}}{V_{\text{collagen}} + V_{\text{nHA}}}
\]

where \(m_{\text{collagen}}\) and \(V_{\text{collagen}}\) is the mass and volume of collagen in the slurry respectively, \(m_{\text{nHA}}\) and \(V_{\text{nHA}}\) is the mass and volume of nHA in the slurry respectively.
3.2.8 Fourier Transform Infra-Red spectroscopy

Fourier Transform Infra-Red (FTIR) spectra were collected to analyse and compare the chemical characteristics of all scaffold samples. FTIR analysis was carried out as described in Chapter 2, using a Spectrum One FTIR (Perkin Elmer, UK). FTIR provides information about the chemical bonding or molecular structure of materials, whether organic or inorganic. Scaffolds were finely cut and mixed with potassium bromide before being pressed into a transparent sample. Spectra were collected between wavenumbers 4000 and 400 cm⁻¹.

3.2.9 Energy Dispersive X-Ray analysis (EDX)

The presence and distribution of chemical elements throughout the material was detected using energy dispersive X-ray analysis (EDX). A field emission scanning electron microscope (FE-SEM; Hitachi S-4300, Berkshire, UK) with attached EDX spectrometer application (Oxford Inca, Oxfordshire, UK) in analyzer mode, was used to characterise the X-rays released from the material following interaction with an incident electron beam. This characterisation is possible as each element has a unique atomic structure, with discrete energy levels, from which the X-rays are emitted and thus individual elements can be identified by analysing the energy of their X-rays.

3.2.10 Degradation

Scaffolds (S–100 and I–High) were immersed in PBS (Sigma, Dublin) and incubated at 37°C for 0, 1, 3, 7, 14, 28 and 42 days in order to investigate their degradation properties. Wet compression testing was conducted on scaffold
samples \((n = 4)\) at each time point as described previously (section 3.2.3). Scaffolds were also freeze-dried \((n = 3)\) and their dry weights measured using the Mettler Toledo MX5 weighing balance. The percentage weight loss was calculated using the following equation:

\[
\% \text{W}_l = \left(\frac{(W_i - W_d)}{W_i}\right) \times 100
\]

Where \(W_l\) is the weight loss (mg), \(W_i\) is the initial weight of scaffolds and \(W_d\) is the degraded weight at the given time point.

### 3.2.11 Statistical Analysis

All data was analysed for significance \((p \leq 0.05)\) using one-way analysis of variance (ANOVA) tests to compare group means. Post hoc tests to determine significant differences between group means were performed using the Tukey test.

### 3.3 Results

Collagen-nHA (coll-nHA) composite scaffolds were synthesised using the (1) powder, (2) *in situ*, (3) suspension and (4) immersion methods in order to determine the optimum technique. Aggregated nHA particles as prepared in Chapter 2 (without optimisation) were used for the powder method, whereas the *in situ*, suspension and immersion methods used the non-aggregated particles that were synthesised using Darvan dispersant, under optimal conditions.

Composite scaffolds fabricated using the powder method displayed a significant increase in compressive modulus as shown in Figure 3.1a, however, SEM images
revealed the agglomerated particles, which were not homogeneously distributed throughout the scaffold (Figure 3.1b) and so this fabrication method was eliminated in all further experiments.

![Graph](image.png)

**Figure 3.1** (a) Young’s modulus of coll-nHA composite scaffold (powder method) vs. collagen only control, showing a significant increase in Young’s modulus following the addition of 50 wt % aggregated nHA particles (b) Scanning Electron Microscopy (SEM) image of the coll-nHA composite scaffold showing aggregated nHA particles assembled on the collagen fibres.

The addition of low amounts of non-aggregating nHA particles (synthesised using Darvan dispersant agent) *via* the three alternative methods - suspension, *in situ*, and immersion, led to composite scaffolds with different compressive moduli as can be seen in Figure 3.2. Adding 10 wt % nHA by the suspension method (S-10) did not significantly alter the Young’s modulus when compared to the collagen only control. The compressive modulus was improved in the scaffold group produced *via* the immersion method (I-Low; soaked in the low concentration nHA suspension for 4 days). The surprising result from this initial study was the significant reduction in Young’s modulus of the composite scaffold produced using
the *in situ* method (10 wt % added). The sequential addition of the calcium and phosphate precursors to the blending collagen slurry prior to lyophilisation led to an adverse mechanical response compared to the collagen only control, and for this reason this method was eliminated from further study.

**Figure 3.2** Young's modulus of composite scaffolds produced using the suspension, *in situ* and immersion techniques (incorporating 10 wt % nHA using the suspension and *in situ* method, and the lowest concentration nHA solution for the immersion method; I-Low).

The problems associated with both the powder and *in situ* methods warranted the elimination of these techniques, and further investigations focused on the use of the suspension and immersion methods, firstly analysing the effect of incorporating increased quantities of nHA particles into the scaffolds on the Young's modulus.

Using the suspension method, up to 500 wt % nHA (S-500) could be added into the coll-nHA scaffolds, with a concentration dependent increase in Young's modulus observed as shown in Figure 3.3. There appears to be a threshold of 200 wt % nHA
(S-200) required before sufficient nHA is present to cause a significant improvement in the compressive modulus. The nHA content in coll-nHA scaffolds produced using the immersion method was not as easy to control or vary as with the suspension method. Figure 3.3 also shows coll-nHA scaffolds produced using the immersion technique in high and low concentration nHA suspension, I-Low ([Ca] 0.001M) and I-High ([Ca] 0.01 M), in comparison to scaffolds produced using the suspension method and collagen only controls.

Significant increases in modulus were achieved using both methods, and the immersed coll-nHA scaffolds displayed a concentration effect similar to the suspension scaffolds. At lower nHA concentrations a 2.5 fold increase was observed (I-Low) in comparison to the collagen only, and this was further improved (12 fold increase) when higher concentrations of nHA were used (I-High). The S-500 scaffold achieved an 18 fold increase in stiffness compared to the collagen only control. This graph demonstrates the ability to tailor the mechanical properties of coll-nHA scaffolds by varying the amount of nHA added using these methods.
Figure 3.3 Young's modulus of a range of composite scaffolds using the suspension method (50 to 500 wt % nHA) and the immersion method (I-Low and I-High).

The amount of nHA present in the coll-nHA composite scaffolds was quantified using weight analysis. Figure 3.4 shows how the weight of nHA present in the composite scaffolds increases as expected from S-50 to S-500 (0.59 ± 0.09 to 5.23 ± 0.04 mg). The immersed scaffolds I-Low and I-High had a quantity of nHA similar to the S-50 and the S-100 scaffolds respectively according to this analysis, and this is the maximum amount of nHA that was incorporated using the immersion method.
Figure 3.4 Graph of the quantity of nHA present in the range of scaffolds determined using weight analysis.

Another method of interpreting the amount of nHA in the range of composite scaffolds is to use a calcium assay which detects the amount of calcium present, and hence the amount of the calcium phosphate nHA present in each construct (Figure 3.5). This absorbance-based method shows results similar to those of the weight analysis, namely that the amount of nHA increases from S-50 to S-500 as expected, and that the immersion technique can incorporate a quantity similar to the S-50 (1-Low) and S-100 (1-High) scaffolds produced via the suspension method.
Figure 3.5 Graph of the relative quantities of nHA present in the various coll-nHA scaffolds, based on the content of calcium as determined using a calcium assay.

Scanning Electron Microscopy (SEM) was used to image a range of scaffolds, from collagen only to coll-nHA scaffolds with various amounts of nHA (selected images shown: collagen only, S-100, S-500 and I-High). Figure 3.6 is a series of low power (x200 magnification) images, showing the pore structure of the scaffolds, before and after nHA incorporation. The images show that the open, interconnected porous structure with homogeneous pores of the collagen control is maintained in the composite scaffolds, even after the addition of 500 wt % nHA particles. Figure 3.7 shows a series of high power images taken at x1000 magnification of the same range of scaffolds. This closer inspection reveals no evidence of micron-sized particles sitting on the collagen fibres as observed previously when using aggregated nHA powder. However, examination of the immersion scaffolds does indicate a widespread distribution of sub-micron sized
particles, and there is no evidence of this in scaffolds produced using the suspension method.

Figure 3.6 Low power Scanning Electron Microscope (SEM) images (x200 magnification) of a range of collagen and coll-nHA scaffolds, all displaying the porous nature of the constructs.
Figure 3.7 High power Scanning Electron Microscope (SEM) images (x1000 magnification) of a range of collagen and coll-nHA scaffolds, all displaying the detailed surface features of the constructs.

Scanning electron microscopy of the constructs indicated that a high porosity was present in all scaffold samples, and this was verified using quantification calculations. Figure 3.8 shows the percentage porosity of composite scaffolds, compared to the collagen only control (99.5 ± 0.01 %). Scaffolds fabricated using the suspension method retained a porosity of 99.0 ± 0.02 %, following addition of 500 wt % nHA. The immersed scaffold, I-High, maintained a porosity of 98.9 ± 0.2 %. 
Figure 3.8 Graph of scaffold percentage porosities, showing a slight decrease with nHA addition although all scaffolds retain porosity values above 98.9%.

Fourier Transform Infra-Red (FTIR) spectra in Figure 3.9 show the presence of nHA in the coll-nHA composite scaffolds made by both methods (S-100 and I-High). Characteristic peaks for hydroxyapatite are located in the region of 500-600 cm\(^{-1}\), which is representative of the asymmetric bending of the \((\text{PO}_4)^{3-}\) group, while the stretching band of the \((\text{PO}_4)^{3-}\) group is found at 1063 cm\(^{-1}\). In addition, characteristic peaks for collagen are seen at 2800-2950 cm\(^{-1}\) (C-H stretching), 1652 cm\(^{-1}\) (C=O group), and 3420 cm\(^{-1}\) (N-H stretching). The spectra for the composite scaffolds also showed a characteristic peak at 1556 cm\(^{-1}\) which is due to the COO-stretch [313], this peak is evident in both the suspension and immersion scaffolds.
Figure 3.9 Fourier Transform Infra-Red (FTIR) spectra of coll-nHA composite scaffolds fabricated using the suspension and immersion methods (S-100 and I-High) compared to a collagen scaffold and commercial HA powder control spectra.

Energy Dispersive X-Ray analysis (EDX) was used to provide further characterisation and identification of elements present within the collagen and composite scaffolds. The EDX graphs show that collagen only scaffold (Figure 3.10 a) contains carbon, oxygen and sulphur, and these elements are common to all scaffolds. In addition, the suspension scaffold, S-500, has large amounts of calcium and phosphate and a trace amount of NaCl (Figure 3.10 b). Compared to this, the immersed scaffold also has high quantities of calcium and phosphate, but contains large amounts of NaCl and traces of potassium (Figure 3.10 c).
Figure 3.10 EDX spectra for (a) Collagen only control scaffold, (b) S-100 and (c) I-High showing the presence of nHA (Ca and P) in the composite scaffolds, but additionally a large presence of NaCl in the immersed scaffold I-High.

An additional, EDAC cross-linking method was applied to the scaffolds to further improve their mechanical properties. The increase in modulus is shown in Figure 3.11 for the collagen only control (0.30 ± 0.09 vs. 1.67 ± 0.63 KPa), S-100 (0.38 ± 0.02 vs. 2.27 ± 0.77 KPa) and S-500 scaffolds (5.50 ± 1.70 vs. 11.3 ± 0.84 KPa).
Figure 3.11 Graph showing the increase in Young's modulus achieved using an additional, chemical (EDAC/NHS) cross-linking treatment

The degradation properties of scaffolds produced by the suspension and immersion methods were compared by soaking each group in PBS at 37°C for 0, 1, 3, 7, 14, 28 and 42 days. S-100 and I-High samples were used in this study as these scaffolds contained the same quantity of nHA, thus allowing for a comparison between the two methods to be made. Figure 3.12 demonstrates how the mechanical viability of both scaffolds decreases at a similar rate; over the 42 day incubation period the Young's moduli of the S-100 and I-High scaffolds were found to decrease by 65 ± 7 % and 59 ± 17 % respectively. Correspondingly, Figure 3.13 shows similar decreases in weight of both scaffold groups over the 6 week time period, showing that both scaffold groups are degrading in a similar manner and over a similar timescale. The S-100 scaffolds experience a percentage weight loss of 33.0 ± 2.6 %, and the I-High scaffolds demonstrate a similar loss of 27.5 ± 15.0 % over the 42 day investigation.
Figure 3.12 Graph showing the degradation of mechanical properties of composite scaffolds after incubation in PBS at 37°C for up to 6 weeks.

Figure 3.13 Graph showing the decrease in weight of composite scaffolds as they degrade over time, up to 6 weeks.

3.4 Discussion

The aim of this study was to develop and evaluate different methods of incorporating nHA particles (<100 nm) into collagen scaffolds for use in bone tissue engineering. Previous studies have indicated the potential benefit of combining collagen type 1 with HA. Xie et al. (2004) reported an enhancement of
osteoblast differentiation using both collagen and HA independently; with an acceleration of osteogenesis obtained when the two materials were combined [189]. Additionally, a composite coll-HA matrix when seeded with human-like osteoblast cells, showed better osteoconductive properties compared to conventional HA, inducing calcified matrix deposition \textit{in vitro} [96]. The biocompatibility of coll-HA composites has been demonstrated in both humans and animals [118]. More recent research has focused, with moderate success, on the use of nanoparticles of HA (nHA), including their incorporation into composite scaffolds [121]. Therefore, in this study, novel methods were applied to produce resorbable coll-nHA composite scaffolds with improved mechanical properties; using the powder, \textit{in situ}, suspension and immersion methods.

Preliminary analysis excluded both the powder and \textit{in situ} methods due to the aggregation of nHA via the powder method and the reduced mechanical viability of scaffolds produced using the \textit{in situ} technique. A study by Liu \textit{et al.} (2009) showed that using the \textit{in situ} method to develop a coll-nHA scaffold led to a gradient effect, with a heterogeneous distribution of nHA crystals and this may suggest a reason for the detrimental effect of this method on mechanical properties of the composite construct [330]. Promising initial results were obtained for both the suspension and immersion methods, and therefore these methods were selected for further analysis.
Results from the suspension and immersion methods show that the mechanical properties of the composites synthesised by each of the methods could be tailored by varying the nHA content. A significant improvement in Young's modulus was achieved following addition of 200 wt % nHA (S-200), and it appears that a threshold of nHA is required for this improvement to occur. This is probably due to the nano-sized dimensions of the particles involved, and therefore, the necessity of a network of these particles to cause a reinforcing effect. The greatest increase in compressive modulus (18 fold vs. collagen control) was obtained by adding 500 wt % nHA using the suspension method. This result highlights the reinforcing role that the ceramic plays. Previous difficulties have been reported with increasing the filler content in composite constructs, often leading to irregular distribution of the ceramic and a reduction of the mechanical properties of the resultant scaffold [129, 181, 329]. These problems have been overcome by using nano-sized particles and the novel approaches discussed in this chapter. A micro-computed tomography (µ-CT; resolution 8 μm, Scanco Medical 40 MicroCT system, Bassersdorf, Switzerland) reconstructed image of the S-500 composite scaffold shows the distribution of the nHA particles throughout the construct in comparison to a collagen only control (Figure 3.14) [331].
Figure 3.14 Micro-computed topography reconstructed image showing the distribution of nHA in the S-500 composite scaffold. The inset shows an image of a collagen only scaffold, demonstrating the inability to detect the collagen fibres using this technique [331].

Previous work done in our lab using commercial (micron-sized) HA showed a 2 fold increase in compressive modulus when 200 wt % HA was added vs. a collagen only control, while the use of 200 wt % nano-sized HA particles (S–200) in this thesis led to a 3.5 fold increase compared to a collagen only scaffold [331]. In addition, composite scaffolds produced using the commercial HA scaffolds could not incorporate the same higher levels of ceramic as was possible when using nHA particles combined with the novel suspension technique. Therefore, the use of nHA particles enabled the incorporation of higher quantities of reinforcing ceramic, thus further improving the mechanical stiffness, without adversely affecting the porosity or brittleness of the resultant scaffolds.
The Young’s modulus values of the scaffolds can be significantly improved by further cross-linking using an EDAC/NHS chemical treatment. DHT treatment causes the collagen molecule to undergo a condensation reaction, removing water molecules bound to the amino acid side groups, thus leaving these side groups free to form inter- and intramolecular bonds. The EDAC chemical treatment targets the amine groups of the collagen molecule and leads to the formation of additional isopeptide bonds. Scaffolds are cross-linked using both DHT and EDAC treatments for subsequent in vitro analysis (Chapter 4).

The amount of mineral that the cells are in contact with will also affect cellular behaviour [332]. The variation of nHA content was more controllable using the suspension method, and higher quantities could be incorporated easily. There is an upper limit to the concentration of the nHA suspension used in the immersion technique (approximately 100 wt %), and hence to the amount of nHA that can be added in this way. This is more than likely due to larger particles forming at higher initial calcium and phosphate precursor concentrations during the synthesis and immersion period, and only a limited quantity of these aggregated particles can penetrate into the porous collagen sponge during the immersion procedure as required [333]. This is supported by results from weight analysis and calcium content assays, both of which show that higher levels of nHA can be added to the composite using the suspension method.
The porosity of the scaffolds remained at approximately 99% after nHA addition, which is very useful for use in bone tissue engineering. High porosity has been reported as a significant factor in scaffold design, due to the necessity to encourage cell infiltration into the centre of the construct, the subsequent removal of waste materials from this region and to allow vascularisation. Construct porosity is usually compromised to achieve higher mechanical properties, but the suspension technique presented in this thesis, and the use of nano-sized particles allows the high porosity to be maintained [51, 114, 328, 334].

SEM demonstrated that all composites displayed an open and interconnected pore structure, similar to the collagen only control scaffold. Higher magnification SEM images showed the formation of nHA aggregates (~1 μm) in the scaffold immersed in higher concentration (I–High), which may be due to the long soaking period (4 days). Energy dispersive X-Ray analysis (EDX) substantiated the FTIR finding, i.e. confirming the presence of nHA in the coll-nHA scaffolds. An EDX map of the S-100 scaffold is shown in Figure 3.15, demonstrating that a homogeneous distribution of calcium and phosphate is achieved throughout the suspension construct. However, the immersed scaffolds were shown to have a significant level of sodium chloride present, which is related to the specific immersion technique, as it was not observed in the suspension scaffolds. Some NaCl would arise as a by-product from the nHA synthesis procedure, although more NaCl would be taken up by the scaffold when it is immersed in PBS during the immersion technique.
Figure 3.15 Representative Energy Dispersive X-Ray (EDX) map of a composite scaffold produced using the suspension method, showing the widespread distribution of calcium (green), phosphate (blue) and sodium (red) overlying a corresponding SEM image.

The ability of the scaffold to degrade over time is another essential feature required of bone graft substitutes, and the loss in weight and ensuing decrease in mechanical properties over time illustrates the capacity of these coll-nHA scaffolds produced using the suspension and immersion methods in this regard. Serious problems have been reported concerning the lack of resorbability of micron-sized hydroxyapatite, but these issues are overcome by the use of nanoparticles [304]. Biodegradability is one of the most important features of scaffolds for use as a bone graft substitute. Ideally the implanted construct degrades at a rate which allows it maintain mechanical integrity as new bone tissue is deposited in its place [171].
Considering all aspects of scaffold development and characterisation shown in this study, the best production technique appears to be the suspension method, in which reproducible, homogeneous constructs can be fabricated, with an ability to easily control the nHA content (up to 500 %). These scaffolds are mechanically stiffer, whilst retaining high porosity, resorbability and an interconnected porous structure suitable for cellular proliferation.

3.5 Conclusion

Bone regeneration requires scaffolds that possess suitable mechanical and biological properties. This study has led to the successful development of two novel techniques for the synthesis of resorbable coll-nHA composite scaffolds. The suspension and immersion methods created significantly stiffer scaffolds with high degrees of porosity (>98.9 %). It was found that coll-nHA composite scaffolds produced by the suspension method were up to 18 times stiffer than the collagen control (5.50 ± 1.70 kPa vs. 0.30 ± 0.09 kPa respectively). The suspension method was also more reproducible, and the quantity of nHA incorporated could be varied with greater ease than with the immersion technique.
Chapter 4: Biological evaluation of Collagen - NanoHydroxyapatite (coll-nHA) composite scaffolds

4.1 Introduction

Porous, resorbable scaffolds with improved mechanical properties have been developed in Chapter 3 using the two main constituents of bone; collagen and nano-hydroxyapatite (nHA) [335]. Structurally, these collagen-nanohydroxyapatite (coll-nHA) composite scaffolds have potential for use in bone tissue engineering, but their biocompatibility, and ability to support bone formation in vitro must be established [171]. Scaffolds containing collagen, hydroxyapatite, and a combination of the two materials have displayed excellent biocompatibility in previous studies [47, 101, 336, 337]. In particular, the use of nHA has been shown to enhance osteoblast adhesion, proliferation and differentiation when added to scaffolds [135, 136, 338].

Biocompatibility can be assessed using in vitro analysis by investigating cellular attachment, proliferation and differentiation parameters. It also serves as a good indication of the aptitude of the construct for in vivo use. The initial attachment and adhesion of cells to the scaffold is very important as the success of this first phase will influence the cell’s subsequent capacity to proliferate and to differentiate [19]. Cellular adhesion is very closely related to the surface characteristics of the
construct, including the topography, chemistry and surface energy, and therefore the presence of nHA is likely to have a large impact on this parameter [339].

Longer term, the osteogenic potential of the composite scaffolds can be analysed by examining the expression of bone formation markers in addition to the deposition and subsequent mineralisation of extracellular matrix (ECM). Alkaline phosphatase (ALP) is a bone enzyme and its activity is a hallmark of the osteoblast lineage and is upregulated during cell maturation, before the onset of mineralisation [340]. Extracellular matrix (ECM) is deposited and mineralised as cells finish proliferating. In 2D culture, matrix maturation occurs after 14 days in culture, while mineralisation of the matrix can be observed by 28 days [20]. However, the 3D situation is more complicated as the matrix conditions will affect the osteogenesis timeline.

4.1.1 Aim

The specific aim of this chapter was to biologically evaluate the coll-nHA composite scaffolds developed in Chapter 3. Specifically the S-100 and S-500 scaffolds were investigated to determine the impact of nHA content and mechanical stiffness on the biological performance vs. a collagen control. Short term in vitro studies were used to investigate initial cellular attachment and proliferation, while longer term studies examined the osteogenic potential of the composite scaffolds.
4.2 Materials and Methods

4.2.1 Scaffold fabrication

The scaffolds investigated in this chapter were made by the suspension method as described in Chapter 3 (S-100 and S-500 – containing 100 and 500 % nHA respectively vs. a collagen control). Briefly, a collagen slurry was fabricated by blending fibrillar collagen (Integra, New Jersey, USA) with 0.5 M acetic acid in a reaction vessel cooled to 4°C by a WK1250 cooling system (Lauda, Westbury, NY, USA) using an overhead blender (IKA Works Inc., Wilmington, NC). For the composite scaffolds, nHA particles were synthesised, washed, resuspended in deionised water and added to the slurry during the blending process. Slurries were de-gassed under a vacuum to remove air bubbles, 67.25 ml of the slurry was pipetted into a steel pan (12.7 cm by 12.7 cm) and placed in a freeze-dryer (VirTis Co., NY, USA). Slurries were freeze dried at a cooling rate of 0.9°C/min and a final freezing temperature of -40°C. Scaffolds were cross-linked and sterilized using a dehydrothermal (DHT) treatment by placing them into a vacuum oven (Vacucell 22, MMM, Germany) at 0.05 bar for 24 hours at a temperature of 105°C. Cylindrical scaffold samples (12.7 mm diameter, 4 mm height) were further cross-linked with 14 mM N-(3-Dimethylaminopropyl)-N’- ethylcarbodiimide hydrochloride and 5.5 mM N-Hydroxysuccinimide (EDAC/NHS) in distilled water. The scaffolds were initially hydrated in phosphate buffered saline (PBS, Sigma-Aldrich) prior to being immersed in a solution of EDAC/NHS for two hours [42]. Residual EDAC was removed by washing the scaffolds (two 30 min washes) in PBS using an orbital shaker.
4.2.2 Cell culture and seeding

Scaffold samples were seeded with $2 \times 10^6$ MC3T3-E1 pre-osteoblast cells (ATCC, Manassas, VA). $1 \times 10^6$ cells suspended in 100 μl of media were seeded on one side of the scaffold. The scaffolds were placed in an incubator for 15 minutes, after which time the scaffolds were turned over and a further $1 \times 10^6$ cells were seeded on the other side. Following another 15 minutes of incubation, each well of a six well plate (Sarstedt, Nuembrecht, Germany) was filled with 5 ml of media. Constructs were cultured in α-minimum essential medium (BioSera, East Sussex, UK) supplemented with 2 % penicillin/streptomycin (Sigma-Aldrich, Germany), 1 % L-glutamine (Sigma-Aldrich, Germany) and 10 % foetal bovine serum (BioSera, East Sussex, UK) for the short term study (up to 7 days).

For the long term study (up to 28 days), scaffolds were maintained in the same standard proliferative medium for 48 hours before the addition of osteogenic factors (this is then assigned as day 0). Osteogenic media was prepared by the addition of ascorbic acid (50 μg/ml), β-glycerolphosphate (10 mM) and dexamethasone (100 nM) to standard proliferative media. Constructs were cultured under standard conditions (37°C, 5 % CO$_2$), and the media was replenished every 3–4 days. At each end time-point constructs were flash frozen in liquid nitrogen and stored at -80°C until analysis (unless they were assessed with the AlamarBlue™ assay first, section 4.2.4). In addition, $n = 3$ constructs were placed into 10 % formalin for processing prior to histological analysis (section 4.2.5 for further details).
4.2.3 Hoechst DNA Assay

Cell number was quantified by using the Hoechst 33258 assay (Sigma-Aldrich, Germany) [341]. Constructs (n = 4) were thawed and digested in 1 ml Qiazol (Qiagen, West Sussex, UK). 40 μL of the lysate was mixed with 800 μL of a working dye solution containing the Hoechst dye (Sigma-Aldrich, Germany). 210 μL of this mixture was pipetted into each well of a 96 well plate to provide triplicates for each sample. The fluorescence of the samples was measured at 460 nm after excitation at 355 nm (Wallac Victor3V 1420 multilabel counter, Perkin Elmer Life and Analytical Sciences, Belgium). Fluorescence readings were compared to a standard curve to quantify cell number.

4.2.4 AlamarBlue™ analysis

Cell activity was evaluated using AlamarBlue™ (BioSource, Belgium) which is a non-endpoint, non-toxic assay [342, 343]. This assay utilises a colorimetric change of solution that is based on an oxidation-reduction (REDOX) reaction. The change in colour of the AlamarBlue™ solution from blue (resazurin) to pink (resorufin) is a chemical reduction of growth medium resulting from metabolic activity of the cultured cells [343, 344]. After 1, 2 and 7 days of incubation, constructs (n = 6) were transferred to 6-well plates containing 10% AlamarBlue™ in 5 ml supplemented media. The plates were placed on an orbital shaker (Biosan, Riga, Latvia) and incubated for 2 hours. 100 μl of the reduced supernatant was then plated in triplicate into a 96-well plate, Absorbance was read on a spectrometer (Titerek Multiskan MCC/340 spectrometer, Titertek, Germany) at 540 nm and 620 nm. Percentage reduction of the AlamarBlue™ solution was then determined
according to the manufacturer’s specifications using a standard curve. Constructs were subsequently flash frozen and stored at -80°C for additional analysis.

4.2.5 Histological Assessment

Constructs (n = 3) were fixed in 10 % formalin for 30 minutes, processed in a tissue processor (ASP300, Leica Microsystems, Germany), embedded in paraffin wax (Leica Microsystems, Germany) and sectioned using a rotary microtome (RM2255, Leica Microsystems, Germany) at 10 μm thickness. Slides were placed overnight in an oven at 60°C. Sections were then hydrated to water in a series of ethanol baths prior to staining with haemotoxlin & eosin (section 4.2.5.1), Von Kossa (section 4.2.5.2) or alizarin red (section 4.2.5.3). Digital images of all stained sections were obtained using an imaging system (NIS Elements Basic Research Version 3.0, Nikon, Japan) in conjunction with a microscope (Nikon Eclipse 90i, Nikon, Japan).

Haematoxylin & eosin Staining (H&E)

Sections were stained in haematoxylin for 5 minutes and in 0.5 % eosin for 3 minutes to examine cell distribution. Haematoxylin stains cell nuclei a dark purple and eosin stains the scaffold pink.

Von Kossa Staining

Sections were also stained for the presence of mineralisation using Von Kossa staining which is highly specific for phosphate deposition, staining mineralised deposits black. 5 % silver nitrate (Sigma Aldrich, Steinheim Germany) solution
was pipetted onto the surface of the scaffold section following rehydration and left under a lamp source for 1 hour. Slides were washed 3 times in distilled H$_2$O and incubated with 5 % sodium thiosulphate (Sigma, St Louis, MO 63103, USA) for 2 minutes. Slides were again washed and stained with 0.1 % nuclear fast red for 5 minutes (Sigma Aldrich, St Louis, MO 63103, USA) as background staining for the scaffold.

*Alizarin Red Staining*

Alizarin red staining was also used to examine mineralisation. It stains calcium a bright red colour. Sections were stained in 2 % alizarin red S (Sigma Aldrich, St Louis, MO 63103, USA) for 2 minutes. This stain can also be used to quantify mineralisation on the scaffolds. The level of mineralisation was quantified by using 10 % cetylpyridinium chloride to absorb the alizarin red stain from stained sections ($n = 6$) \[345\]. Two construct slices per slide were quantified, leaving the two remaining sections for examination under the microscope. 400 µL of cetylpyridinium chloride solution was pipetted onto the slides and the stain was desorbed for 15 minutes. 100 µL was pipetted in triplicate in a 96 well plate. Absorbance readings at 572 nm were obtained (Wallac Victor$^2$V 1420 multilabel counter, Perkin Elmer Life and Analytical Sciences, Belgium). Readings for the cetylpyridinium solution were subtracted to eliminate its effect. As the level of staining increases, the absorbance reading increases, thus enabling quantification of the mineralisation in comparison to the qualitative histological data.
4.2.6 Alkaline Phosphatase

Alkaline phosphatase (ALP) was chosen as a marker of early bone formation. ALP provides a high level of phosphate ions thereby creating a local environment conducive to the nucleation of hydroxyapatite mineral. ALP activity of cells on the collagen, S-100 and S-500 constructs was measured at days 0, 7, 14, 21 and 28 using a p-nitrophenyl phosphate (pNPP) assay which is based on the conversion of p-nitrophenyl phosphate into p-nitrophenol. This reaction yields a yellow water soluble end product that can be calibrated to ALP concentration. Constructs \( n = 4 \) were placed in individual eppendorf tubes and incubated with 200 µl of ALP lysis buffer (0.1 M sodium acetate anhydrous, 2 % triton-X-100). Constructs were manually digested using forceps, thoroughly mixed with ALP lysis buffer and incubated on ice for 1.5 hours. Manual mixing of the solution was repeated continually using forceps. Samples were centrifuged at 10,000 g for 10 minutes at 4 °C. 50 µl of the supernatant was plated out in triplicate in a 96-well plate. 100 µl of pNPP substrate solution (10 mM) was added to each well. Each 96-well plate was covered in aluminium foil and incubated at 37 °C for 1 hour. After 1 hour, 100 µl of 0.3 M NaOH solution was added to each well to stop the reaction and the plates were read on a spectrometer at 405 nm (Wallac Victor³V 1420 multilabel counter, Perkin Elmer Life and Analytical Sciences, Belgium).

4.2.7 Scanning Electron Microscopy (SEM)

Constructs were imaged after 0, 7, 14, 21 and 28 days in culture using a scanning electron microscope (Tescan Mira XMU, Tescan USA Inc, Pennsylvania, USA). Constructs were prepared by fixing in 3 % gluteraldehyde for 2 hours, followed by
dehydration, in 10 minutes stages, through a series of ethanols (10 %, 30 %, 50 %, 70 %, 95 %) followed by 30 minutes in 100 % ethanol. The constructs were stored in 100 % ethanol until they were dried using a critical point dryer (Quorum E3000 CPD, Kent, UK).

### 4.2.8 Statistical analysis

Results are expressed as mean ± standard deviation. Two-way analysis of variance (ANOVA) followed by a pair wise multiple comparison procedure (Tukey test) was used. Statistical significance was declared at p ≤ 0.05.

### 4.3 Results

This investigation was split up into short term (up to 7 days) and long term (up to 28 days) studies. The short term study was used to determine cell attachment and proliferation rates, and to determine which constructs (collagen, S-100 and S-500) had the most potential to be investigated further in the long term study. The long term study was used to assess the ability of the constructs to induce mineralisation.

(i) Short Term Analysis

### 4.3.1 Cell number – DNA Hoechst assay

The number of cells on the collagen, S-100 and S-500 scaffolds is displayed in Figure 4.1 following 24 hours and 7 days of culture in standard media. Cell attachment calculations were based on the percentage of cells present 24 hours
after seeding 2 x 10^6 MC3T3-E1 cells. A cell attachment of 94% was achieved on the S-500 scaffold group which was significantly higher than the 50% attachment observed on both of the collagen and S-100 groups. By day 7, the S-500 group still maintained statistically higher cell numbers compared to the collagen and S-100 groups (Figure 4.1).

![Figure 4.1](image.png)

**Figure 4.1** Graph showing cell numbers at 24 hours and 7 days on collagen, S-100 and S-500 scaffolds. The results show significantly more cells are present on the S-500 composite scaffold at each time point.

### 4.3.2 Metabolic activity

The AlamarBlue™ assay showed that after 24 hours in culture, cells cultured on the S-100 and S-500 scaffolds have significantly higher metabolic activity than on the collagen scaffolds. By day 7, the cells on the collagen scaffold have recovered and there is no difference in metabolic activity on all three scaffold types (Figure 4.2).
Figure 4.2 Graph showing metabolic activity on the collagen, S-100 and S-500 scaffolds after 24 hours and 7 days in culture.

4.3.3 Cell distribution

Cell distribution in the three scaffold types was examined after H&E staining. The images in Figure 4.3 show the distribution of cells throughout the constructs after 24 hours and 7 days in culture. The stained slices were taken at a depth of 300 μm from the surface, and the cells can be seen to have migrated into the scaffold after 24 hours. By 7 days, they appear to have infiltrated further into the construct. The cells seem to be distributed homogenously with no evidence of cell encapsulation along the edges the constructs.
Figure 4.3 Haematoxylin and eosin stained slices of the scaffolds. The images show the migration of cells from the surface of the constructs after 24 hours and 7 days in culture (arrows demonstrate cell infiltration). All images were taken at x4 and have the same scale bar (500 µm) as in the first image.

(ii) Long Term Analysis

The osteogenic potential of the collagen, S-100 and S-500 scaffolds were investigated up to 28 days in osteogenic media, following 48 hour pre-culture in standard proliferative media.

4.3.4 Cell number

Figure 4.4 shows cell numbers on the collagen, S-100 and S-500 scaffolds. There is no significant difference between the three scaffold groups and high cell numbers are maintained up to day 28.
4.3.5 Histological Assessment

Three different types of staining techniques were used to examine the collagen, S-100 and S-500 constructs histologically. H&E staining was used to examine cell distribution, whilst Von Kossa and alizarin red staining were used to look at the levels of mineralisation.

*Haematoxylin and eosin (H&E)*

Haematoxylin and eosin staining examined cell distribution. All images in Figure 4.5 were taken at a depth of 600 μm from the scaffold surface at x4 magnification. Cells can be seen throughout the collagen, S-100 and S-500 constructs. By day 7 some evidence of encapsulation (cells forming an area of high cell density around the circumference of the scaffold) can be seen occurring along the scaffold edge and this is particularly prominent for the S-100 constructs. By day 28 the degree of
encapsulation increases for all constructs, however again, it is most noticeable for the S-100 construct. As the scaffolds did not contract by day 28 an open pore structure was observed at all time points.

**Figure 4.5** Haematoxylin and eosin stained slices of the scaffolds. The images show the migration of cells from the surface of the constructs at days 0, 7, 14, and 28. All images were taken at x4 and have the same scale bar (500 μm) as in the first image.
Von Kossa staining

Von Kossa stains for mineral by staining phosphate black. As expected, there was no mineral in the collagen only scaffold at day 0, a small quantity in the S–100 construct, and a larger amount observed in the S–500 construct (Figure 4.6). An increase in staining intensity, which is suggestive of an increase in the mineral content, is observed in the S–100 construct at day 21 and at day 28 on the collagen scaffold. This would suggest that mineralisation is occurring earlier on the S-100 constructs. There is no change in staining for the S-500 constructs from day 0 to day 28.
Figure 4.6 Von Kossa stained slices of collagen, S–100 and S–500 constructs at days 0, 7, 14, 21 and 28. The brown/black staining shows the presence of phosphate. All images were taken at x4 and have the same scale bar (500 µm) as in the first image.
Alizarin Red Staining

Alizarin red staining is another technique used to detect the presence of mineral. Figure 4.7 reveals a similar trend to the one that was observed using Von Kossa staining. At day 0, mineral can be detected on the S-500 construct only, by day 28 the staining increases slightly. For the S-100 construct, staining increases at day 21, whereas for the collagen scaffold it increases at day 28.

The onset of mineralisation is especially difficult to observe in the S–500 construct histologically due to high nHA content. Therefore, an absorbance based assay was used to quantity the amount of mineral in each construct at each time point up to day 28. Figure 4.7 also shows the mineral content in the constructs at each time point, relative to the blank collagen scaffold at day 0. Although not statistically significant, the results show increased mineralisation in the S-100 and S-500 at each time point.
Day 14

Day 21

Relative mineralisation
(Azarn red quantification, Abs 572 nm)

Collagen Only  S - 100  S - 500

Collagen Only  S - 100  S - 500

Relative mineralisation
(Azarn red quantification, Abs 572 nm)

Collagen Only  S - 100  S - 500

146
Figure 4.7 Alizarin red stained slices, and the measured mineral content graphs of collagen, S-100 and S-500 constructs at days 0, 7, 14, 21 and 28 relative to collagen at day 0. The red staining in the images represents the presence of calcium, and all images were taken at x4 with the same scale bar (500 \( \mu \)m) as in the first image.

4.3.6 Scanning Electron Microscopy (SEM)

SEM was used to examine the constructs (Figure 4.8). Low power magnification was used to image the pore structure of the constructs (day 0 images), while cell morphology was examined using higher magnifications (day 7, 14, 21 and 28 images). ECM deposition can be observed on all construct surfaces from day 7 which remains visible up to day 28. At the low power images it was hard to identify individual cells as they were flat and overlapped each other so that they appeared as a large sheet. However, on closer inspection, the cells have a planar morphology with cytoplasmatic projections that are strongly attached to the construct. This is observed in the day 21 and 28 images.
Figure 4.8 SEM images of seeded scaffolds at days 0, 7, 14, 21 and 28, showing the deposition of mineralised extracellular matrix (ECM) and presence of cells. The planar morphology and cytoplasmatic processes of the cells is visible at higher magnifications (days 21 and 28).
4.3.7 Alkaline phosphatase

Alkaline phosphatase (ALP) enzyme is a marker of the osteoblast lineage. It is known to be upregulated during cell maturation, before the onset of mineralisation. The p-nitrophenyl phosphate (pNPP) assay provides an indication of the activity of the ALP enzyme in the collagen, S-100 and S-500 constructs over a 28 day period (Figure 4.9). Initial activity is higher on S-500 than on the collagen and S-100 scaffolds, although a general increase in ALP activity over time is observed on each construct (not significant for the collagen only control). By day 14 there is significant increase in ALP activity on S-500 compared the other groups implying that the S-500 scaffold supports differentiation at an earlier time point. A significant increase is observed by day 21 on the S-100 construct.

![Graph showing the expression of alkaline phosphatase on each construct group, normalised to cell number following 0, 7, 14, 21 and 28 days in culture.](image)

Figure 4.9 Graph showing the expression of alkaline phosphatase on each construct group, normalised to cell number following 0, 7, 14, 21 and 28 days in culture.
4.4 Discussion

The short term study (up to 7 days) investigated the cell attachment and proliferation rates on coll-nHA composite scaffolds, while the long term study (up to 28 days) explored the ability of the scaffolds to support bone tissue formation.

Many studies have shown the ability of both collagen and hydroxyapatite to enhance cellular activities. Zhu et al. (2006) showed that surface treating titanium implants with collagen and nHA improved cell attachment and motility due to increased wettability and surface roughness, and both of these aspects are likely to affect cell behaviour in the scaffolds used in this study [314]. Other studies have shown that cell attachment is enhanced by the use of nano-sized HA particles, compared to conventional micron-sized HA [126, 306, 346, 347]. Kim et al. (2005) cited the enhanced binding of adhesion molecules such as fibronectin and vitronectin to nHA in composite scaffolds as a reason for increased levels of cell attachment observed on the constructs [348].

Short term results showed that the S-500 scaffold had cell attachment rates of 94 %, whereas the collagen and the S-100 scaffolds had lower rates of 50 %. This may be due to the high affinity of cells for the nHA particles in the S-500 construct, combined with the absorbent nature of the scaffold compared to the other scaffold groups. Metabolic activity, assessed using an AlamarBlue™ assay, demonstrated that the composite scaffolds induced enhanced cellular activity initially, although no significant difference remained between the groups by day 7 [349]. This initial
difference may result from different attachment rates in the collagen and composite scaffolds, as the secondary, protein mediated attachment phase may occur earlier on the composite scaffolds due to the presence of nHA.

Cells migrated into the construct, and this infiltration was seen to continue with time, leading to homogeneous distribution of cells right to the centre of the composite scaffolds. Good cellular infiltration is an important feature to ensure the success of a bone graft substitute, and this penetration may be influenced by the mechanical integrity of the constructs, which maintains the open porous structure throughout the culture period. Cell mediated scaffold contraction is another issue often experienced when culturing scaffolds *in vitro* for longer periods [41, 42, 350]. The cells act to contract the collagen struts, leading to a breakdown of scaffold viability, and consequently a large reduction in size, affecting permeability and pore size. This contraction effect was not observed in these scaffolds because of the enhanced compressive moduli of the scaffolds due to the reinforcement from the nHA particles, and in particular the EDAC cross-linking treatment.

The positive results obtained in the short term analysis merited more detailed investigation of the three scaffolds. The long term study was conducted in osteogenic media, following an initial pre-culture period (48 hours) in normal proliferative media. The long term cell number results show that all scaffolds maintain high cell numbers throughout the 28 day culture and there is evidence that cells are still proliferating at this late stage. This proliferation is occurring
simultaneous to mineralisation of the deposited ECM, although cell number is usually seen to steady off at an earlier stage on collagen scaffolds. The additional proliferation may be due to the construct retaining its structural viability, and mechanical signalling to the cells. Contraction of the collagen scaffolds was cited as a reason for the differentiation in previous studies; as the cells are forced closer together in a smaller area, leading to a concentration of cell signalling and growth factors, which in turn leads to cell differentiation and ECM mineralisation [351].

Cell distribution up to day 28 demonstrated an encapsulation effect in all scaffold types, although cells have infiltrated within the scaffold. The encapsulation effect is most prominent on the S-100 scaffold and is seen from day 7. Additional staining was carried out to examine mineralisation using Von Kossa and alizarin red. Mineralisation occurred throughout the scaffolds; by day 21 on the composite scaffolds, and by day 28 on the collagen only constructs, as shown using both Von Kossa and alizarin red staining. The presence of nHA in the composite scaffolds, particularly S-500, made it difficult to analyse the deposited mineral. Therefore, the alizarin red quantification technique was applied to measure. The alizarin red quantification results show that the composite scaffolds have a higher initial mineral content as expected due to the nHA. However by day 7, the mineral content decreases, which may be attributed to degradation of the scaffold. As the media is replaced, the leached nHA particles would be removed. Therefore, the increase in mineralisation noted from day 7 to day 14 and beyond is most likely due to the cell mediated deposition of mineral.
SEM was used to study construct macrostructure and to assess cell morphology. SEM revealed the planar morphology of the cells and their cytoplasmatic projections which were strongly attached to the composite material. The SEM images (Figure 4.10) show the ability of the cells to bridge gaps and pores in the scaffold, and the images also highlight the nano-sized features of the S-500 scaffold.

Figure 4.10 SEM images of the S-500 scaffold at day 28, showing (a) the ability of the cells to bridge gaps (magnification = x1670) and (b) the attachment of cell processes to the scaffold surface (magnification = x16870).

Okada et al. (2009) found that large sheets of nHA, and large grain sizes were required for cells to attach well and adopt this planar shape. They hypothesised that the small crystal planes in the nano-sized particles restricted the clustering of integrins and focal adhesion points, subsequently leading to cell apoptosis. However, this was not observed in this investigation, and this may be due to the
presence of the collagen fibres, and the favourable pore architecture of the nano-composite struts leading to the enhanced attachment and cellular behaviour [352].

We also examined gene expression, specifically the expression of ALP, which is a mid-stage marker of osteogenesis. ALP activity is seen to begin earlier on the S-500 composite scaffold in this study, with significantly higher expression per cell number by day 14. This indicates that cellular differentiation is occurring earlier on the S-500 scaffold vs. S-100 and the collagen only control. By day 21 the S-100 scaffold demonstrates a peak in ALP expression, which implies that this construct is also inducing osteoblastic differentiation before the collagen only construct which does not display a significant increase in ALP expression over the 28 day culture period. Ngiam et al. (2009) showed that the presence of nHA significantly enhanced cell numbers initially, although they found that the expression of ALP was not significantly different between the scaffold groups after longer culture periods [353]. On the other hand, Keselowsky et al. (2004) found that the presence of hydrophilic hydroxyl (−OH) functional groups enhance the recruitment of structural and signalling components involved in cell adhesion, and that the increased adhesion achieved initially translates into increased mineralisation over time [354].
4.5 Conclusion

The coll-nHA composite scaffolds displayed excellent biocompatibility, showing high initial cell attachment (S-500) and proliferation over the short term analysis. Cells migrated well throughout all the constructs, resulting in increased mineralisation of deposited ECM and higher levels of differentiation of the pre-osteoblast cells on the composite scaffolds vs. the collagen control construct over the 28 day culture period. These \textit{in vitro} results show that the coll-nHA scaffolds have potential for use as a bone graft substitute.
Chapter 5: Investigation of the potential of nHA particles to transfecct in 2D and to sustain release in 3D using coll-nHA gene activated composite scaffolds

5.1 Introduction

Gene therapy is a technique to deliver small DNA sequences to cells or tissues to correct a genetic defect or treat a disease. Gene therapy can be applied to the field of bone regenerative medicine as a method for enhancing the expression of osteogenic tissue inductive factors [12]. In this approach, cells are transfected (i.e. specific DNA sequences are introduced into cells) and osteogenic genes are expressed, leading to improved healing and bone formation [13]. Successful transfection requires a delivery system to carry the plasmid DNA (pDNA) into the scaffolds, and two general delivery approaches are using viral and non-viral methods [200]. Viral methods include the use of retroviral, lentiviral and adenoviral vectors, and these approaches generally lead to high transfection efficiencies, although their clinical use is limited due to cytotoxicity and immunogenicity concerns [234]. On the contrary, non-viral approaches such as the use of calcium phosphate (CaP) particles, liposomes and polymers are safer and not limited by the size of the DNA plasmid they can deliver. However, the transfection
efficiency of these approaches needs to be improved. CaP particles are widely used and investigated because they are cost-effective and relatively easy to prepare, they can be used to transfect a wide range of mammalian cells \textit{in vitro} and also avoid any issues such as immune responses and high toxicity associated with other techniques [262]. Calcium phosphate mediated transfection was introduced by Graham and Van der Eb in 1973, although poor transfection efficiency and concerns about the reproducibility of this approach have been expressed. Recent modifications of this method have been reported to deliver reporter genes, such as green fluorescent protein (GFP) and luciferase, in addition to the delivery of therapeutic genes including bone morphogenic proteins BMP-2, BMP-5 and BMP-9 and alkaline phosphatase for bone tissue regeneration, although an improvement in the transfection efficiency is still required [14, 201, 223]. Reporter genes are typically used to provide quick and effective feedback during the optimisation of a delivery system, and can be both visual and quantitative. Generally, once the delivery system has been optimised, therapeutic genes are then applied to cells for the induction of a desired healing response [12]. Mesenchymal stem cells (MSCs) are considered to be a promising platform for cell and gene therapy, and the manipulation of these cells \textit{in vitro} has attracted much interest as a source of cells for developing novel tissue-engineered constructs [355]. In addition, MSCs serve as a target cell for \textit{in vivo} gene therapy-based applications, and therefore, the transfection of rat MSCs is investigated in this study instead of the MC3T3-E1 pre-osteoblast.
The use of 3D scaffolds for gene delivery has shown potential, as this gene activated matrix approach allows for controllable and sustained localised delivery of pDNA both \textit{in vitro} and \textit{in vivo} [13, 356]. This method overcomes limitations associated with traditional growth factor delivery including short half-life, large dose requirement, high cost, the need for repeated applications, and poor distribution [12, 357].

5.1.1 Aim

The specific aims of this study were to investigate the ability of the nHA particles developed using Darvan dispersant in Chapter 2 to transfect cells. Additionally, the capacity of this non-viral delivery vector incorporated into the composite scaffolds developed in Chapter 3 was assessed as a gene activated matrix for sustained gene expression vs. a collagen only scaffold.

5.2 Materials and Methods

5.2.1 Plasmid propagation

Two plasmids (pDNA) were used in this study, pMaxGFP (Amaxa, Lonza Cologne AG, Germany) and pGaussian-Luciferase (pGLuc; New England Biolabs, Massachusetts, USA), encoding the reporter genes, green fluorescent protein (GFP) and luciferase. These particular pDNA samples were selected for their ability to provide a quick and reliable assessment of the cellular transfection achieved using the various methods described below. GFP is expressed in the cell cytoplasm, and
can be analysed easily using fluorescence microscopy. Luciferase expression can be measured quantitatively using a luciferase assay (section 5.2.9).

Plasmid amplification was carried out by transforming One Shot® TOP10 Chemically Competent E. coli bacterial cells (Biosciences, Ireland) according to the manufacturer’s protocol. The transformed cells were cultured on LB plates with 50 µg/ml kanamycin (Sigma-Aldrich, Ireland) as the selective antibiotic. Bacterial colonies were harvested and inoculated in LB broth (Sigma-Aldrich, Ireland) overnight for further amplification. The harvested bacterial cells were then lysed and the pDNA was collected using mini- and maxiprep kits (Qiagen, West Sussex, UK). Nucleic acid content was analysed using NanoDrop 1000 spectroscopy, taking the 260/280 ratio and 230 nm measurement to determine the ng/µl measurement. Plasmids in this study were used at a concentration of 1 µg/2 µl in TE buffer.

5.2.2 CaP-pDNA complex synthesis

The CaP nanoparticles were precipitated by adding 100 µl phosphate solution containing 0.012 M Na₂HPO₄.12H₂O, 5 mM NaOH, 50 mM HEPES buffer and 140 mM NaCl into 100 µl 0.02 M CaCl₂.2H₂O. The phosphate solution was prepared in the presence or absence of Darvan dispersing agent – no_D (0 % v/v Darvan), low_D (0.016 % v/v Darvan), med_D (0.066 % v/v Darvan) and high_D (0.1 % v/v Darvan; quantity used in Chapter 2) in order to determine the effect of Darvan addition on transfection efficiency. 138 µl of filtered (0.2 µm) CaP solution was then added to 6 µl pDNA (3 µg, pMaxGFP was used for the 2D transfection and
complex analysis, or pGLuc which was used in the 3D transfection study) and 6 µl 250mM CaCl₂ (total volume 150 µl). To ensure that the incubation of pDNA in CaCl₂ solution alone is not sufficient for successful transfection, 6 µl pDNA and 6 µl 250mM CaCl₂ in 138 µl deionised water was utilised as a negative control, while a lipid (lipofectamine) is used as a positive control (section 5.2.5) in the transfection experiments.

5.2.3 Binding analysis

A Quant-iT™ PicoGreen® dsDNA kit was used to determine the pDNA binding efficiency of CaP particles synthesised with and without Darvan (no_D, low_D, med_D and high_D). The PicoGreen reagent was prepared according to the manufacturer's protocol. Briefly, 100 µl Quant-iT PicoGreen dsDNA reagent was added to 19.9 ml TE buffer and this solution was protected from light during preparation and use. The CaP-pDNA complexes were prepared and centrifuged at 10,000 g for 10 minutes. 350 µl of the supernatant from each sample was mixed with 350 µl of the PicoGreen reagent and 200 µl samples were plated in triplicate in a 96 well plate. Fluorescence of the samples was measured (excitation 480 nm, emission 520 nm) and DNA concentration was deduced using a standard curve.

5.2.4 CaP-pDNA complex analysis

The average particle sizes of freshly prepared CaP-pDNA complexes (no_D, low_D, med_D and high_D) were measured (n = 3) using dynamic light scattering (DLS) (ZetaSizer 3000 HS, Malvern instruments, UK) as described in section 2.2.4. Zeta Potential (ZP) readings (n = 3) were also conducted on freshly prepared
suspensions to determine the stability of the CaP-complexes. This was done using a ZetaSizer 3000 HS (Malvern instruments, UK), as described in section 2.2.6.

5.2.5 Lipid-pDNA complex preparation (positive control)

The commercial lipid transfection agent lipofectamine (Invitrogen, Biosciences, Dublin, Ireland) was used as a positive control in this study, according to the manufacturer’s protocol. Lipofectamine is a commonly used transfection reagent, which transfects cells by altering the plasma membrane to allow the DNA to enter the cytoplasm. A preliminary investigation showed that a lipid:pDNA ratio of 2:1 yielded the optimum GFP gene expression in 2D, and consequently this ratio was used for all following experiments. The lipid-pDNA complex was prepared as follows: 12 µl lipofectamine was incubated in 63 µl OptiMEM serum free media (Invitrogen, Biosciences, Dublin, Ireland) for 5 minutes at room temperature before being added to a solution containing 6 µl pDNA in 69 µl OptiMEM. This solution (total volume 150 µl) was incubated at room temperature for 20 minutes before use.

5.2.6 Cell culture

MSCs were isolated from 8 week old female Fischer 344 rats as described. After euthanasia, marrow was flushed from the tibia and fibula with phosphate buffered saline (PBS) and a single cell suspension was recovered. After centrifugation (600x g, 10 minutes) cells were plated at 120 x 10^6 cells/cm², in complete rat MSC growth medium [10 % foetal bovine serum (FBS; Hyclone), 45 % F12-Ham and 45 % α-MEM (Biosciences, Ireland) supplemented with antibiotics (100 U/ml
penicillin G and 100 μg/ml streptomycin sulphate; Gibco). Flasks were incubated at 37°C in 5 % CO₂/90 % humidity. After 8 days colonies became compact and cells were detached with 0.25 % trypsin/EDTA and re-plated at 2000 cells/cm². Subsequently, cultures were passaged at 5-day intervals and expanded to passage 5 for experimentation. The MSCs were cultured in Dulbecco's Modified Eagles Medium (Sigma-Aldrich, Germany) supplemented with 2 % penicillin/streptomycin (Sigma-Aldrich, Germany), 0.6 % L-glutamine (Sigma-Aldrich, Germany), 10 % FBS (BioSera, East Sussex, UK), 0.6 % glutamax (Biosciences, Ireland) and 1.2 % non essential amino acids (Biosciences, Ireland).

5.2.7 2D Transfection study

MSCs were plated in adherent 12 well plates at a density of 100,000 cells/well. Media was changed 24 hours after plating, and 1 hour later this media was removed again for the transfection to be conducted. 150 μl of the prepared pDNA-complex solutions (no_D, low_D and positive and negative controls) were then added to the wells and incubated for 15 minutes before 2 ml of media was added. OptiMEM serum free media was required for the wells containing lipofectamine (lipid) transfected cells, and this solution was removed 6 hours after transfection and replaced with regular media. Cells were assessed for transgene expression 24 and 48 hour post-transfection, using fluorescence microscopy (Leica DM IL, Laboratory Instruments and Supplies, Co. Meath, Ireland) to detect GFP expression from transfected cells. Images were captured using an attached camera (Leica DFC 420 C, Laboratory Instruments and Supplies, Co. Meath, Ireland).
5.2.8 3D Transfection study

Coll-nHA scaffolds (S-100 and S-500) were prepared as described previously (sections 3.2.1 and 3.2.2) and cross-linked before use as described in section 4.2.1. A collagen only scaffold served as a control. The pDNA-complexes (no_D, low_D and lipid) were added by soak loading which involved placing the scaffolds in a dry 6 well plate and adding 75 µl of the pDNA-complex solutions drop-wise to the scaffold surface. The scaffold was incubated for 15 minutes, turned, and the remaining 75 µl was added to the other side and incubated for an additional 15 minutes. The cell suspension was prepared during this time and 400,000 cells were seeded onto each side, with 15 minute incubation periods following each addition. 5 ml of media was then added to the well and the scaffolds were cultured in normal culture conditions (section 4.2.2).

5.2.9 Luciferase Assay

A LumiFlex GLuc assay (New England Biolabs, Isis Ltd, Ireland) was used to quantify the expression of luciferase from MSCs seeded on collagen, S-100 and S-500 constructs. The GLuc assay solution was prepared by adding 50 µl of the 100x substrate into 800 µl of the GLuc assay buffer. This solution was vortexed to mix, and stored in a tightly sealed, light protected container. Aliquots (25 µl) of culture media were plated in triplicate in a 96 well plate with 50 µl of GLuc assay solution and luciferase expression was determined using luminescence measurements (Wallac Victor2™ 1420 multilabel counter, Perkin Elmer Life Sciences, Finland).
5.3 Results

5.3.1 Analysis of the effect of Darvan on binding efficiency

The results in Figure 5.1 demonstrate the effect of Darvan content on the ability of the CaP to bind to the pDNA; the highest CaP-pDNA binding was achieved using the CaP particles with no Darvan (no_D; 99.74 ± 0.03 %), and the introduction of Darvan reduced the binding efficiency in a dose dependent manner (low_D: 57.4 ± 0.6 %, med_D: 15.4 ± 1.3 %, high_D: 11.1 ± 0.9 %). There was no pDNA binding detected in the negative control sample.

![Graph showing the percentage of pDNA binding to CaP particles with different amounts of Darvan dispersant present](image)

Figure 5.1 Graph showing the percentage of pDNA binding to CaP particles with different amounts of Darvan dispersant present

(no_D: 0 %, low_D: 0.016 %, med_D: 0.066 %, high_D: 0.1 %).

5.3.2 Analysis of the effect of Darvan on average particle size

The average particle sizes of the CaP-pDNA complexes are shown in Figure 5.2. The effect of Darvan addition is evident, as the addition of low_D (0.016 % v/v) leads to a reduction in size from 4512.5 ± 456 nm (no_D) to 264.9 ± 12.4 nm. This
average particle size is reduced in a dose dependent manner; med_D: 167.2 ± 22, high_D: 102.1 ± 1.6 nm.

Figure 5.2 Graph showing the average particle size of CaP-pDNA complexes with different amounts of Darvan dispersant present.

5.3.3 Zeta Potential (ZP)

ZP measurements of the CaP particles and CaP-pDNA complexes provide an indication of the stability of the suspensions. Figure 5.3 shows an increase in stability of the CaP particles, and the CaP-pDNA complexes with increasing Darvan content; no_D-pDNA: 16.27 ± 0.93, low_D-pDNA: 27.9 ± 3.64, high_D-pDNA: 36.48 ± 6.62. The ZP of the negative control (pDNA in CaCl₂) is shown in comparison to the groups containing the CaP particles, highlighting the stabilising effect the CaP particles have on the suspension.
Figure 5.3 Graph demonstrating the ZP of the CaP particles (no_D, low_D and high_D), and of their corresponding CaP-pDNA complexes, in comparison to the pDNA without CaP particles present (negative control).

5.3.4 2D Transfection study

The series of images in Figure 5.4 provides a visual demonstration of the ability of the CaP-pDNA complexes to transfect cells with pMaxGFP (24 and 48 hours post transfection). From the earlier complex analysis results, the no_D and low_D CaP-pDNA complexes were selected to be investigated in the transfection study. These results indicate that applying pDNA in CaCl₂ solution alone (negative control) to the cells did not lead to transfection, while positive transfection is observed in the cells treated with the lipid (positive control), although there is evidence of cell death (very bright green cells) in this sample at 48 hours. The use of the no_D and low_D suspensions both yielded positive transfection, with higher transfection efficiency demonstrated in the low_D treated cells.
Figure 5.4 Fluorescent images demonstrating transfected MSCs expressing GFP using the no_D, low_D and lipid (positive control) methods. No transfection was observed in the pDNA in CaCl₂ (negative control) group.
5.3.5 Luciferase release study

The results obtained in the 2D GFP study demonstrated that the no_D and low_D groups were effective CaP-based methods for transfection, and therefore these techniques were investigated further in 3D, using the composite scaffolds as a template for transfection. The pGLuc plasmid was used to transfect the MSCs seeded on the constructs, enabling the quantitative measurement of luciferase expression on each scaffold. Figure 5.5 shows the lipid-mediated expression (positive control) of luciferase from MSCs seeded on the S-100 and S-500 scaffolds vs. a collagen control. The collagen scaffold experienced significantly higher levels of luciferase expression up to day 7, while S-100 and S-500 showed a more sustained release of luciferase expression, with significantly more expression observed at day 10 on S-100 than on the collagen scaffold.

![Graph demonstrating luciferase expression from MSCs seeded on collagen, S-100 and S-500 constructs using the lipid (positive control) as the delivery agent. (The symbol indicates when the culture media was changed).](image)

**Figure 5.5** Graph demonstrating luciferase expression from MSCs seeded on collagen, S-100 and S-500 constructs using the lipid (positive control) as the delivery agent. (The symbol indicates when the culture media was changed).
The lipid method (positive control) demonstrated that the 3D scaffold has an effect on gene expression, indicating that the addition of nHA into the collagen scaffold resulted in a more sustained expression profile. The ability of the CaP particles to transfect cells in a 3D matrix was then investigated. Results show that a similar trend to the lipid method was observed when the MSCs were transfected using the no_D particles to deliver the pGLuc, as shown in Figure 5.6. Increased luciferase expression was obtained on the collagen scaffold at day 7, while MSCs on the S-100 and S-500 constructs experienced a more sustained release of luciferase vs. the collagen control, with the S-100 scaffold demonstrating significantly higher expression at day 10.

![Graph demonstrating luciferase expression from MSCs seeded on collagen, S-100 and S-500 constructs using no_D as the delivery agent.](image)

**Figure 5.6** Graph demonstrating luciferase expression from MSCs seeded on collagen, S-100 and S-500 constructs using no_D as the delivery agent.

In addition, the expression of luciferase from MSCs on the constructs also revealed a similar trend to the other methods when low_D particles were used as the
delivery agent (Figure 5.7). Significantly higher expression was achieved on the S-100 and S-500 constructs at day 10, again validating the ability of the composite scaffolds to support sustained gene expression vs. the collagen control.

![Graph demonstrating luciferase expression from MSCs seeded on collagen, S-100 and S-500 constructs using low_D as the delivery agent.](image)

**Figure 5.7** Graph demonstrating luciferase expression from MSCs seeded on collagen, S-100 and S-500 constructs using low_D as the delivery agent.

### 5.3.6 Analysis of the effect of Darvan on 3D luciferase expression

The 3D results indicate that significantly higher levels of sustained release were achieved using the S-100 construct, and therefore this construct was used to compare the expression achieved using no_D and low_D complexes (Figure 5.8). There is no significant difference at any time point between the luciferase expression achieved using the two methods. Sustained gene release is obtained *via* both methods, demonstrating the potential of CaP-based transfection.
5.4 Discussion

The optimisation of CaP-mediated gene delivery is important in order to fully exploit the potential of this cost-effective and safe method for transfecting a wide range of cells. Efficient binding of the pDNA to the CaP particles is required to maximise the quantity of pDNA that is delivered into the cells, as investigations have shown that naked DNA (i.e. no delivery agent) cannot transfec cells. Results from this study have shown that while the Darvan dispersing agent influences the binding, approximately 60 % of the pDNA can bind to the CaP particles synthesised using 'low_D' (0.016 % v/v Darvan). This is important, as the average complex size measurements showed that Darvan is required to prevent aggregation. Therefore, while the CaP particles synthesised without Darvan had the best pDNA binding efficiency, the use of these particles yielded complexes with an average...
size of over 4.5 μm, compared to the nano-sized complexes obtained when Darvan is used. The size of the complex is important for transfecting cells, with many studies showing the enhanced transfection efficiency of smaller particles [262, 263, 272]. A balance is required therefore, between optimum binding, and average particle size, and for this reason the CaP particles synthesised with a low Darvan content were used in subsequent investigations (eliminating med_D and high_D). The zeta potential (ZP) of the complexes provided an insight into the stability of the CaP-pDNA suspensions, and the results showed that the presence of Darvan tended to increase the ZP value, and therefore the stability of the suspensions. From this initial analysis, the CaP particles synthesised using the low Darvan content demonstrated the most potential as a delivery agent for transfection, and an initial transfection study was utilised to investigate the ability of these particles to transflect rat MSCs in 2D culture.

This 2D transfection study was conducted using GFP expression from MSCs as a qualitative indication of the capacity of CaP particles as a delivery vector. These results showed that both no_D and low_D CaP-pDNA complexes successfully transfected the MSCs, with the low_D showing the best transfection efficiency, assessed using this qualitative method. The method of adding precipitated CaP particles to pDNA incubated in a CaCl$_2$ solution, as is used in this study, has recently been shown to lead to higher transfection efficiency than the original method of forming the CaP particles in the presence of pDNA (co-precipitation) [262, 277, 358]. This may be due to the pDNA inhibiting calcium phosphate
precipitation, as it binds to the calcium ions [263]. Additional techniques such as glycerol shock treatment are required for transfection when the pDNA is added prior to CaP formation [263]. Other CaP based transfection methods reported in literature have used very high Ca/P ratios (usually two orders of magnitude higher than that of any calcium phosphate compound) to achieve transfection, although transfection at these conditions may be predominantly due to the nano-size of the particles formed at these high ratios [124]. The use of Darvan in this study prevents the particles from aggregating while maintaining a stoichiometric ratio (1.67), thereby producing complexes in the nano-sized range, capable of entering the cells by endocytosis. A comparative transfection study looking at the effect of Ca/P ratio was carried out, using the stoichiometric ratio ‘low_D’ method, as described in section 5.2.2, and a suspension precipitated with an increased ratio Ca/P = 334 (i.e. 200X low_D). The GFP expression results are shown in Figure 5.9, illustrating the enhanced transfection efficiency achieved using the stoichiometric ratio, low_D technique.
Figure 5.9 The effect of Ca/P ratio is demonstrated by the GFP expression achieved using low_D and a suspension with 200 times the Ca/P ratio of low_D.

The 2D transfection results also showed that the lipid (lipofectamine – positive control) treated cells demonstrated high transfection efficiency, although this method was found to be quite toxic, leading to increased cell death compared to the other transfection methods (Figure 5.10) [359]. This correlates with other findings in literature, with cell toxicity of 50 % reported by Corsi et al. (2003) following lipofectamine-mediated transfection [360].
Figure 5.10 Fluorescence image demonstrating cell death (bright green cells) in lipid-treated MSCs.

The necessity of additional parameters involved in the synthesis of the CaP-pDNA complexes was also investigated using pMaxGFP and the 2D transfection model. Results demonstrated that the pre-incubation step (pDNA incubated in CaCl$_2$ before use), filtration of the CaP particles, and the use of HEPES buffer in the precipitation reaction were all required to enhance cellular transfection. Pedraza et al. (2008) also showed that the use of the pDNA in CaCl$_2$ pre-incubation step and filtration of the CaP particles improved transfection [262]. The filtration step mechanically removes any aggregated particles, ensuring that the suspension that is added to the cells is under the size limit (<200 nm) for endocytosis to proceed. The presence of excess calcium ions (by pre-incubating the pDNA in CaCl$_2$) has been shown to enhance transfection, both with CaP-mediated transfection, and when using alternative pDNA delivery systems [361-364]. The calcium ions effectively condense the DNA structure by forming ionic bonds with the phosphate groups of
DNA, as calcium has a small hydrodynamic radius and a high surface to charge area. The pDNA is therefore protected until it enters the cell, while remaining functional.

This condensation of pDNA is also important when cells are transfected in 3D, as it needs to remain protected until it enters the cells. Studies have used polymers to encapsulate the pDNA molecules to sustain gene expression from scaffolds, although the CaP particles have been shown to accomplish this on their own [357]. Therefore, these CaP-pDNA complexes no_D and low_D have potential for transfecting cells in a 3D environment, in addition to in 2D culture.

The 3D transfection studies were conducted using composite scaffolds developed in Chapter 3, S-100 and S-500 vs. a collagen control. A preliminary study was conducted using pMaxGFP lipid-pDNA complexes to investigate if these scaffolds offered a suitable template for transfection to occur. Figure 5.11 shows the expression of GFP (in green) from transfected MSCs seeded on the S-500 scaffold at two different magnifications (x20 and x40). The expressed protein is observed in the cell cytoplasm, and the non-transfected cells are seen in red (labelled with PKH26 and nuclei stained with DAPI).
Following this validation of the 3D transfection (using GFP) protocol, the pGLuc plasmid was used in order to quantify the gene expression (reporter gene luciferase) from MSCs seeded on the composite S-100 and S-500 scaffolds vs. a collagen control. The use of pGLuc plasmid instead of pMaxGFP provides a more accurate method for comparison of the different constructs, in addition to enabling a comparison between the use of no_D and low_D to be conducted.

The pGLuc transfection study involved soak-loading the S-100, S-500 and collagen control scaffolds with the CaP-pDNA complexes optimised in the 2D study. Lipid-mediated transfection (positive control) was also carried out, and these results indicated that higher initial expression is achieved on the collagen control scaffold, but that the expression was prolonged on the S-500 and particularly on the S-100.
scaffold up to 10 days in culture. This trend was also observed applying the CaP-based transfection delivery agents, no_D and low_D, and there was no significant difference between the expression achieved using the two methods. The sustained gene expression on the coll-nHA composite scaffolds vs. the collagen control may be due to the presence of the nHA particles within the scaffolds, as these particles would interact with the pDNA complexes and prolong the cellular uptake of the pDNA, thereby sustaining the expression of the reporter gene. This sustained release profile is desired for many in vivo applications requiring a prolonged and steady healing response, such as in bone regenerative medicine.

5.5 Conclusion

The results from this study indicate the potential of nano-hydroxyapatite fabricated using the processes described in Chapter 2 combined with the coll-nHA composite scaffolds developed in Chapter 3 for use as a gene activated matrix. Efficient transfection of rat MSCS was achieved using dispersant-aided calcium phosphate-based delivery methods. Similar levels of transfection were obtained in the lipid treated cells (positive control) in 2D culture, although there was evidence of cell toxicity with this technique. The 3D transfection study demonstrated that the S-100 and S-500 composite scaffolds supported sustained gene expression up to day 10, thereby validating their aptitude for use in bone tissue regeneration.
Chapter 6 Discussion

6.1 Introduction

Bone tissue engineering applies the principles of engineering and life sciences towards the development of biological substitutes that can replace, restore, or improve bone tissue function [1]. In BTE a 3D scaffold is used to provide a suitable template for new tissue formation, and ideal scaffolds should be biocompatible with osteoconductive and osteoinductive properties, allowing for cells to attach, proliferate and form ECM [48]. The scaffold should be porous, biodegradable, and possess sufficient mechanical integrity to support tissue formation [47, 171, 327]. Collagen based scaffolds meet many of these requirements, but lack clinical success due to their limited mechanical properties. This thesis aimed to address this issue, by reinforcing the collagen scaffolds with the bioactive ceramic nHA (synthesised in Chapter 2). The use of nano-sized particles was hypothesised to improve the Young’s modulus value (compressive properties) of the scaffolds, while maintaining their high porosity and biocompatibility (Chapters 3 and 4).

The healing response obtained from BTE scaffolds can be improved significantly by the addition of signals or cytokines to the constructs to invoke or enhance a desired cellular response, and this can be achieved by the addition of growth factors or preferably specific genes which target bone formation. Gene therapy can be a valuable tool to avoid the limitations of local delivery of growth factors,
including short half-life, large dose requirement, high cost, need for repeated applications, and poor distribution [201]. The concept of a gene delivery vector contained within a biodegradable scaffold is a relatively recent development in the field of regenerative medicine and this system has been coined as a 'gene activated matrix' by Bonadio and collaborators (1999) who developed the first GAM system [13]. Therefore, this thesis aimed to develop a GAM delivery system using the collagen-nHA composite scaffolds (Chapter 3) as a template, and the synthesised nHA particles (Chapter 2) as the non-viral transfection agent (Chapter 5). Details of the results obtained and their implications are discussed below.

The work described in Chapter 2 has led to the development of reproducible non-aggregating nHA particles which can be used in a suspension, or freeze-dried to obtain a fine powder. This was achieved following the optimisation of synthesis parameters such as the concentration of the initial calcium and phosphate solutions, the order of addition, the pH of the reaction, the use of sonication and ultimately the application of a dispersing agent, Darvan. It was found that the addition of 0.1 % Darvan to the precipitation reaction produced nano-sized particles (<100 nm) in a stable suspension. In the literature, it is noteworthy that although many studies report the synthesis or use of nano-sized HA, these studies are actually working with aggregated, micron-sized clusters of nanocrystalline HA, unlike the disperse particles produced using the novel method described in this study [138, 145, 157, 164, 167, 322, 365, 366]. X-ray diffraction is used in these papers to provide a crystallite size within the nano-range, but often the microscopical investigation
demonstrates the agglomerated nature of the “nano-particles” applied in those studies.

There are many advantages associated with using nano sized particles, over conventional HA. Firstly, the enhanced mechanical properties that can be obtained on addition of nHA to composite scaffolds is highly relevant to the aim of this thesis [141]. Additional benefits include the bioresorbability of the nHA, unlike larger HA particles which do not degrade in vivo for many years, and the ability to overcome scaffold brittleness associated with the use of conventional HA [356, 367-369]. In addition to the enhanced material properties, the use of nHA has also been shown to induce increased cellular responses, which is mainly due to the increased surface area of the bioactive material [127, 128, 346]. In a comparative study between the addition of nano- and micro-sized particles of HA to a polymer scaffold, Heo et al. (2009) found that adding the nHA particles yielded significantly stronger constructs, with enhanced biological properties, and attributed these improvements to the disperse and hydrophilic nature of the nHA [135]. Fukui et al. (2008) demonstrated that the combination of nHA and collagen resulted in enhanced bone tissue response when implanted in an in vivo rabbit model in comparison to collagen only, and collagen-calcined HA scaffolds [370].

However, the fabrication of non-aggregated nHA is difficult, often requiring the use of extensive ageing steps and high temperatures [122, 143, 144]. Cationic surfactants such as CTAB and SDS have been employed to prevent particle
agglomeration, although time consuming ageing and heating steps are still necessary [167, 308, 371]. The synthesis presented in this thesis provides a cost-effective, quick and simple technique for the production of disperse nHA particles, suitable for use in a number of clinical applications.

The nHA produced in this study was then incorporated into collagen scaffolds (Chapter 3), based on the hypothesis that the ceramic particles would reinforce the collagen fibres and generate constructs with improved mechanical properties suitable for use as a bone graft substitute. Four methods were developed to fabricate coll-nHA composite scaffolds based on the freeze-drying technique optimised in our lab [49, 94]. Characterisation of the scaffolds produced by each method demonstrated that despite achieving a high porosity, the powder (adding dried, aggregated nHA particles into the collagen slurry before freeze-drying) and in situ (conducting the nHA synthesis in the collagen slurry before freeze-drying) methods held the least potential based on the mechanical and material properties of their resultant scaffolds. On the contrary, the immersion (soaking porous collagen scaffolds in nHA suspension) and suspension (adding suspended nHA particles to the collagen slurry before freeze-drying) techniques allowed for the fabrication of a range of composite scaffolds with intrinsic properties highly suitable for use in bone tissue engineering. Of the two methods however, the suspension method had a number of advantages. From a production view point, the suspension method was less time-consuming, more reproducible and higher nHA content could be incorporated easily. In addition, the suspension method provided the ability to
accurately control the nHA content, thereby offering the capacity to tailor the mechanical properties of the resultant scaffold. The results demonstrated that up to 500 wt % nHA could be added to the collagen scaffold homogeneously, resulting in a scaffold with 18 times the Young’s modulus of the collagen control, while maintaining the high porosity (99 %) and biodegradability suitable for \textit{in vivo} bone tissue regeneration.

Reports in the literature have long demonstrated the advantages of using composite scaffolds, exploiting the benefits of each individual material to produce a superior product. Many studies, including research in our laboratory, have combined collagen with calcium phosphates and HA to improve the mechanical properties which limit the clinical use of collagen scaffolds [10, 120, 142, 188, 197, 329, 331, 372, 373]. The incorporation of nano-sized HA into collagen scaffolds has proved more difficult however, with papers reporting various issues, such as the aggregation of the particles during fabrication, inhomogeneous particle distribution throughout the scaffold, and a limit to the nHA content added [330, 374-376]. For example, Sachlos \textit{et al.} (2006) developed a coll-nHA scaffold by placing a collagen membrane between calcium and phosphate solutions, allowing the precipitation reaction to occur in the collagen scaffold [377]. However, no reference is made to the content of nHA in the scaffold, or the gradient effect that likely occurred during this synthesis (i.e. calcium rich side vs. phosphate rich side).

The suspension method presented in this thesis overcomes these problems, offering a fast, adaptable and reproducible technique for the fabrication of a range of coll-
nHA scaffolds. The S-100 and S-500 composite scaffolds (containing 100 and 500 % nHA added via the suspension technique respectively) were selected for biological analysis for different reasons. The S-100 scaffold had the same Young’s modulus value as the collagen only scaffold, so this scaffold was analysed to determine the effect of the nHA particles on biological performance. However the S-500 scaffold had 500 wt % nHA content and a significantly improved Young’s modulus, and so this construct were evaluated to assess the increased stiffness and nHA content.

*In vitro* studies were conducted in order to determine the osteogenic potential of the S-100 and S-500 composite scaffolds, and the results obtained demonstrated that both of the composite scaffolds displayed excellent biocompatibility. The short-term investigation examined the behaviour of pre-osteoblastic MC3T3-E1 cells seeded on the constructs, and showed that cell attachment was significantly higher on the S-500 construct. The addition of nHA to scaffolds has been shown to influence cell attachment, with significant increases attributed to the hydrophilicity and serum protein adsorption abilities of the nHA [121, 325, 378]. Li *et al.* (2009) demonstrated the relationship between high cell density on regions of higher calcium content and substrate stiffness, and this correlates with what was observed in this thesis, with higher cell attachment on the stiffer S-500 construct than on S-100 [332]. Additionally, the cells were shown to infiltrate into the centre of the composite constructs after 7 days in culture. This is an important aspect to
consider, as encapsulation of the cells around the circumference of the construct would lead to a necrotic central region, and inhomogeneous tissue growth [379].

Results from the long term study revealed that the composite scaffolds provided a suitable environment for cellular proliferation, differentiation, matrix deposition and mineralisation, thereby indicating the potential of these constructs to support bone-like tissue formation. High power scanning electron microscopy showed matrix deposition, and the planar morphology of the cells on the surface of the construct, demonstrating their affinity for the composite material. The osteoinductive properties of the HA has been shown in previous studies to influence cellular behaviour. Lin et al. (2007) reported the ability of the ceramic to induce expression of osteo-specific genes, enhancing the differentiation of cells [380]. In addition, Habibovic et al. (2006) stated that increasing the specific surface area of the HA further improves the osteoinductivity [381]. The cell-mediated in vitro matrix deposition has been shown to further improve the mechanical properties of constructs, thereby moving towards the Young’s modulus of host bone (>10 GPa) [41, 382]. Initial bone graft substitutes aimed to achieve mechanical properties resembling that of native tissue, stating that this was required for successful integration of the scaffold in the defect site [171, 383]. Recently however, there has been a shift in opinion in this regard; with emphasis placed on generating compliant constructs, and ensuring they have sufficient mechanical properties to preserve structural integrity and functionality during in vivo implantation and long-term performance. In addition, it is worth mentioning
that current standard treatments for repair of load bearing tissues require the use of added supports such as fixation and sutures to bear mechanical loading during healing and rehabilitation [31, 384]. Furthermore, the scaffold is regarded as a template for guiding tissue growth, and therefore the influence of the scaffold’s intrinsic mechanical properties on ECM deposition and tissue regeneration takes precedence over the load-bearing ability of the scaffolds when implanted.

Following the development of coll-nHA composite scaffolds suitable for bone tissue regeneration, the ability of these scaffolds for gene therapy application was explored. The hypothesis in this investigation was that the nHA particles developed in Chapter 2 could be applied as a non-viral vector, using the composite scaffolds as a gene activated delivery system to achieve sustained gene expression.

Initial results provided an insight into the parameters required to enable nHA-based transfection, indicating that nHA synthesised using a low amount of Darvan (0.016 %) showed the most potential to attain efficient gene expression. CaP based gene delivery provides an attractive alternative to other viral (retrovirus and adenovirus methods have high transfection efficiency but bring with them major in vivo safety concerns) and non-viral (polyplex and lipoplex methods display moderate transfection efficiency but exhibit cytotoxic effects at high doses) vectors because it is quick, cost-effective, able to transfect a wide range of cells in vitro, and avoids immune responses and high toxicity issues [263-265]. However, further research is required to improve the reproducibility and enhance the transfection efficiency to
levels obtained using viral methods [237]. The results presented in this thesis correlate with the findings reported by Pedraza et al. (2008) regarding the improvement in transfection efficiency obtained by applying a filtration step and incubating the pDNA in calcium chloride prior to nHA particle addition.

Using these optimised techniques, the nHA-pDNA complexes were incorporated into composite scaffolds to develop a gene activated matrix (GAM). Using a luciferase reporter gene, the gene expression from MSCs seeded on the GAM was measured over a ten day culture period to obtain a release profile. Results have demonstrated that a high expression is achieved using the collagen only scaffold up to 7 days, indicating the suitability of this scaffold for use when a burst release profile is required, i.e. a large quantity of gene delivered over a short time scale. However, the composite scaffolds displayed a more sustained gene expression profile, with stable expression maintained in the MSCs up to 10 days in culture. This prolonged gene expression is more suitable for many clinical applications in which a controlled, sustained delivery of a therapeutic gene is desired. For bone tissue regeneration, therapeutic genes such as BMP-2 and BMP-7 would stimulate cells and enhance new tissue formation in the implant [385-387]. These genes require a suitable delivery system however, and the lack of a system that can adequately mimic both the physical properties and release kinetics of bone matrix remains a major handicap [388].
Natural polymer scaffolds have been investigated for their ability to deliver gene therapy in a direct localised manner [356]. The combination of gene therapy and biocompatible scaffolds suitable for tissue engineering applications exploits the power of genetic cell engineering to provide the biochemical signals which influence proliferation or differentiation of cells. Further research is required to advance the 3D delivery methods, as it is crucial to fully understand the mechanisms involved in these delivery systems in order to fully exploit its benefits to the fullest extent. Bonadio et al. (1999) showed that in vivo healing of a canine defect model could be achieved using a collagen matrix with naked pDNA delivery [13]. Hydroxyapatite scaffolds have also been investigated as a gene delivery system, although results indicated that incomplete healing was achieved due to the poor infiltration that occurred in this scaffold [389]. This result illustrates the importance of the matrix material, and it is has been shown that the properties of the composite scaffolds developed in this thesis meet the requirements for successful delivery. These requirements include the ability of the matrix to allow cell infiltration in a biocompatible environment, the ability to degrade at an appropriate rate and the ability to cross-link or modify the matrix to tailor for a range of a clinical applications [229, 356]. The use of complexed-pDNA has enhanced gene expression from 3D matrices in comparison to the use of naked DNA, and this thesis has shown that nHA particles are capable of achieving this sustained 3D delivery [235, 286, 357]. This finding correlates with the work of Krebs et al. (2009) and Endo et al. (2006) which showed that the addition of
calcium phosphate particles enhanced the transfection efficiency, and hence the healing response achieved *in vivo*.

In particular, the GAM developed in this thesis possesses inherent properties suitable for use in bone tissue regeneration, and several review papers hypothesise that there is a great potential for applying gene therapy to enhance bone tissue formation [12, 390]. It is envisaged that these scaffolds will be used to deliver bone-specific therapeutic genes in our laboratory in the near future.
6.2 Future work:

- This thesis has shown the advantages of incorporating nHA particles into collagen scaffolds. Additional aspects of scaffold preparation which are worth examining include altering the freeze-drying process to attain larger pores, and the addition of polymer microcapsules which may be synthesised to contain growth factors or drugs for therapeutic applications.

- The osteogenic potential of the composite scaffolds developed in Chapter 3 was investigated using the pre-osteoblastic MC3T3-E1 cells. A number of alternative cell lines (rat or human MSCs, amniotic stem cells) are currently under investigation in the research group, and the behaviour of these cells on the composite scaffold merits examination. Ongoing work in the lab is also examining the co-culture of MSCs and endothelial cells to induce angiogenesis, and these composite scaffolds offer an alternative template to study vascularisation.

- An *in vitro* investigation of the proteins secreted by cells on the composite scaffolds would provide further insight into their activity, and the effect of the composite material on protein adsorption is also of interest.

- This thesis has demonstrated the ability of the nHA particles to deliver pDNA into cells, and of the coll-nHA composite scaffolds to act as a gene matrix delivery system. The next step would be to apply the developed technology to the delivery of therapeutic genes. The logical application of this system would be to target osteo- and angiogenesis with specific pDNA to induce a therapeutic healing response. A comparative study into the
response achieved using traditional growth factor delivery vs. the gene
activated matrix approach would be very valuable.

• Ultimately the composite scaffolds, and gene activated matrices developed
in this thesis are optimised *in vitro* for application *in vivo*. The promising
results obtained in this thesis indicate that these bone graft substitutes merit
investigation, to evaluate the potential healing that can be induced in an *in
vivo* animal model.

6.3 Conclusions

• A novel precipitation technique was developed for the synthesis of non-
aggregating nHA particles. Synthesis parameters such as concentration of
the initial reactants, order of addition and the use of Darvan dispersant were
optimised to produce a stable, reproducible suspension of nHA particles
under 100 nm in dimension.

• The incorporation of a suspension of nHA particles into collagen scaffolds
yielded a range of porous, biodegradable scaffolds with improved
mechanical properties relative to the collagen only control. The nHA
particles were homogeneously distributed throughout the construct,
reinforcing the collagen fibres.

• *In vitro* analysis of the composite scaffolds demonstrated their excellent
biocompatibility. Short term analysis revealed that enhanced cell
attachment was obtained due to the presence of the nHA particles and the
stiffer constructs, while long term examination revealed the osteogenic potential of the composite scaffolds, thereby establishing the aptitude of these constructs for use in bone tissue regeneration.

- The ability of the nHA particles to deliver pDNA to cells was then exploited. The procedure for synthesising the nHA-pDNA complexes was optimised, determining the effect of Darvan, and the reaction parameters required for efficient transfection.

- The optimum nHA transfection methods were then applied to the composite scaffolds to generate gene activated matrices as 3D delivery systems. MSCs seeded on the composite constructs demonstrated sustained gene expression following nHA-based transfection.
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