Phosphonated Polymers for Nanofibrous Tissue Scaffolds

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<td>Molecular weight cut off</td>
</tr>
<tr>
<td>NMP</td>
<td>Nitroxide mediated polymerisation</td>
</tr>
<tr>
<td>NMR</td>
<td>Nuclear magnetic resonance</td>
</tr>
<tr>
<td>PAA</td>
<td>Poly(acrylic acid)</td>
</tr>
<tr>
<td>PCC</td>
<td>Pyridinium chlorochromate</td>
</tr>
<tr>
<td>PCL</td>
<td>Polycaprolactone</td>
</tr>
<tr>
<td>PCL-b-PAA</td>
<td>Polycaprolactone-b-poly(acrylic acid)</td>
</tr>
<tr>
<td>PDI</td>
<td>Polydispersity</td>
</tr>
<tr>
<td>PMDETA</td>
<td>N,N,N’,N”,N”-Pentamethyldiethylenetriamine</td>
</tr>
<tr>
<td>PtBA</td>
<td>Poly(tert-butyl acrylate)</td>
</tr>
<tr>
<td>PVPA</td>
<td>Poly(vinylphosphonic acid)</td>
</tr>
<tr>
<td>PVPA-co-AA</td>
<td>Poly(vinylphosphonic acid-co-acrylic acid)</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>------------</td>
</tr>
<tr>
<td>RI</td>
<td>Refractive index</td>
</tr>
<tr>
<td>ROP</td>
<td>Ring opening polymerisation</td>
</tr>
<tr>
<td>SEM</td>
<td>Scanning electron microscopy</td>
</tr>
<tr>
<td>SFF</td>
<td>Solid freeform fabrication</td>
</tr>
<tr>
<td>SG1</td>
<td>$N$- tert-butyl-$N$-1-diethylphosphono-(2,2-dimethylpropyl)</td>
</tr>
<tr>
<td>Sn(Oct)$_2$</td>
<td>Tin (II) 2-ethylhexanoate</td>
</tr>
<tr>
<td>tBA</td>
<td>tert-Butyl acrylate</td>
</tr>
<tr>
<td>TFA</td>
<td>Trifluoroacetic acid</td>
</tr>
<tr>
<td>THF</td>
<td>Tetrahydrofuran</td>
</tr>
<tr>
<td>T-T</td>
<td>Tail-to-tail</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
</tr>
<tr>
<td>VPA</td>
<td>Vinylphosphonic acid</td>
</tr>
<tr>
<td>εCL</td>
<td>ε-Caprolactone</td>
</tr>
<tr>
<td>μCT</td>
<td>Micro-computed tomography</td>
</tr>
</tbody>
</table>
Abstract

This thesis, entitled “Phosphonated polymers for nanofibrous tissue scaffolds”, was written by Peter James Youle at the University of Manchester for the degree of Doctor of Philosophy and was submitted in 2016.

The work contained within concerns itself with the synthesis and characterisation of phosphonated polymers intended for application as nanofibrous tissue scaffolds for improving the healing of bone; it is based on previous work performed in the University of Manchester that identified poly(ε-caprolactone) (PCL) nanofibres coated with poly(vinylphosphonic acid-co-acrylic acid) (PVPA-co-AA) as a promising material for enhancing bone healing. This thesis initially focuses on the characterisation of a commercially sourced PVPA-co-AA by defining its composition and molar mass using quantitative $^{31}\text{P}^{[\text{H}]}\text{NMR}$ and aqueous gel permeation chromatography. A method of synthesising the copolymer by free radical polymerisation, with controlled rates of monomer addition, was developed to produce PVPA-co-AA copolymers with a range of compositions. This work was published in *Macromolecules*, a copy of the paper is included in the appendices for ease of reference.

Additionally, nanofibres of PVPA-co-AA were then formed by electrospinning and crosslinked with ethylene glycol; the subsequent nanofibres were found to be water-stable and retained their structure after hydration and subsequent drying.

A block copolymer, polycaprolactone-$b$-poly(acrylic acid) (PCL-$b$-PAA), was synthesised by four-step ATRP and two-step NMP based approaches, with the block character of the resulting copolymer being demonstrated by GPC and dynamic light scattering.

The PCL-$b$-PAA was subsequently used as a compatibiliser for PCL and PVPA-co-AA emulsions, which were used to create composite nanofibres by electrospinning. These nanofibres were in turn characterised by scanning electron microscopy and compared to nanofibres formed using a surfactant, Span® 80, and the original dip-coated nanofibres.

Finally, a small portion of work was undertaken to develop phosphonated PCL analogues, by attempting to synthesise phosphonated ε-caprolactone monomers.
Declaration

No portion of the work referred to in the thesis has been submitted in support of an application for another degree or qualification of this or any other university or other institute of learning.
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Dedication

Thanks to my supervisors for help given and insight supplied.

Thanks to everyone involved in the “Bone Void Filler” project: Dr Rachel Zhong and Rebecca Dey for comradery in chemistry; Dr Eric Wang for explanations of cell biology; and Dr Ian Wimpenny for teaching me how to use the electrospinning apparatus and SEM, as well as general troubleshooting.

Thanks to Keith Nixon for running and maintaining the GPC laboratory, the staff in the Microanalysis laboratory for running so many samples, even when already busy, and the NMR staff for their expertise on the quantitative $^{31}\text{P}^{[1]}\text{H}$ NMR method, and those working in Mass Spectroscopy, who were always happy to lend a hand and offer advice.

Special thanks to Robert Woolfson, Stephen Worrall and the rest of NoWNano, for moral support, kind words, commiserations and happy times.

Especial thanks to Fern and Kai, for always being loving, supporting and there for me.
1. Introduction

In the UK there are approximately 850,000 bone fractures reported every year. The majority of these heal without the need for further intervention. However, it is estimated that 5-10% of these fractures do not heal effectively: they are known as non-union fractures. A cross-sectional study in Scotland over five years found an annual incidence of 18.94 non-union fractures per 100,000 members of the population. These fractures often coincide with underlying health conditions, such as diabetes or osteoporosis, and have a significant negative effect on the quality of life of sufferers.

The work in this thesis is concerned with the development of phosphonate-containing polymers for nanofibrous tissue scaffolds. Previous work has identified an electrospun poly(ε-caprolactone) (PCL) scaffold with a coating of poly(vinylphosphonic acid-co-acrylic acid) (PVPA-co-AA) as a promising system for bone tissue scaffolds. It is hoped that the materials resulting from this project will be evaluated for in vivo testing, with a view to further development.

In this introductory chapter there will be a brief overview of bone tissue and tissue engineering techniques and materials, as well as a more in-depth consideration of electrospinning, PCL and PVPA as they are of direct interest for the work that follows. This is followed by an explanation of some key analytical techniques, an assessment of the prior art and the aims and objectives of the work undertaken in this thesis.

1.1 Bone Tissue

The focus of this work is on developing bioactive, phosphonate-containing polymers for bone tissue scaffolds based on PVPA-co-AA. In order to do this, it is useful to understand the basics of bone composition and biology, as well as the contexts and scenarios in which bone tissue scaffolds will be used.

1.1.1 Function

The principal purpose of bone tissue is to form the bones of the skeleton, which has the purpose of providing support and protection to other organs and tissues of the body, as well as providing a mechanical structure to allow movement. In addition, bone tissue has a role in metabolism and maintaining homeostasis by providing a reservoir of minerals and ions (particularly \( \text{Ca}^{2+} \)) and sequestering toxic heavy metals.
1.1.2 Structure

In terms of structure, bone tissue can be classified and considered in a number of ways, which will be outlined here, starting from a macroscopic level before looking more closely at the microstructure, sub-microstructure and nanostructure of bone tissue (see Fig. 1). Bone can be defined as either cortical or cancellous, which have the same composition but differ in their structure and function.

80% of bone by mass is cortical bone, which reflects the fact that it has a low porosity (5% by volume) and hence is mechanically strong, providing the bulk of the resistance to compression and bending forces. This corresponds to the low cellular infiltration and metabolic activity for cortical bone. The interior of bone mass is cancellous bone, which is highly porous (50-90% by volume), has a much higher surface area and is much more metabolically active. These differences are obvious on visual inspection of bone cross-sections and when considering their mechanical properties, as can be seen in Table 1.

<table>
<thead>
<tr>
<th></th>
<th>Compressive Strength (MPa)</th>
<th>Young’s Modulus (GPa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cortical bone</td>
<td>131-224</td>
<td>17-20</td>
</tr>
<tr>
<td>Cancellous bone</td>
<td>5-10</td>
<td>0.05-0.1</td>
</tr>
</tbody>
</table>

On a microscopic level, bone tissue is formed into osteons, which differ for the different types of bone. Cortical osteons are cylindrical, with an approximate diameter of 200 μm and length of 400 μm and run parallel with the long axis of the bone. They have a central lacuna (the Haversian Canal, containing blood vessels and nerve fibres) wrapped in up to 20 concentric layers of bone material. The osteons are sheathed and connected by an organic matrix known as ‘cement lines’. Cancellous osteons appear to be less organised, with sinusoids allowing direct contact between the lamellae and bone marrow, rather than a central cavity.

On a sub-microscopic level are the lamellae which can be woven (primary) or lamellar (secondary). In woven bone the organic matrix is randomly orientated within the plane, while in lamellar bone the matrix forms layers which run perpendicular to each other and hence have greater strength.

On the nanoscale bone again has two components: the extracellular matrix (ECM) formed primarily of type I collagen, and a mineral component of hydroxyapatite. Collagen is a natural polymer that forms fibrils which are organised into an array, leaving holes that can be filled by
nano-platelets of hydroxyapatite crystals. Bone is a composite material with the hydroxyapatite providing hardness and strength, while the collagen provides toughness and a degree of flexibility.

Figure 1. An overview of the substructure of bone tissue. Adapted from Rho et al.⁹

As can be seen from Fig. 2, the biology of bone is complex, and not fully understood, but a basic understanding is included here. Two main cell lines are present and required for the normal function and remodelling of bone tissue: osteoprogenitor cells and osteoclasts.⁶

Figure 2. A schematic of bone remodelling, showing the complexity of the system and highlighting important cells and signalling molecules. Adapted from Tang et al.¹⁰
Osteoprogenitor cells are responsible for the formation and maintenance of bone and sit in vacancies in the interior of the bone until they receive signals to migrate, proliferate and differentiate into osteocytes or osteoblasts. Osteoblasts sit on the surface of the bone producing the ECM and promoting its mineralisation. Once the bone is formed they remain on the bone surface, reverting to osteoprogenitor cells or maturing into osteocytes, which sit within the bone tissue itself, maintaining the ECM and secreting signalling molecules.\textsuperscript{6,10}

Osteocytes perform the opposite function and break down bone tissue. On receiving a signal they bind to the surface of the bone, forming a pocket where they lower the local pH in order to dissolve the hydroxyapatite and secrete enzymes to digest the exposed ECM.

These cell types work in tandem, constantly breaking down and rebuilding bone in a process known as remodelling. This allows bone to respond to changing demands (e.g. higher density in response to greater stress), prevents microfractures from propagating and aids calcium homeostasis.

1.1.3 Bone Healing

![Figure 3. Bone at various stages of regeneration. Adapted from Schmidt-Bleek K. et al.\textsuperscript{11}](image)

In addition to normal remodelling, bone cells need to respond to trauma (i.e. fractures) to repair the damage, without leaving scar tissue which would compromise the strength of the bone.\textsuperscript{11}

There are 4 phases to normal bone healing (see Fig. 3), outlined below:

1. Initial response: a haematoma forms at the site of the injury, the blood clot allows cells to migrate and proliferate and is a source of signalling molecules.\textsuperscript{12}

2. Soft callus formation: chondrocytes produce cartilage and form the soft callus which secures the site of the fracture. Blood vessels infiltrate the callus to provide nutrients and enable the recruitment of more cells.
3. Hard callus formation: osteoblasts gradually ossify the cartilage into woven bone which affixes the fracture site more securely.  
4. Remodelling: the woven bone is gradually remodelled over a period of 3-5 years into normal bone as described previously, completing the healing process with the bone being restored to its original strength and condition.

1.1.4 Problems in Bone Healing

The process of bone healing can be perturbed by a number of factors such as disease, unstable fixation or critical size defects. In many diseases the complex metabolism of bone remodelling is disturbed, which can result in a loss of bone density, decreased mineralisation or a loss of extracellular matrix. These conditions can also be localised or present throughout the body; they can be congenital, or be influenced by environmental factors such as dietary deficiencies or infection. An overview of common bone diseases and their effects is presented in Table 2.

<table>
<thead>
<tr>
<th>Disease</th>
<th>Effects on bone tissue</th>
</tr>
</thead>
<tbody>
<tr>
<td>Osteoporosis</td>
<td>An imbalance between bone resorption and formation leading to increased bone porosity and a corresponding loss of bone strength. Systemic effect.</td>
</tr>
<tr>
<td>Paget’s disease</td>
<td>Excessive breakdown and remodelling of bone tissue, leading to misshapen and weakened bones. Generally localised.</td>
</tr>
<tr>
<td>Osteonecrosis</td>
<td>Cellular death, followed by bone degradation which is often caused by a loss of blood supply to the affected area. Can also be a side effect of bisphosphonate medication. Localised and tends to occur more at joints.</td>
</tr>
<tr>
<td>Rickets</td>
<td>A deficiency of vitamin D weakens bones, increases the likelihood of fractures and can cause deformity of long bones.</td>
</tr>
<tr>
<td>Skeletal fluorosis</td>
<td>Excessive levels of fluoride in the body lead to the formation of CaF$_2$ in bone tissue, increasing its hardness and making it brittle.</td>
</tr>
<tr>
<td>Osteosarcoma</td>
<td>The most common type of malignant bone cancer usually treated with radio/chemotherapy followed by surgery to remove the affected bone.</td>
</tr>
</tbody>
</table>

Even healthy individuals can have problems with bone healing if they have a severe break, resulting in a non-union fracture. It is estimated that 5-10% of fractures fall into this category and will not heal well without medical intervention. Many of these are due to unstable fixation, where the two ends of the bone have a large degree of movement between them, this disrupts the formation of the soft callus, preventing the bone from healing. Unstable fixation is usually treated by holding the bones in place with bone screws and plates, which can be made from a variety of materials.
More complex are cases of critical size defects, where the gap between the ends of the broken bone is large enough to prevent healing altogether: instead each end of the bone remodels. In these cases it is necessary not only to fix the bones in place, but to promote the healing of the bones. One of the best established techniques for achieving this is Ilizarov surgery, where the bones are held in place by an external frame and are gradually moved apart as they heal towards each other.25

An alternative approach is to fill the gap with a material that will promote healing of the bone (i.e. an osteoconductive material). Originally such approaches used grafts of bone tissue either from other bones from the patient (autografting), from tissue donors (allografting) or from other species (xenografting). Each of these broad classes of bone graft materials come with their own advantages and disadvantages, summarised in Table 3, and can take a variety of forms.

Autografts are often considered to be the gold standard for bone grafts as they are the best at promoting bone healing and there are no issues with compatibility or rejection.26 However, there is a risk of donor site morbidity and complications, as well as a limited supply.27 The graft material is typically harvested from intraoral sites (e.g. the chin) or extraoral sites (e.g. the iliac crest) and includes the periosteum in order to ensure a good blood supply to the graft.28,29

Allografts are typically harvested from cadavers, thereby avoiding donor site morbidity, and processed into a usable material by a number of steps, often including freeze-drying. However, they tend to be less efficacious than autografts, can elicit an immune response from the patient and have a small risk of disease transmission to the patient.28,29

Xenografts are often derived from porcine, bovine or coral sources and have a potentially unlimited supply.26 Xenografts can elicit a severe immune response,29 and so have to be heavily processed to remove antigenic moieties. These processing steps result in xenografts having diminished osteoconductivity compared to autografts and allografts.26,29

<table>
<thead>
<tr>
<th>Table 3. A summary of the properties of bone grafts.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Autograft</td>
</tr>
<tr>
<td>Osteoconductive</td>
</tr>
<tr>
<td>Osteoinductive</td>
</tr>
<tr>
<td>Osteogenic</td>
</tr>
<tr>
<td>Compatible</td>
</tr>
<tr>
<td>Donor morbidity</td>
</tr>
<tr>
<td>Availability of supply</td>
</tr>
</tbody>
</table>
1.1.5 Summary
To conclude, bone tissue is a complex, composite tissue that is capable of self-repair under normal conditions. However, in a number of disease states and in extreme trauma this ability is impaired and intervention is required. This intervention often takes the form of the insertion of a bone graft to encourage healing across the break. A number of options exist for bone graft materials, all of which have strengths and weaknesses, and there is a clinical need to develop novel bone graft materials with improved qualities.

1.2 Tissue Engineering
Tissue engineering is an interdisciplinary area of science and engineering, taking contributions from medical and life sciences, materials science and chemistry, with the aim of producing materials capable of repairing, or replacing, damaged tissues and organs.\textsuperscript{30} There is a huge variety of approaches for bone tissue engineering, but generally they are either scaffolds, which are highly porous solids fabricated from metals, ceramics or polymers; or matrices, which are often soft hydrogels. Additionally, they can be made of synthetic or biologically-derived materials and can include biological entities, such as proteins, or even whole cells.\textsuperscript{10, 26, 31} This work concerns bone tissue scaffolds and so will focus on their properties, rather than those of other tissue scaffolds or matrices.

1.2.1 Typical Properties of Bone Tissue Scaffolds
For a bone tissue scaffold to promote effective healing, it needs to exhibit the following properties:\textsuperscript{32, 33}

- Biocompatibility – be non-toxic and non-allergenic.
- Porosity – to enable diffusion of nutrients and waste products.
- Osteoconductivity – to allow bone tissue to grow into/onto the scaffold.
- Osseointegration – to bind and integrate into existing bone tissue.

These features are necessary for a successful bone tissue scaffold. The scaffold cannot be harmful to cells as that would prevent successful tissue engineering; it needs to be porous to enable cells to enter the scaffold and for blood vessels to infiltrate; and it needs to encourage the ingrowth of bone so that the break heals. Additionally, it is desirable for the scaffold to have the following properties:

- Osteoinductivity – to recruit immature cells and promote differentiation into osteoblasts.
- Biodegradability – the scaffold breaks down gradually under physiological conditions to produce harmless by-products.
- Mechanical strength – capable of performing the load-bearing function of the bone while it heals.

These features should be present in an ideal tissue scaffold. An osteoinductive scaffold would allow for faster healing over a scaffold that is only osteoconductive as there would be more cells to produce the ECM and undertake mineralisation. A scaffold that degrades at the same rate as the bone heals would mean that, once the healing is complete, the tissue would be restored entirely to its original condition. If the scaffold’s strength is matched well to the bone then the patient can continue normal activities in an uninhibited manner whilst the bone heals.

1.2.2 Materials for Bone Tissue Scaffolds

A wide variety of materials have been used for bone tissue scaffolds and a brief overview will be given here, and summarised in Table 4; the simplest way to categorise them is firstly by whether they are natural or synthetic, and then into inorganic or organic.

<table>
<thead>
<tr>
<th>Table 4. Categories of materials for tissue engineering with some examples.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Natural</strong></td>
</tr>
</tbody>
</table>
| Organic | Silk $^{36}$  
 Chitosan $^{37}$  
 Collagen $^{38}$  | Polycaprolactone $^{3, 39}$  
 Polylactic acid $^{40}$ |
| Inorganic | Calcium triphosphate $^{41}$  | Bioactive glasses $^{42}$  
 Hydroxyapatite  
 Metals |

The category of natural materials includes any material that is found in nature. These materials are usually harvested from a natural source and processed; chitosan for example is sourced from the chitin of shellfish. These natural materials are usually non-toxic to cells and promote good surface adhesion. However, they can elicit immunological responses and their mechanical properties tend to be insufficient. To an extent these problems can be overcome by careful processing of the materials to remove antigenic components and by crosslinking, respectively.

Any material that is not present in nature is considered to be a synthetic biomaterial, though many mimic aspects of natural materials to try to gain the associated advantages, such as good cell adhesion. This presents a much larger toolbox of materials, each with their own advantages and disadvantages. In addition to the properties of each material it is common to use composite
materials, often pairing an organic, biodegradable polymer with an inorganic, bioactive ceramic in a way reminiscent of bone tissue.\textsuperscript{41, 42}

### 1.2.3 Biological Adjuncts for Bone Tissue Scaffolds

In addition to the material, biological adjuncts are also often used to enhance the bioactivity of the scaffold. A large variety exists including proteins, carbohydrates, RNA and stem cells. Perhaps the best known and most widely used of these adjuncts are the bone morphogenic proteins (BMPs), a family of proteins that are known to act as signalling molecules. They encourage indigenous mesenchymal stem cells to differentiate into osteoblasts,\textsuperscript{36, 45, 46} and are a licensed treatment for delayed union and non-union of long bone fractures in their own right.\textsuperscript{47} There exists a range of strategies for deploying these entities, such as inclusion in the bulk material, core-shell particles, conjugation to polymers and coatings.\textsuperscript{48}

### 1.2.4 Methods of Scaffold Fabrication

In addition to the choice of materials, how a scaffold is produced is also a significant factor. Porosity, on multiple length scales, is extremely important for tissue scaffolds to allow the diffusion of nutrients, signalling molecules and waste products; for the migration of cells into the scaffold; and to allow vascularization and the growth of new tissue.\textsuperscript{34, 36} However, very few of the materials used are intrinsically porous; therefore the porosity needs to be introduced during the fabrication step. A large number of techniques have been proposed and developed for this function, a brief overview will be provided here.

![Solid freeform fabrication (SFF)](image)

Solid freeform fabrication (SFF) is a family of techniques for producing 3D scaffolds with features down to micron scale depending on the method used.\textsuperscript{10, 51} There are a variety of methods, and an equally large number of materials to which they can be applied, including ceramics, polymers and metals; in addition it is possible to include biological entities in some methods, an overview of
some SFF techniques can be seen in Table 5 and an example of 3D printing in Fig. 4. SFF methods generally produce scaffolds through layer-by-layer deposition, polymerisation, sintering or binding of materials. What sets SFF methods apart is the degree of control that is afforded over the structure of the tissue scaffold, the appropriate size, shape and porosity can all be included in the design of the biomaterial. Furthermore, using SFF is it possible to use computer-aided design and manufacturing, in combination with scanning technologies such as MRI, to tailor tissue scaffolds for individual patients (see Fig. 4).

Other methods of inducing porosity can achieve smaller pore sizes than SFF, but it is harder to create scaffolds of the desired shape and size on a macroscopic scale.

Perhaps the simplest method of inducing porosity in scaffolds is solvent casting with particulate leaching. The material, typically a polymer, is dissolved in a suitable organic solvent and an insoluble solid, a porogen, is added. The mixture is poured into a mould and the solvent is allowed to evaporate, leaving a solid polymer embedded with the porogen, which can be leached out by the application of water, leaving a porous solid. This method is simple and suitable for a wide range of polymers, but the thickness of the scaffolds made is this way is limited to a few millimetres; any thicker and the porogen cannot be leached out completely.33

Another method of inducing porosity is the freeze-drying of polymer emulsions; which have the advantage of not using porogens, and hence can form thicker structures. Briefly, the polymer is dissolved in a suitable solvent and an anti-solvent is then added, typically water; the emulsion is frozen in liquid nitrogen to preserve the microstructure and the solvent removed by freeze-drying to leave a porous 3D architecture. A similar method, thermally induced phase separation, uses a poor solvent to achieve a similar effect.42 Rapid cooling of the solution induces a phase separation into a polymer-rich solid phase and a polymer-poor liquid phase, which is removed by freeze-drying.

Rather than forming a solid with voids, an alternative to making a porous scaffold is to make a 3D network of fibres. The simplest way to produce such fibres is wet casting, where a polymer solution is extruded into a coagulation bath of non-solvent; a filament of polymer and solvent forms and solidifies as the solvent diffuses out into the non-solvent bath.33 A non-woven mesh is created as the continuous polymer filament is deposited at the bottom of the vessel. Fibres produced in this manner can be as fine as a few micrometres in diameter.
Table 5. An outline of some basic SFF techniques and their properties.

<table>
<thead>
<tr>
<th>Method</th>
<th>How it works</th>
<th>Suitable Materials</th>
<th>Feature size (μm)</th>
<th>Inclusion of Biological moieties</th>
<th>Inclusion of Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>3D Printing</td>
<td>A layer of powder is spread onto a piston and cohered by a binder delivered by an inkjet printer. The stage is lowered and the process repeated until the 3D structure is complete.</td>
<td>Polymers, Ceramics</td>
<td>100</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Fused deposition modelling</td>
<td>Molten polymer is extruded from a print head and solidifies on cooling. 3D structure built up layer by layer.</td>
<td>Thermoplastic polymers. Ceramics can be included as composites</td>
<td>100</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>Stereolithography</td>
<td>The surface of a bath of polymer is selectively polymerised by the application of light. The polymerised layer is moved away from the surface and the 3D structure is built up layer by layer.</td>
<td>Polymers that can be photopolymerised</td>
<td>1</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Selective laser sintering</td>
<td>A similar process to 3D printing, but uses a laser to fuse the powder by melting.</td>
<td>Polymers, Ceramics, Metals</td>
<td>100</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>3D plotting</td>
<td>A viscous liquid is extruded into a liquid medium of equal density. The extruded liquid solidifies through cooling or by crosslinking.</td>
<td>Polymers, Ceramics</td>
<td>100</td>
<td>Yes</td>
<td>Yes</td>
</tr>
</tbody>
</table>
1.2.5 Electrospinning

An alternative method to producing non-woven fibre networks is electrospinning, which can form fibres of smaller diameter than wet spinning and will be explained here in greater detail as it is the fabrication method used in this project. The simplest way to consider electrospinning is as an extension of the principles of electrospray technologies (see Fig. 5), which are commonly employed as methods of ionisation for mass spectroscopy (MS) methodologies.\(^5^3\)

![Figure 5. Schematic of an electrospray ionisation system. Adapted from Cech et al.\(^5^4\)](image)

In electrospray, the solution to be sprayed is passed along a very fine needle connected to a high voltage source, a Taylor cone with an excess of charge builds up at the tip of the needle. Charged droplets are formed from the tip of the Taylor cone, which are moved away from the needle by the electric field. As these droplets travel towards the plate the solvent evaporates, reducing the size of the droplets. Within the droplets, there is competition between repulsion of the charges and the surface tension. As the droplet decreases in size, the repulsion begins to dominate; from here there are two proposed mechanisms. Coulombic fission assumes that the droplet divides into smaller droplets, until each droplet holds only a single ion. Ion evaporation conversely assumes that ions are ejected directly from the droplet to reduce the charge density.\(^5^4\)

The theory is similar for electrospinning polymer solutions. Consider the situation above, but with a solution of much higher viscosity and surface tension. Rather than droplets being emitted from the Taylor cone, a stable stream of polymer solution is jetted from the tip towards a grounded plate for a short distance. As the solvent evaporates the charge density increases, but the jet is unable to separate into droplets due to its high surface tension, resulting in the jet ‘whipping’ randomly and stretching out (see Fig. 6). The solvent continues to evaporate until a polymeric filament remains, which is collected onto the grounded plate where the charge is earthed.\(^5^5-5^7\)
The ‘whipping’ motion of the jet is referred to as bending instability and stretches the polymer fibre, reducing the final diameter to as small as 50 nm.

The apparatus required for electrospinning, at its most basic, consists of a syringe pump, a syringe and needle, a high voltage source and a grounded collector (see Fig. 6). However, for such a simple technique there is a wide variety of parameters to control and optimise, as described in Table 6. Fundamentally, these can be described as solution, process or atmospheric conditions. These variables must be kept within certain bounds for the electrospinning to be successful, which will vary from system to system, but within these limits the variables can be changed to alter the diameter and morphology of the polymer nanofibres. The most common parameters to be varied are the concentration, flow rate and voltage as they are the easiest to control and generally have the greatest effect on the nanofibres.

More complex systems can be used to achieve control of further parameters, resulting in finer control of fibre diameters and more interesting morphologies or arrangements. For example, the electrospinning apparatus can be placed inside temperature- and humidity-controlled boxes to control these parameters. Another factor that can be controlled is the collector geometry; collectors are usually stationary, flat sheets resulting in randomly aligned nanofibres. For some applications it is desirable to have aligned nanofibres, these can be created by using a rotating drum collector. If the drum rotates at very high speed the fibres are aligned around the circumference of the drum.
Table 6. Some key parameters for electrospinning and their effects on the morphology and diameter of the resulting nanofibres.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Effect on Nanofibre</th>
</tr>
</thead>
<tbody>
<tr>
<td>Concentration</td>
<td>Increasing the concentration of the polymer solution increases the viscosity, this leads to an increase in the fibre diameter and a reduction in beading.</td>
</tr>
<tr>
<td>Flow rate</td>
<td>Increasing the voltage increases the volume of material in the jet and hence increases the diameter of the fibres, but if it is too high it results in beading.</td>
</tr>
<tr>
<td>Voltage</td>
<td>If the voltage is too high, beading is observed. The relationship between the voltage and fibre diameter seems to be a minor effect.</td>
</tr>
<tr>
<td>Distance between needle and collector</td>
<td>If too far, or too close, then the fibre will be beaded. If too close then the fibre will still be wet when it hits the collector, resulting in ribbons and spreading.</td>
</tr>
<tr>
<td>Conductivity</td>
<td>Typically altered by adding salts or ionic surfactants, reduces the incidence of beading and generally decreases fibre diameter (though there are exceptions).</td>
</tr>
<tr>
<td>Humidity</td>
<td>High humidity can cause circular pores to appear on the surface of the fibres in certain conditions.</td>
</tr>
<tr>
<td>Temperature</td>
<td>Increasing the temperature decreases the viscosity of the solution, decreasing the fibre size; it can also lower the polydispersity of the nanofibre diameters.</td>
</tr>
</tbody>
</table>

A large number of polymers have been processed into nanofibres by electrospinning, as outlined in Table 7. Blends of polymers have also been spun together to make composites. Additionally, other materials have been included into the nanofibres such as proteins, carbon nanotubes and hydroxyapatite, which is of particular relevance for bone tissue scaffolds.59, 60

Table 7. Summary of polymers that have been successfully electrospun and the conditions used.59, 61

<table>
<thead>
<tr>
<th>Polymer</th>
<th>Solvents used</th>
<th>Typical concentrations used</th>
</tr>
</thead>
<tbody>
<tr>
<td>Polystyrene</td>
<td>THF, DMF, Chloroform</td>
<td>18-35 wt%</td>
</tr>
<tr>
<td>Polyacrylonitrile</td>
<td>DMF</td>
<td>15 wt%</td>
</tr>
<tr>
<td>Nylon</td>
<td>Formic acid</td>
<td>10 wt%</td>
</tr>
<tr>
<td>Polyaniline52</td>
<td>Chloroform/DMF</td>
<td>0.5-2 wt%</td>
</tr>
<tr>
<td>Poly(acrylic acid)53</td>
<td>Water/DMF</td>
<td>4-10 wt%</td>
</tr>
<tr>
<td>Collagen</td>
<td>Hexafluoro-2-propanol</td>
<td>8 wt%</td>
</tr>
<tr>
<td>Polycaprolactone64</td>
<td>Chloroform, toluene, DCM</td>
<td>10 wt%</td>
</tr>
<tr>
<td>Polyvinyl alcohol</td>
<td>Water</td>
<td>1-16 wt%</td>
</tr>
<tr>
<td>Polylactic acid</td>
<td>DMF, DCM</td>
<td>5-14 wt%</td>
</tr>
</tbody>
</table>
Electrospun nanofibre structures have a high surface area, high degrees of porosity and pore interconnectedness, high aspect ratio and tend to have high tensile strength. These properties make them suitable not only for tissue scaffolds, but for other applications as well. For example, catalyst supports, wound dressings, filtration and fabric applications. Accordingly, a number of scale-up methodologies are being developed in anticipation of the demands of these industries.  

1.3 Polymers of Interest

In the course of this project two polymers were of particular interest, namely PCL and PVPA. Accordingly, they will be described in detail in this section.

1.3.1 Poly(ε-caprolactone)

PCL is a synthetic polyester that has a number of qualities that make it suitable for tissue engineering applications. It is hydrophobic, semi-crystalline, has a low melting point (59-64°C) and is soluble in a wide range of organic solvents, making it suitable for many fabrication technologies; additionally, it is easy to blend with other hydrophobic polymers, which means that degradation kinetics can be tailored to an extent. PCL is commercially available and is easily synthesised, the most common synthetic route is the ring opening polymerisation (ROP) of ε-caprolactone (see Fig. 7); a number of initiators and catalysts are available for this reaction.

![Figure 7. Synthesis of PCL by ROP of ε-caprolactone.](image)

PCL fulfils the most important characteristics of tissue scaffolds in that it is non-toxic and completely biodegradable. Furthermore, it degrades over the course of years rather than weeks or months, as for polyglycolide or poly(D, L-lactide), which is a much better fit for the timescales of bone regeneration. Polyesters are degraded by hydrolysis, forming the alcohol and carboxylic acid; this shortens the chain length until water-soluble oligomers are formed, which diffuse away from the scaffold. As shown in Fig. 8, there are 3 modes for the degradation of polymers: surface, bulk and auto-catalytic.
Extremely hydrophobic polyesters undergo surface degradation. Water can only penetrate the surface layers of the polymers, so these are degraded first while those in the centre are unaffected until they are exposed. In bulk degradation the polymer chains are broken throughout the structure, leading to uniform loss of molecular weight. The structure maintains its shape, but gradually weakens until it falls apart. The final model is bulk degradation with autocatalysis, as the polymer chains are broken, acidic moieties are produced and remain trapped in the scaffold, lowering the pH and accelerating the degradation. This leads to the structure losing molecular weight and mass faster in the centre than on the surface, effectively hollowing out the structure.\textsuperscript{66}

Which of these mechanisms occurs depends on the relative rates of water diffusing into the polymer, and hydrolysis products diffusing out of the polymer. If reaction products leave faster than water can penetrate the polymer matrix, then surface degradation is observed. Conversely, if water enters faster than diffusion products can leave, then autocatalysis can occur. If the rates are comparable, then bulk degradation is the result.\textsuperscript{66}

Another consideration is the action of enzymes on the polymer; esterases (enzymes that hydrolyse ester bonds) can in principle accelerate the breakdown of polyesters. However, no such acceleration has been observed for PCL either \textit{in vitro} with isolated enzymes in buffer, or \textit{in vivo} in whole animal studies,\textsuperscript{68} indicating that the degradation of high molecular weight PCL is driven purely by hydrolysis.

PCL is thought to be degraded in two steps; initially PCL undergoes bulk degradation.\textsuperscript{69} This idea is supported by the fact that the molecular weight of the PCL decreases linearly with time, but without any mass loss; this indicates polymer chains randomly breaking into smaller chains, rather than oligomeric units that can be lost from the surface. Additionally, altering the shape of the polymeric scaffold had no effect on the degradation rate, ruling out a surface degradation

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\textbf{Figure 8.} Models for the degradation of scaffolds: a) surface degradation; b) bulk degradation; c) bulk degradation with autocatalysis.\textsuperscript{66}
mechanism. Once a sufficiently low molecular weight polymer has been reached (below 3,000 g mol\(^{-1}\)) a second stage begins, with cells taking up the polymer by endocytosis and degrading the polymer by an intracellular mechanism.

Another factor to be considered is the ultimate fate of the products of this degradation: are they retained in the body, or metabolised and excreted? PCL has been shown to be highly bioresorbable, i.e. almost all the by-products are excreted from the body. This was proven using radiolabelled PCL injected into rats; after 135 days, 92% of the radioactive tracer was recovered and the radioactivity of the animal tissues had returned to background levels, demonstrating that nearly all of the PCL metabolites are excreted from the body with no bioaccumulation.

There are 3 synthetic pathways to PCL, all from different starting materials: step polymerisation, ROP and free radical ROP.

![Figure 9. Synthesis of PCL by the condensation of 6-hydroxycaproic acid. The reaction is an equilibrium driven by the removal of water from the reaction vessel.](image)

Step polymerisation of 6-hydroxycaproic acid (see Fig. 9) is often performed at high temperatures and/or low pressures to remove the water that is produced. Enzymes, such as lipases, have also been used to catalyse this reaction. This method is rarely used as the resulting polymers tend to have low molar masses and high polydispersities.

The most reliable, and widely used, method for the synthesis of PCL is the ROP of ε-caprolactone. Many catalysts and initiators are available, and the mechanisms that they use can be classed as anionic, cationic, monomer-activated or coordination-insertion. This methodology is the most developed and can be used to obtain polymers of higher molecular weight and lower polydispersity.

One of the most common systems for ROP uses stannous (II) 2-ethylhexanoate (often called tin octanoate or Sn(Oct)\(_2\)) as the catalyst and a range of nucleophiles (typically alcohols) as initiators (see Fig. 10). The polymerisation proceeds via the coordination insertion mechanism and works well, yielding polymers of high molecular weight. Additionally, Sn(Oct)\(_2\) is commercially available, easy to handle and compatible with a wide range of initiators and reaction conditions. However, the polymerisation requires high temperatures (around 100°C) which can lead to high polydispersities by the promotion of intramolecular and intermolecular transesterification.
Figure 10. a) Overall reaction scheme for ROP using Sn(Oct)$_2$; b) formation of the active species, this equilibrium persists throughout the reaction. Therefore increasing the concentration of the alcohol increases the rate of polymerisation, while increasing the concentration of carboxylic acids decreases the rate; c) the propagating chain.\(^\text{73}\)

ROP of εCL with Sn(Oct)$_2$ proceeds by a coordination-insertion mechanism (see Fig. 11); the monomer coordinates to the active species via the carbonyl oxygen, prompting the coordinated alkoxide to attack the carbonyl carbon. The ester carbonyl then coordinates to the tin (II) centre, the carbonyl reforms and the ring opens, reforming the active species.\(^\text{73}\)

Figure 11. Coordination-insertion of εCL into the stannous-alkoxide bond, resulting in the ROP of the lactone ring.\(^\text{73}\)
The third method of synthesising PCL is little used and is a bit more unusual: free radical ROP of 2-methylene-1,3-dioxepene (MDO). A radical adds to the end of the alkene forming a tertiary carbon radical, this rearranges and undergoes ring opening before propagating (see Fig. 12). The tertiary carbon can also propagate, resulting in a polymer with an alkyl backbone with pendant acetals. Interestingly, the ratio of propagation from the tertiary radical versus ring opening depends on the size of the ring and the associated strain. Seven-membered rings undergo 100% ring opening, while six-membered rings undergo only 85% ring opening. Additionally, this approach has been used to make copolymers with vinyl acetate, vinylphosphonic acid (VPA) and dimethyl vinylphosphonate (DMVP).

1.3.2 Poly(vinylphosphonic acid)

Phosphonic acid containing polymers are polyelectrolytes of intermediate strength and have found applications as corrosion resistant coatings for metals, surfactants and dental materials and are of growing interest to groups researching conductive membranes for fuel cells, waste water remediation and wound healing applications. PVPA is the simplest of these acids, bearing a phosphonic acid directly on the polymer backbone, and forms part of the key bioactive copolymer under investigation in this work. PVPA is formed either by direct polymerisation of VPA or by polymerisation of a vinylphosphonate ester followed by hydrolysis (see Fig. 13).

Direct polymerisation is the most straightforward approach and the most successful method reported in the literature is that of Bingöl et al (see Fig. 14). In their method, vinylphosphonic acid (VPA) and 2,2'-azobis(2-methylpropionamidine) dihydrochloride (AAPH), a water-soluble azo-
type initiator, are dissolved in a small quantity of water and heated to 80 °C under an atmosphere of argon for 3 hours. After purification, this resulted in PVPA with a reported $M_w$ of 62,000 determined by static light scattering.

Bingöl et al. characterised the PVPA by $^1$H and $^{13}$C NMR, which indicated that the polymer was highly regio-irregular. The PVPA was found to be atactic and contained a large number of head-to-head (H-H) and tail-to-tail (T-T) linkages in the polymer chain. Usually free radical polymerisations result almost exclusively in head-to-tail (H-T) linkages. This implies that the polymerisation mechanism for VPA is highly unusual; Bingöl et al. suggested that the VPA forms anhydrides during the course of the reaction (see Fig. 15), these anhydrides can be polymerized in sequence via a 5- or 6-membered transition state, yielding H-H and H-T linkages respectively. Bingöl et al. also demonstrated that, while VPA is a diprotic acid with $pK_a$ s of 2.74 and 7.34, the phosphonic acids in the PVPA act as monoprotic acids. It is suggested that, due to the close proximity of the phosphonic acid groups, the charge density is too high to allow dissociation of the second proton in PVPA.

Another study by Voit and co-workers involved the synthesis of PVPA via both the acid and ester, DMVP in this case, and examination by NMR. PVPA synthesised directly from the acid was
atactic and contained 17% H-H/T-T linkages; PVPA from the ester however, had only 8% H-H/T-T linkages and was predominantly isotactic. Irregularities occurred at a higher rate for the acid route, but were still present for the ester. This implies that, though the cyclopolymerisation mechanism is likely to be having an effect, it is not the sole cause of the H-H/T-T linkages because the ester, which cannot form cyclic anhydrides, still exhibits regio-irregularities.

Though VPA is speculated to form anhydrides spontaneously under the polymerisation conditions, there is no direct spectroscopic evidence for this. The anhydrides can however be formed deliberately by including dehydrating agents such as acetic anhydride in the reaction mixture (see Fig. 16). In this way a copolymerisation can be performed using VPA, VPA anhydride and VPA dianhydride. These conditions yielded PVPA of higher molecular weight with greater monomer conversion compared to reactions performed in ethyl acetate. Furthermore, NMR analysis indicated that PVPA derived from VPA anhydride-rich reactions possessed a greater degree of region-irregularities.

The same research team also found, through $^{31}$P NMR, that anhydrides persist in the PVPA chains for long time periods even in aqueous solution. Examination of PVPA membranes with solid state NMR has found that inter and intramolecular anhydrides form when PVPA is dried under high temperature and low pressure.

PVPA is insoluble in most organic solvents, meaning that polymerisation of VPA has to take place in water, ethanol or methanol to prevent precipitation from limiting the molecular weight of the polymer. This restricts the initiators, and comonomers, that can be used in the polymerisation. Polymers of vinylphosphonate esters, however, are much more soluble in other solvents, allowing for much greater flexibility in reaction conditions. PVPA formed by the free radical polymerisation of vinyl phosphonate esters tend to have a more regular microstructure and tacticity compared to PVPA from the acid route; additionally, they usually have a lower molecular weight. This is because vinylphosphonate esters are vulnerable to intramolecular chain transfer to the alkoxy group, resulting in esters being incorporated into the polymer backbone (see Fig. 17). These are
cleaved during the hydrolysis of the ester groups to reveal the PVPA, resulting in shorter polymer chains.\textsuperscript{89}

![Figure 17. Mechanism for the chain transfer to the alkoxy group, resulting in esters in the polymer backbone.\textsuperscript{89}](image)

An alternative to free radical polymerisation, that is not available to VPA due to its labile protons, is anionic polymerisation. Using sec-BuLi and diphenylethylene (DPE) as an initiator system in anhydrous conditions at low temperatures diisopropyl vinylphosphonate (DIVP) was successfully polymerized to PDIVP, which was in turn hydrolysed to PVPA with molecular weights up to 800,000 g mol\textsuperscript{-1}.\textsuperscript{90} Interestingly, the degree of regioirregularity is much lower than for polymers made by free radical chemistry, and they are predominantly isotactic. Furthermore, anionic polymerisation was used to make polystyrene-b-PVPA block copolymers from a polystyrene macroinitiator, showing that more complex architectures are possible using anionic polymerisation.

The final step is to convert the phosphonate esters into phosphonic acids via hydrolysis; this reaction is usually performed at low pH and elevated temperatures.\textsuperscript{91} However, these conditions are non-selective and will hydrolyse all esters that are present. A more selective approach is to convert the phosphonate ester to the trimethylsilyl phosphonate with trimethylsilyl bromide (TMSBr) under anhydrous conditions (see Fig. 18). The silyl ester hydrolyses under the action of water, yielding the phosphonic acid. Furthermore, this has been shown to be selective for phosphonate esters over carboxylate esters, though care needs to be taken while concentrating the samples.\textsuperscript{92, 93}
As has been mentioned, phosphonic acid-containing polymers have been investigated for a variety of applications; of particular interest for this work is their use in dental or bone tissue applications. They contain a high concentration of phosphonate groups and it is thought that they can encourage the deposition of bone mineral, hydroxyapatite, through biomimicry.

Collagen cannot mineralise spontaneously; non-matrix phosphoproteins are required to begin this process by sequestering CaPO$_4$ into an amorphous mineral phase. Experiments have shown that demineralised collagen grids do not remineralise, while those incubated with a mixture of PAA and PVPA solutions rapidly underwent intra and extra-fibrillar mineralisation.

As well as promoting the formation of bone mineral, PVPA has been found to inhibit the degradation of ECM by matrix metalloproteins (MMPs). Demineralised dentin beams were coated in PVPA, which was immobilised by crosslinking the polymer with 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride. Beams treated in this way had slower loss of Young’s modulus and mass, with MMP inhibition comparable to that of commercial inhibitors. This inhibition is thought to be due to PVPA chelating to the metal centre of MMPs (usually zinc or calcium ions), thereby preventing their action.

1.3.3 Summary

PCL is a well-known biodegradable polyester with a range of properties that make it of great interest for biomaterials applications; it is usually synthesised by ROP and has a range of catalysts and initiators available for this purpose.

PVPA conversely is a relatively little known polymer that has a variety of uses, most intriguingly as a biomaterial. It can be synthesised from the acid by free radical polymerisation or from the ester by free radical or anionic polymerisations. Previous work has found that PVPA can aid the mineralisation and retention of collagen fibres, indicating that it is suitable for bone tissue engineering applications.
1.4 Analytical Techniques

In order to make rational, informed decisions about which polymers to use it is necessary to analyse them and define their relevant qualities. A variety of analytical techniques have been used and an overview of the key methods will be given below.

1.4.1 Nuclear Magnetic Resonance

NMR is a very powerful spectroscopic technique, which can give information on the environment, bonding, conformation and relative abundance of nuclei in a sample.

Spin ½ nuclei (such as $^1$H, $^{19}$F and $^{31}$P) can have two orientations. Ordinarily there is no energetic difference between these two states, but in a magnetic field one state lies parallel to the magnetic field, while the other is antiparallel. The parallel spin is lower in energy and hence has a higher population than the antiparallel. If radiation at an appropriate frequency (and hence energy) is introduced, nuclei will transition from the parallel to the antiparallel state and an absorption will be observed at that frequency. The energy difference between the spin states is related to the magnetogyric ratio of the nuclei in question and the degree of shielding. Electrons around the nuclei generate a magnetic field which shields it from the effects of the external magnetic field. Nuclei with greater electron density (i.e. near to electropositive nuclei) have more shielding; hence appear at lower energies in the spectrum.

This adsorption is quickly saturated as the populations of the spin states reach parity, it is then necessary to allow the system to relax back to the normal level before repeating the irradiation.

Desirable traits of nuclei for NMR spectroscopy include: highly abundant isotopes, high magnetogyric ratio and reasonable relaxation times. These properties allow high resolution spectra to be generated quickly.

1.4.2 Gel Permeation Chromatography

GPC is a separation technique that is commonly used to determine the molecular weight and distribution of polymer samples. Unlike other forms of chromatography, it separates molecules by hydrodynamic volume, rather than intermolecular interactions, making it suitable for separating polymer species which are of different sizes but identical chemistries. The stationary phase of the column consists of porous beads, typically silica or crosslinked polymers, with a controlled range of pore sizes. Smaller particles can enter more pores than larger particles; they therefore have a larger space available to them and hence take longer to move down the column. Ideally there should be no interactions between the polymer and the packing material. Once passed through
the column there is a detector, most commonly refractive index (RI) or ultraviolet (UV), these measure the mass concentration of the polymer at a given time, producing a GPC trace.

A calibration curve can be generated using low-polydispersity polymer standards. Hence the molecular weight of a species eluted at any time is known by comparison to the calibration curve. GPC is a fast and simple analysis technique that can be used to determine the $M_n$, $M_w$ and polydispersity of a polymer sample. However, there are some caveats and factors to bear in mind when considering GPC data. GPC does not separate species by molecular weight, but by hydrodynamic volume; therefore the method is only quantitative when the polymer standards used for the calibration curve are of the same type as the sample being tested. The column imposes limits on the size of polymer that can be distinguished: the size exclusion limit of the column. All particulates larger than the largest pore will be excluded from the column and hence will travel through the same volume and coelute with one another at the solvent front. Enthalpic interactions can be limited by choice of packing material and media, but can accelerate or retard the progress of the polymers through the column; this is especially problematic for charged species.

As long as these factors are kept in mind while considering the data, GPC remains a powerful and effective method for the determination of molecular weight distributions for polymer samples.

1.5 Prior Art

This work follows up a series of studies performed by Downes et al.\textsuperscript{3-5} in the School of Material Science at the University of Manchester. Based on the observation that polyphosphonates encourage the deposition of hydroxyapatite,\textsuperscript{94, 98, 99} they investigated the effect of a synthetic polyphosphonate, PVPA-co-AA, on PCL scaffolds. PCL nanofibre mats were produced by electrospinning, dip-coated in PVPA-co-AA and stabilised by heat treatment over 24 hours (see Fig. 19).\textsuperscript{3}

![Figure 19. Nanofibrous PCL/PVPA-co-AA scaffold formed into a bead (left) and as seen by SEM (right). Adapted from Downes et al.\textsuperscript{3}](image-url)
Initial work focused on the physical properties of this scaffold. Using SEM the mean fibre diameter was determined to be 269 ± 102 nm. The stability of the PVPA-co-AA on the scaffold in aqueous media was also measured, and an interesting difference was found between samples that had been heat-treated and those that had not. Samples were incubated in culture media over 21 days and the concentration of phosphorus was measured using Energy-Dispersive X-ray Spectroscopy (EDX). After 2 days the concentration had dropped sharply for both samples and after 7 days the untreated scaffold was entirely free of PVPA-co-AA (see Fig. 20).

![Figure 20. Change in phosphorus content for the PCL/PVPA-co-AA scaffold on incubation in aqueous media as measured by EDX. Adapted from Downes et al.](image)

The phosphorus concentration in the heat-treated sample reached a limiting value of 1 wt%. It was speculated that the heat treatment caused some of the PVPA-co-AA chain to become trapped within the PCL matrix. However, it is also possible that the treatment dehydrated the PVPA-co-AA, forming intra and intermolecular phosphonic anhydrides which are known to be persistent in water, mechanically tying the PVPA-co-AA to the scaffold. A number of other physical characteristics were also measured; of particular importance are the hydrophilicity of the scaffold and its mechanical properties, as summarised in Table 8. The coated scaffold had a much lower water contact angle, meaning that it is more hydrophilic and making it easier for cells to attach to the scaffold.

<table>
<thead>
<tr>
<th></th>
<th>PCL alone</th>
<th>PCL/PVPA-co-AA scaffold</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water contact angle (°)</td>
<td>123.3 (±10.8)</td>
<td>43.3 (±1.2)</td>
</tr>
<tr>
<td>Compressive Strength (MPa)</td>
<td>14 (±1.2)</td>
<td>72 (±4.9)</td>
</tr>
<tr>
<td>Young’s Modulus (GPa)</td>
<td>0.9 (±0.1)</td>
<td>3.9 (±0.9)</td>
</tr>
</tbody>
</table>

The compressive strength and Young’s modulus of the scaffold were enhanced by coating the PCL with PVPA-co-AA and were found to lie between those of cortical and cancellous bone. However,
it should be noted that these measurements were taken on dry samples and that in wet (i.e. in vivo) conditions the PVPA-co-AA will be dissolved, severely compromising any contribution to mechanical strength.

Additionally, initial biological experiments found that the coatings were biocompatible and encouraged the attachment and proliferation of osteoblast cell lines, while discouraging the growth of osteoclasts. This selective response was probed further, finding that the PVPA-co-AA coated scaffolds showed improved viability, more mineralisation and an increase in the production of enzymes and proteins associated with bone formation for osteoblasts. Moreover, it was found that PVPA-co-AA led to a decrease in osteoclast cell numbers, with a corresponding increase in cell apoptosis, comparable to Alendronate, a commercially available bisphosphonate drug. This selective osteoclast toxicity is hypothesised to be due to the accumulation of non-hydrolysable analogues of ATP being taken up by osteocytes and inhibiting key molecular pathways, leading to apoptosis. This is analogous to the action of bisphosphonate drugs prescribed for osteoporosis, but with the advantage of being localised, reducing side-effects; a structural comparison between biologically occurring pyrophosphate, a generic bisphosphonate and the P-O-P motif in PVPA-co-AA shows that this hypothesis is plausible (see Fig. 21).\textsuperscript{100,101}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure21.png}
\caption{Comparison of the structures of pyrophosphate, a generic bisphosphonate and the P-O-P motif that occurs in PVPA-co-AA.}
\end{figure}

The effects of the scaffold on the rate of repair of critical size defects was also investigated.\textsuperscript{5} PCL and PCL/PVPA-co-AA scaffolds were prepared as before and tested in vitro on mouse calvaria bone defects. After 35 days the PCL/PVPA-co-AA scaffolds had 63.57% bone fill, compared to 29.64% for PCL alone and micro-computed tomography (\textmu CT) images of the calveria show an obvious difference in mineralisation between the scaffolds (see Fig. 22). Additionally, EDX found higher phosphorus and calcium concentrations in the scaffolds containing PVPA-co-AA, indicating a higher rate of hydroxyapatite deposition in these scaffolds.
The mechanisms for this increase in the rate of mineralisation were hypothesised to be due to three factors:

- Increased wettability of the scaffold, making it easier for cells (e.g. osteoblasts) to attach to the nanofibres, increasing the rate of proliferation of osteoblasts and enabling them to mineralise the scaffold more easily.

- Chelation of Ca$^{2+}$, phosphonate groups form a ‘bone hook’ trapping Ca$^{2+}$ ions on the surface, thereby increasing the local concentration and encouraging further deposition of hydroxyapatite.

- Selectively inducing the apoptosis of osteoclasts, preventing the resorption of the newly formed bone.

Though the mechanical properties and biological effects of this scaffold have been explored, as summarised in Table 9, the investigation of the chemical properties has been more tentative. The PVPA-co-AA used in the scaffolds has been sourced from a commercial supplier and is poorly defined, with no information concerning the purity, composition, molecular weight or distribution of the polymer available. In order for the work to be repeated and further experiments to be performed, these factors need to be defined and a robust synthetic route developed.
Table 9. A summary of the prior art comparing the suitability for bone tissue applications of electrospun PCL nanofibres with a PVPA-co-AA coating and those without.3-5

<table>
<thead>
<tr>
<th></th>
<th>Uncoated PCL nanofibres</th>
<th>PCL/PVPA-co-AA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hydrophilicity</td>
<td></td>
<td>+</td>
</tr>
<tr>
<td>Mechanical strength</td>
<td></td>
<td>+</td>
</tr>
<tr>
<td><em>In vitro</em> bone fill</td>
<td></td>
<td>+</td>
</tr>
<tr>
<td>Collagen</td>
<td></td>
<td>+</td>
</tr>
<tr>
<td>Alkaline phosphatase</td>
<td></td>
<td>+</td>
</tr>
<tr>
<td>Osteocalcin</td>
<td></td>
<td>+</td>
</tr>
<tr>
<td>Osteoprotegerin</td>
<td></td>
<td>+</td>
</tr>
<tr>
<td>Calcium deposition</td>
<td></td>
<td>+</td>
</tr>
<tr>
<td>Osteoblast proliferation</td>
<td></td>
<td>+</td>
</tr>
<tr>
<td>Osteoclast apoptosis</td>
<td></td>
<td>+</td>
</tr>
</tbody>
</table>

1.6 Aims and Objectives

The overall aim of this project is to produce materials for use as bone tissue scaffolds that are biocompatible, biodegradable and osteoconductive; the polymer should be capable of being used alone, as part of a polymer blend or as a component in a composite material.

The work presented here is concerned with the chemistry of this project, and has the following aims and objectives:

1. Characterization of the PVPA-co-AA used previously by Downes et al.3-5 This includes determining the purity, molar mass distribution and composition of the polymer.
2. Develop a synthesis of the PVPA-co-AA and synthesise a series of PVPA-co-AA polymers with a range of compositions for further biological evaluation.
3. Improve the production of the nanofibrous PCL/PVPA-co-AA scaffold by developing a suitable compatibiliser (i.e. PCL-b-PAA) to enable emulsion electrospinning.
4. Explore different approaches to create suitable bone tissue scaffolds based on this methodology (i.e. crosslinked PVPA-co-AA nanofibres and directly phosphonated PCL).
2 Characterisation of Commercial PVPA-co-AA

2.1 Introduction

The commercial PVPA-co-AA used in previous studies was not characterised in terms of its chemical properties. This means that further studies, or others attempting to replicate the experiments, may have different findings as they may use PVPA-co-AA of a different purity, composition or molecular weight. Therefore work was performed to characterise the commercial PVPA-co-AA, to ensure that any further experiments are using the same copolymer. Additionally, the methods developed here may find use in the purification and analysis of further PVPA-co-AA polymers.

2.1.1 Dialysis

Dialysis is a purification method that is particularly popular for the purification of proteins, but has also been used successfully for synthetic polymers and nanoparticles. This method is conceptually similar to filtration, but on a shorter length scale. The solution to be purified is placed in a semipermeable membrane bag, the small molecule impurities diffuse outwards into the external solution while the larger particulates are retained.

Many types of membrane are available, allowing the use of a wide variety of solvents, but the most popular system uses cellulose acetate for the membranes and water as the solvent. This has a number of advantages as water is inexpensive, easy to obtain and non-toxic. In addition to material choices, the molecular weight cut-off (MWCO) of the tubing needs to be considered. Materials with a molecular weight greater than the MWCO will be retained, while those with lower molecular weights will be lost, it is therefore important to consider the relative sizes of the desired product and the impurities. It is recommended that the MWCO be at least 50-100 times greater than the molecular weight of the impurities, to ensure fast and complete removal, and at most 50-80% of the molecular weight of the macromolecule, to prevent unacceptable product losses.

The MWCO is experimentally determined for globular polymers (such as proteins) under neutral pH conditions; where the polymers or conditions differ markedly from this then the actual sizes of retained macromolecules will vary. For example, highly charged species such as polyelectrolytes will be extended into rods rather than spheres, and hence will be retained at lower MWCOs than would otherwise be expected.

The cellulose ester membrane is typically purchased as tubing, which needs to be soaked and washed with water prior to use to restore flexibility and remove any preservative. The solution to
be purified is then placed in the tubing which is sealed with polypropylene clips capable of resisting the large pressures that can build up due to osmosis. The bag is then placed in a relatively large volume of water, with stirring to promote fast diffusion. Diffusion is a passive, concentration gradient driven process and it is necessary to change the water several times to maintain this gradient. After a sufficient length of time has passed the impurities will have been reduced to statistically insignificant levels and the desired macromolecule recovered from the solution remaining in the dialysis tubing.

Overall, dialysis is a suitable method for the purification of polymers, especially if they are water-soluble, as it is effective, cheap and simple to perform. However, it is a relatively slow process and uses large quantities of solvent, which can be problematic if less benign solvents are required.

2.1.2 $^{31}$P$\text{[}^1\text{H}]$ NMR

The $^{31}$P nucleus is well suited to NMR study as it is spin $\frac{1}{2}$, has a moderately high magnetogyrar ratio, is 100% abundant and covers a wide range of chemical shifts, allowing for easy distinction between nuclei. Unlike $^1$H NMR however, the chemical shift is affected more by bond overlap and hybridisation than by deshielding by adjacent nuclei. Furthermore, normal $^{31}$P$\text{[}^1\text{H}]$ NMR is not quantitative due to a long relaxation time, $T_1$, leading to resonance saturation and the broadband decoupling causing an uneven enhancement of the signals due to the Nuclear Overhauser Effect (NOE).

2.1.3 Aqueous GPC

Aqueous GPC is very similar to GPC run in organic solvents, but it is further complicated by the fact that the polymers to be examined usually interact more strongly with one another and the column material, which leads to shifts in elution volumes. It is therefore common to use salts and buffered solutions to try to screen these intra and intermolecular interactions, in an attempt to attenuate these effects. It is also advantageous to use calibration standards that are as similar as possible to the polymer being investigated, so that any unpreventable effects occur to both equally.

2.1.4 Aims and Objectives

To characterise the commercial PVPA-co-AA by a number of spectroscopic and chromatographic methods to determine:

- The purity of the commercial PVPA-co-AA, and to develop purification methodology,
- The composition of the commercial PVPA-co-AA,
- The molecular weight of the commercial PVPA-co-AA.
2.2 Experimental

2.2.1 Materials
Commercial PVPA-co-AA (40 wt% aqueous solution, First Scientific Dental GmbH) was purified by dialysis (see below). Dialysis tubing (12,000-14,000 g mol$^{-1}$ MWCO, 17.5 mm inflated diameter, Medicell Membranes Ltd.) was soaked in water for 20 minutes and rinsed directly before use. D$_2$O (99.9 atom % D, Sigma-Aldrich), tetrahydrofuran (THF, 99.9%, Fisher), diethyl ether (≥99%, Aldrich), ethyl acetate (≥99.5%, Sigma-Aldrich), acetone (≥99.5%, Sigma-Aldrich), acetonitrile (≥99.5%, Sigma-Aldrich), acetic acid (≥99.7%, Fluka), dimethylformamide (DMF, ≥99.9%, Sigma-Aldrich) and trimethyl phosphate (TMP, ≥99%, Sigma-Aldrich) were used as supplied.

2.2.2 Instrumentation
Attenuated total reflectance- Fourier transform infrared spectroscopy (ATR-FTIR) was performed using a Thermo Scientific Nicolet iS5 FTIR Spectrometer equipped with an iD5 ATR attachment.

NMR was performed on a Bruker Avance 400 MHz instrument. $^1$H NMR was performed at 400 MHz, $^{13}$C NMR at 101 MHz and $^{31}$P at 162 MHz.

Aqueous GPC was performed using a Waters 515 HPLC pump with TSK gel columns (5000 and 6000 Å pore size) and an ERC-7515A RI detector, at 40°C using 0.1 M NaH$_2$PO$_4$ buffer solution as eluent with a flow rate of 0.5 mL min$^{-1}$.

Elemental analysis was performed using a Thermo Scientific FLASH 2000 CHNS/O Analyser and a Thermo iCap 6300 Inductively Coupled Plasma Optical Emission Spectroscopy (ICP-OES) instrument. Results quoted are accurate to within 0.3 wt%.

2.2.3 Procedures

Purification of PVPA-co-AA by Precipitation
The commercial PVPA-co-AA solution (5 mL) was evaporated to dryness under reduced pressure to give the PVPA-co-AA as a yellow solid (2 g). This was then dissolved into ethanol (10 mL) and an aliquot of this solution was pipetted into a selection of solvents. The solubility of the PVPA-co-AA was observed and any solids that formed were collected, dried and analysed by NMR.

Purification of Commercial PVPA-co-AA by Dialysis
The commercial PVPA-co-AA was purified by dialysis. Typically, 30 cm of dialysis tubing was prepared by soaking in deionised water for 20 mins and then rinsed to remove any preservative. One end of the tubing was then sealed with a tubing clip and beyond that a figure of eight knot.
The commercial PVPA-co-AA solution (10 mL) was pipetted into the tubing and the open end sealed as above; sufficient air was left in the tubing to give it buoyancy. The tubing was then placed in deionised water (3 L) under gentle stirring. This water was changed after 1 h and after 5 h. After 24 h the tubing was removed from the beaker and the contained solution transferred to a round-bottomed flask, taking care as the contents of the tube were under pressure. This was then evaporated to dryness using a rotary evaporator at 60°C and 15 mbar.

**Quantitative $^{31}\text{P}\{^1\text{H}\} \text{NMR}**

Typically, dry PVPA-co-AA (80 mg) was dissolved in D$_2$O (2 mL) and trimethyl phosphate (TMP, 30 mg) was then added as a marker compound. This sample was analysed using non-standard quantitative $^{31}\text{P}\{^1\text{H}\} \text{NMR},$ this method has a $T_1$ of 35 s, runs 64 scans and uses inverse gated decoupling.

**Determining the Molar Mass and Distribution of Commercial PVPA-co-AA**

Aqueous GPC was used to determine the molar mass distribution of the commercial polymer. This was initially run using TSK-GEL 3000 and 4000 columns (suitable for low mass polymers), but these were switched to TSK-GEL 5000 and 6000 (suitable for higher mass polymers) which were more suitable for the polymers being investigated. Additionally, rather than the usual poly(ethylene glycol) standards PAA-Na standards were used instead, with 0.1 M NaH$_2$PO$_4$ as the eluent.

**2.3 Results and Discussion**

**2.3.1 Purity of the Commercial PVPA-co-AA**

The PVPA-co-AA used for the previous work was used as supplied, without further purification or analysis. The first objective of this research was to establish the purity of the commercial PVPA-co-AA as any impurities, such as residual monomers, could adversely affect the results of any biological testing. A sample of the supplied polymer solution was evaporated to dryness to give a pale yellow solid, which remained tacky after several hours under vacuum. This tackiness indicated that the polymer could be contaminated with a high-boiling liquid impurity.

An FTIR spectrum was recorded for the solid polymer and analysed. The spectrum (Fig. 23) showed the anticipated bands, with a broad O-H absorption centred on 2940 cm$^{-1}$ and a strong C=O absorption at 1696 cm$^{-1}$ for the carboxylic acid. Medium intensity bands from 3300-2500 cm$^{-1}$ and 1500-1375 cm$^{-1}$ indicate the stretching and bending modes of methylene C-H for the polymer backbone. Absorptions at 1150 cm$^{-1}$ and 995-915 cm$^{-1}$ indicate P=O and P-O stretches, showing
that the phosphonic acid is present. The FTIR spectrum matches what would be expected for PVPA-co-AA,\textsuperscript{110} with no obvious bands for impurities, indicating that any impurities that are present only in small quantities or have considerably similar FTIR spectra to the polymer, for example residual alkene-bearing monomers. Such monomers would be indicated by the presence of bands corresponding to C=C bonds, however, these are likely to be obscured by the coincident and stronger C=O absorptions of the carboxylic acids. Therefore IR analysis, while demonstrating that there was no gross contamination of the sample, was not considered sufficient to ascertain if the commercial PVPA-co-AA was of high purity.

![Figure 23. FTIR spectrum of the commercial PVPA-co-AA as supplied.](image)

A sample of the commercial PVPA-co-AA was dissolved in D\textsubscript{2}O and examined by \textsuperscript{1}H NMR (Fig. 24). The methylene protons appear below 2 ppm while the methine protons are shifted downfield due to their proximity to the acidic groups; additionally, at 4.25 ppm there is a broad peak which is indicative of the OH groups of the phosphonic acid. The \textsuperscript{1}H NMR spectrum of the commercial PVPA-co-AA matches what would be expected.\textsuperscript{84, 111} However, the sharp peaks in the spectrum show the presence of impurities.

![Figure 24. \textsuperscript{1}H NMR spectrum of the commercial PVPA-co-AA as supplied, with some impurities indicated.](image)
Of particular interest are the sharp peaks at 6.0 ppm which are indicative of vinyl protons, the most obvious explanation for which is that there are residual monomers present. It can also be seen that there are sharp peaks at 3.6 and 2.2 ppm, which could correspond to alkyl groups adjacent to carboxylic or phosphonic esters, implying that the commercial PVPA-co-AA is synthesised by the ester monomer route, rather than the acid monomer route. Regardless of the identity of the impurities, they could interfere with the scaffold fabrication and the results of the biological studies and hence need to be removed from the PVPA-co-AA.

2.3.2 Purification of the Commercial PVPA-co-AA

Polymers are often purified by precipitation in a selective solvent: the impurities remain dissolved while the polymer precipitates out of solution and is then collected by filtration. This approach was attempted for the commercial PVPA-co-AA with several solvents (Table 10) with limited success.

<table>
<thead>
<tr>
<th>Solvent used</th>
<th>Precipitate formed</th>
<th>Pure</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetic acid</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Acetone</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Acetonitrile</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Diethyl ether</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>DMF</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Ethyl acetate</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>THF</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>

Of the solvents tested, only acetic acid was successful in removing the impurities from the commercial PVPA-co-AA; however, it was unsuitable for the purification of the polymer as it could not be removed from the resulting polymer (see Fig. 25). The persistence of the acetic acid was hypothesised to be due to strong hydrogen bonding between the acidic groups, analogous to that seen in carboxylic acids which results dimer formation, and an associated increase in boiling point.
Figure 25. $^1$H NMR spectrum of the commercial PVPA-co-AA purified by precipitation in acetic acid. There is a large peak at 2.08 ppm for the residual acetic acid. Inset: schematic of a carboxylic acid dimer.

An alternative method of purification is dialysis, which is especially popular for water-soluble polymers such as PVPA-co-AA. Dialysis tubing with a MWCO of 12,000 – 14,000 g mol$^{-1}$ was selected as this would retain the polymer while allowing the outward diffusion of small impurities, such as residual monomers and oligomers at a suitable rate. It was found that the polymer was successfully purified after 24 hours, with the water being changed several times to maintain the concentration gradient.

Figure 26. $^1$H NMR spectrum of the commercial PVPA-co-AA purified by dialysis.

The polymer afforded once the dialysis solution has been dried is hard and off-white in colour, indicating that the polymer is purer; this assertion is supported by the $^1$H NMR spectrum (Fig. 26) which shows no sharp peaks indicative of residual monomer. However, an unexpected broad peak remains at 3.6 ppm, coinciding with the sharp peaks observed previously. If those peaks are indeed due to esteric monomers, such as DMVP, then this remaining peak shows that the polymer ester was not completely hydrolysed and that some of these groups remain, which may affect the polymer’s bioactivity.
The $^{31}\text{P}[^1\text{H}]$ NMR spectrum (Fig. 27) again shows a lack of impurities, along with the expected broad peak at around 30 ppm for the phosphonic acids. Additionally, it shows a peak at 24 ppm corresponding to the formation of phosphonic anhydrides which form during the drying of the copolymer by a dehydration reaction, and can persist for long periods of time even in aqueous solutions.\(^8\)

2.3.3 Determining the Composition of the Commercial PVPA-co-AA

The composition of the PVPA-co-AA (i.e. the proportion of which is AA and the proportion that is VPA) is an important parameter as this could significantly affect the physical and biological properties of the copolymer. To identify this, it is necessary to determine some way to differentiate between the AA and VPA groups within the polymer; the most obvious difference between them is that while both contain carbon, oxygen and hydrogen, only VPA contains phosphorus. Therefore any technique that is capable of quantifying the phosphorus content of the copolymer could potentially be used to determine its composition.

The first technique considered was elemental analysis, performed in the Microanalysis Laboratory in the School of Chemistry. The approach is simply to measure the wt% of the polymer that is carbon, and that which is phosphorus, and convert this into a ratio, P:C. The AA and VPA repeat units have P:C ratios of 0 and 1.29 respectively, as will the homopolymers. Copolymers however, will have an intermediate value, which can be used to calculate their composition. The wt%P and wt%C of the copolymer can be determined from the structures of AA and VPA and their mole fractions, \(n\) and \(m\) respectively:
where $M$ is the total mass of the copolymer being examined, this factor can be dismissed by using the P:C ratio, $R$, which is determined experimentally:

$$ R = \frac{wt\%P}{wt\%C} = \frac{(\frac{m}{m+n} \times 31)}{\frac{2m+3n}{m+n} \times 12} $$

$R$ in turn is converted to the molar ratio of AA to VPA, $n/m$:

$$ n/m = 1 - \frac{2(\frac{12}{31} \times R)}{3(\frac{12}{31} \times R)} $$

This can be used to calculate the mol% VPA in the copolymer:

$$ mol\% \text{ VPA} = \frac{100}{1 + \frac{n}{m}} $$

The second method considered is quantitative $^{31}$P{$^1$H} NMR; normal $^{31}$P{$^1$H} NMR experiments are not quantitative due to the reasons outlined previously, but they can be made to be quantitative if special pulse programs are used.\(^{113}\) The first problem is that the $^{31}$P nucleus has a relatively long relaxation time; this can be counteracted through the use of relaxation agents such as magnesium chloride\(^{114}\) or paramagnetic metal complexes.\(^{115}\) Alternatively, long times between each scan can be used to ensure that the nuclei return to the ground state prior to the start of the next pulse. In these experiments, the delay period has been extended to at least 35 seconds to prevent saturation of the nuclei and reduction of the signal.\(^{108}\) The second issue with $^{31}$P{$^1$H} NMR is the NOE, which can be prevented by using a pulse sequence with inverse-gated decoupling. The number of scans has also been increased to 64, in order to ensure a high signal to noise ratio even for low phosphorus concentrations.
Figure 28. Example of a quantitative $^{31}$P($^1$H) NMR spectrum; a) phosphonic acid peak at 30.3 ppm; b) anhydride peak at 24.5 ppm; and c) TMP marker peak at 2.8 ppm.

Under these conditions the relative peak integrals (Fig. 28) reflect the relative abundances of the species in the copolymer. By using a known mass of a marker compound, TMP, the number of moles of phosphorus in the copolymer (and hence the mass of VPA) can be determined; if the total polymer mass is also known then the mol% VPA of the copolymer can be calculated.

The number of moles per integral, $n$, can be calculated from the mass of TMP used ($m_{\text{TMP}}$), its integral ($I_{\text{TMP}}$) and its molecular weight:

$$n = \frac{m_{\text{TMP}}}{I_{\text{TMP}} \times 140.07}$$

$n$ can then be used to determine the number of moles of VPA, $M_{\text{VPA}}$, and of any anhydride, $M_{\text{Anhydride}}$:

$$M_{\text{VPA}} = I_{\text{VPA}} \times n$$

$$M_{\text{Anhydride}} = I_{\text{Anhydride}} \times n$$

The moles of AA, $M_{\text{AA}}$, can be calculated from the above and the mass of copolymer, $m_{\text{polymer}}$, used:

$$M_{\text{AA}} = \frac{m_{\text{polymer}} - (M_{\text{VPA}} \times 108.03) - (M_{\text{Anhydride}} \times 198.05)}{72.06}$$

Having determined the number of moles of all of the components, it is then possible to calculate the composition of the copolymer, expressed as mol% VPA:
Both the elemental analysis and quantitative $^{31}$P-$^{1}H$ NMR methods have strengths and weaknesses. The elemental analysis method requires minimal sample preparation, but does need to be processed in the microanalysis laboratory and is also susceptible to impurities affecting the result. The NMR approach needs more preparation (i.e. weighing and dissolving the copolymer and TMP), but also gives more information regarding purity and can be used more generally (i.e. calculating monomer conversions). Both methods were used to determine the composition of the commercial PVPA-co-AA and a sample of VPA monomer (Table 11).

<table>
<thead>
<tr>
<th></th>
<th>Commercial PVPA-co-AA</th>
<th>VPA monomer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Quantitative $^{31}$P-$^{1}H$ NMR</td>
<td>34 mol% VPA</td>
<td>97 mol% VPA</td>
</tr>
<tr>
<td>Elemental Analysis</td>
<td>30 (± 1.0) mol% VPA</td>
<td>101 (± 1.7) mol% VPA</td>
</tr>
</tbody>
</table>

The results agree broadly, but are slightly different. For the VPA this difference is due to the presence of impurities as the monomer is supplied at a quoted purity of 97%. The elemental analysis cannot show this, and hence cannot compensate for it. The quantitative $^{31}$P-$^{1}H$ NMR spectrum (Fig. 29) however displays these impurities and can compensate for them, making it the more powerful of the two methods.

![Figure 29. Quantitative $^{31}$P-$^{1}H$ NMR spectrum of VPA, showing TMP at 3.1 ppm, VPA at 15.6 ppm, impurities at 16.5, 17.9, 25.0 and 26.3 ppm](image)

The composition of the commercial PVPA-co-AA was found to be 34 mol% VPA, in later work both elemental analysis and quantitative $^{31}$P-$^{1}H$ NMR will be used to analyse PVPA-co-AA samples to ensure accurate results are reported, but the composition determined by the NMR method will be considered the more accurate figure.
2.3.4 Determining the Molecular Weight of the Commercial PVPA-co-AA

PVPA-co-AA is insoluble in organic solvents, but dissolves readily in water, therefore aqueous GPC was used to determine its molecular weight. However, the copolymer is a polyanion and thus can be expected to interact strongly with the packing material of the column, either being rejected or attracted, depending on the residual charge, and with itself, increasing its hydrodynamic volume and apparent molecular weight. It is therefore necessary to add a buffer to the solution to minimise these molecular interactions; 0.1 M NaH$_2$PO$_4$ was chosen as the eluent as it should be an effective buffer solution. In addition, the PAA-Na used as standards will have the same hydrodynamic diameter as PVPA in this buffer, an advantage that can be expected to be conferred on the PVPA-co-AA.

The aqueous GPC result for the commercial PVPA-co-AA can be seen in Fig. 30, along with the calibration curve. On inspection it can be seen that the distribution of the copolymer is broad and potentially bimodal, this can be attributed to auto-acceleration or other factors, such as monomer addition rates, affecting the copolymerisation. Alternatively, the supplier may have blended multiple batches of polymer to meet a technical specification. The $M_n$ of the polymer was found to be 8,000 g mol$^{-1}$, and the $M_w$ was 31,000 g mol$^{-1}$.

![Figure 30. Aqueous GPC trace for the commercial PVPA-co-AA, with the PAA-Na calibration curve overlain.](image)

Observing the calibration curve, it appears to be linear at long elution times, but begins to turn downwards at short elution times. Conventionally the curve would be expected to be linear until it meets the exclusion limit of the column set, at which point it would turn sharply upwards. This implies that some other mechanism other than size separation is operating, at least for very high masses. Accordingly, these data cannot be considered to be absolutely quantitative, despite this the aqueous GPC method used will give useful qualitative results when comparing PVPA-co-AA polymers with one another.
2.4 Conclusions

The commercial PVPA-co-AA was found to be impure as supplied, and the impurities were hypothesised to be residual ester monomers. Additionally, small quantities of un-hydrolysed ester side groups may persist in the copolymer.

No suitable solvents were found for purification by precipitation, an alternative method was found in dialysis, which gave excellent results but is time-consuming.

Two methods were developed for quantifying the composition of the copolymer: elemental analysis for carbon and phosphorus and quantitative $^{31}$P{($^1$H)} NMR with a known marker compound, TMP. The commercial PVPA-co-AA was found to be 34 mol% VPA by NMR and 30 mol% VPA by elemental analysis. The NMR method is considered to be more reliable, though both methods will be used to examine future samples to ensure accurate results.

Aqueous GPC with 0.1 M NaH$_2$PO$_4$ buffer as eluent was used to determine the molecular weight distribution of the copolymer, with $M_n$ and $M_w$ of 8,000 and 31,000 g mol$^{-1}$, respectively. These results are not absolute, but can be considered to be suitable for comparison with other PVPA-co-AA samples that are measured under the same conditions.
3 Synthesis of PVPA-co-AA

3.1 Introduction

Having characterised the commercial PVPA-co-AA polymer, attention turned to synthesising further examples of the copolymer for use in biological testing. Having a range of compositions available to use would allow experiments to determine if any composition was particularly effective and possibly help to elucidate the mechanism by which the PVPA-co-AA enhances the rate of bone healing. This chapter is concerned with the synthesis of PVPA-co-AA over a wide range of compositions, including each homopolymer, while attempting to control other characteristics. Methods developed in the previous chapter were used to purify and characterise the resulting copolymers.

3.1.1 PVPA-co-AA

Few previous attempts at synthesising PVPA-co-AA have been reported, one such study made nanobeads for the take-up of indium using suspension polymerisation with poly(vinyl alcohol) as a stabiliser and \(N,N'-\text{methylenebisacrylamide}\) as a crosslinker.\(^{117}\) The monomers were placed in a round-bottomed flask with water and poly(vinyl alcohol) under \(N_2\) at 60°C then benzoyl peroxide was added as the initiator. A yield of 69% was reported for the PAA homopolymer sample, with lower yields for increasing VPA feed concentration. Additionally, through elemental analysis they determined that the VPA conversion was lower than for the AA, indicating that VPA is a less reactive monomer. The idea that VPA and its esters have relatively low reactivity is supported by the experiences of others attempting to create copolymers with methyl acrylate,\(^{118}\) N-vinyl pyrrolidone\(^{119}\) and styrene.\(^{120}\)

A patent was also found for the synthesis of PVPA-co-AA and its methacrylic acid analogue: poly(vinylphosphonic acid-co-methacrylic acid).\(^{79}\) Differences in reactivity were overcome by adding the more reactive monomer, AA or methacrylic acid, and the initiator to the reaction vessel gradually. This means that at any one time the concentration of VPA is far higher, compensating for its lower reactivity and increasing its conversion.

3.1.2 Aims and Objectives

To develop a synthesis suitable for preparing a range of PVPA-co-AA copolymers covering the entire range of compositions.
3.2 Experimental

3.2.1 Materials
Deionised water for polymerisation was degassed by purging with N₂ for 1 hour. Dialysis tubing (12,000-14,000 g mol⁻¹ MWCO, 17.5 mm inflated diameter, Medicell Membranes Ltd.) was soaked in water for 20 minutes and rinsed directly before use. Acrylic acid (AA, 99%, Aldrich), vinylphosphonic acid (VPA, 95%, TCI) and 2,2'-azobis(2-amidinopropane) dihydrochloride (AAPH, 97%, Aldrich), D₂O (99.9 atom % D, Sigma-Aldrich) and trimethyl phosphate (TMP, ≥99%, Sigma-Aldrich) were used as supplied.

3.2.2 Instrumentation
NMR and Aqueous GPC analyses were performed as described in Section 2.2.2.

3.2.3 Reactions

Simple PVPA-co-AA Synthesis
VPA (1.46 mL, 2.00 g, 18.5 mmol), AA (2.96 mL, 3.111 g, 43.2 mmol), AAPH (15 mg, 0.0556 mmol) and H₂O (5 mL) were placed in a round-bottomed flask fitted with a reflux condenser under N₂. The flask was submerged in an oil bath preheated to 90°C and left stirring for 24 h. The reaction mixture was allowed to cool to room temperature and purified by dialysis over 24 h. The resulting solution was evaporated to dryness to give a white solid (3.470 g, 68% yield).

PVPA Synthesis
VPA (1.46 mL, 2.00 g, 18.5 mmol) and AAPH (5 mg, 0.018 mmol) were dissolved in H₂O (1 mL) and placed in a 2-necked round-bottomed flask fitted with a reflux condenser under N₂. This was closed with a rubber septum, heated to 90°C and left stirring for 4 h. Once complete, the reaction solution was allowed to cool to room temperature and purified by dialysis over 24 h. The resulting solution was evaporated to dryness to give a white solid (0.903 g, 45% yield).

PVPA-co-AA Synthesis by Gradual Addition of Acrylic Acid and Initiator
VPA (1.46 mL, 2.00 g, 18.5 mmol) was dissolved in H₂O (2 mL) and placed in a 2-necked round-bottomed flask fitted with a reflux condenser under N₂. This was closed with a rubber septum and heated to 90°C with stirring. AA (2.96 mL, 3.111 g, 43.2 mmol) was dissolved in H₂O (3 mL) and AAPH (15 mg, 0.0556 mmol) was dissolved in H₂O (2.4 mL). 1/12 of each of these solutions was added to the reaction vessel every 30 minutes. The reaction was allowed to continue for a further 18 h. Once complete, the reaction solution was allowed to cool to room temperature and purified...
by dialysis over 24 h. The resulting solution was evaporated to dryness to give a white solid (3.809 g, 74% yield).

**PVPA-co-AA Synthesis by Gradual Addition of Acrylic Acid**

VPA (1.46 mL, 2.00 g, 18.5 mmol) and AAPH (7.2 mg, 0.026 mmol) were dissolved in H\(_2\)O (0.5 mL) and placed in a 2-necked round-bottomed flask fitted with a reflux condenser under N\(_2\). This was closed with a rubber septum and heated to 90°C. AA (0.54 mL, 0.571 g, 7.93 mmol) was dissolved in H\(_2\)O (0.5 mL); this was added to the reaction solution at a rate of 0.1 mL every 5 minutes. H\(_2\)O was added as required to keep the reaction mixture stirrable. The reaction solution was then left to stir at 90°C for 18 h. Once complete the reaction solution was allowed to cool to room temperature and purified by dialysis over 24 h. The resulting solution was evaporated to dryness to give a white solid (1.292 g, 50% yield).

### 3.3 Results and Discussion

The first experiment performed used a straightforward polymerisation of VPA and AA using AAPH, a water-soluble azo initiator that has been used to successfully synthesise PVPA homopolymers (Scheme 1).\(^8\) A 30:70 mole ratio of VPA:AA was used to try to recreate an approximation of the commercial copolymer. An acceptable yield of 68% was obtained after dialysis, but there was a great difference in the conversions of the two monomers, with the VPA conversion being 47% compared to an overall monomer conversion of 74%. Furthermore, the monomer feed was 30 mol% VPA while the obtained polymer was only 9 mol% VPA, indicating that this simple approach was unsuitable for the synthesis of PVPA-co-AA over a wide compositional range.

The poor conversion of VPA can be attributed to its low reactivity compared to AA. R. Dey in the Budd group determined that the reactivity ratios of AA (\(r_1\)) and VPA (\(r_2\)) were 4.09 and 0.042.\(^1\) With \(r_1 >> 1 >> r_2\) it is likely that the system will suffer greatly from composition drift, i.e. initially the polymer will be almost exclusively PAA. It is only as AA is depleted from the reaction mixture that VPA is included in the copolymer chain. This will lead to inhomogeneity both within and between polymer chains, with some chains being entirely PAA and others being gradient copolymers. The low reactivity of the VPA monomer can be partially explained by its
cyclopolymerisation mechanism, but other factors should also be considered. Firstly, VPA is less vulnerable to radical attack than AA as its head group is much larger, leading to greater steric hindrance. Secondly, VPA has pK\textsubscript{a}s of 2.74 and 7.34 while AA has a pK\textsubscript{a} of 4.26. VPA is therefore more likely to be deprotonated in the reaction mixture leading to increased repulsive electrostatic forces between the growing chain and the monomers. Thirdly, a radical on the α-carbon of VPA is thought to be less stabilised than the equivalent radical on AA, making it more likely to react with an AA monomer. The relatively poor reactivity of the VPA monomer and its relative instability means that it rarely adds to the propagating chain, and when it does it is likely to go on to attack an AA monomer, all other things being equal.

Therefore to increase the conversion of the VPA, and hence the mol% VPA in the copolymer, other factors must biased in its favour. The methodology of the patent was adopted and the AA and AAPH were added gradually throughout the reaction. Slow addition of the AA means that at any given time the concentration of VPA is much higher, compensating for its low reactivity; and slow addition of the AAPH means that propagating chains are always present in the reaction mixture which should increase the yield. A set of experiments were performed to find the optimum temperature for the polymerisation (Table 12). Reactions were performed at 80, 90 and 100°C, with a VPA:AA ratio of 30:70 and keeping all other conditions the same.

Table 12. Results from the experiments varying the temperature of the reaction. *Indicates that dialysis was run without clips to correctly seal the tubing, hence the low yield. Total monomer conversion and VPA conversion were determined by \textsuperscript{1}H and quantitative \textsuperscript{31}P\{\textsuperscript{1}H\} NMR respectively. \(M_{\text{peak}}\) is used as a comparison as some polymers are too large and fall outside of the range of the calibration curve for the aqueous GPC, meaning that \(M_n\) cannot be calculated accurately.

<table>
<thead>
<tr>
<th>Conditions</th>
<th>Total Conversion (%)</th>
<th>VPA Conversion (%)</th>
<th>Yield (%)</th>
<th>mol% VPA (EA)</th>
<th>mol% VPA (NMR)</th>
<th>(M_{\text{peak}}) (g mol\textsuperscript{-1})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Commercial</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>30 (± 1.0)</td>
<td>34</td>
<td>11,000</td>
</tr>
<tr>
<td>Simultaneous addition</td>
<td>74</td>
<td>47</td>
<td>68</td>
<td>13 (± 0.8)</td>
<td>9</td>
<td>266,000</td>
</tr>
<tr>
<td>80 °C</td>
<td>87</td>
<td>62</td>
<td>27*</td>
<td>21 (± 0.8)</td>
<td>21</td>
<td>319,000</td>
</tr>
<tr>
<td>90 °C</td>
<td>89</td>
<td>70</td>
<td>74</td>
<td>25 (± 1.0)</td>
<td>22</td>
<td>372,000</td>
</tr>
<tr>
<td>100 °C</td>
<td>84</td>
<td>58</td>
<td>73</td>
<td>22 (± 0.8)</td>
<td>20</td>
<td>422,000</td>
</tr>
</tbody>
</table>

Gradual addition of the AA and AAPH leads to a slight improvement in the yield, but the main effect is a dramatic increase in the conversion of the VPA and a corresponding increase in the VPA present in the copolymers. Additionally, the reaction at 90°C was found to have the highest VPA
conversion and content. Further experiments varying the feed ratio were run at this temperature in order to maximise the VPA conversion (Table 13).

Table 13. Results from the experiments varying the feed ratio. * indicates that the reaction was performed by Dr R Zhong, † indicates that the copolymer failed to be purified by dialysis.

<table>
<thead>
<tr>
<th>Feed Ratio (VPA:AA)</th>
<th>Total Conversion (%)</th>
<th>VPA Conversion (%)</th>
<th>Yield (%)</th>
<th>mol% VPA (EA)</th>
<th>mol% VPA (NMR)</th>
<th>( M_{(peak)} ) (g mol(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Commercial</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>30 (± 1.0)</td>
<td>34</td>
<td>11,000</td>
</tr>
<tr>
<td>30:70</td>
<td>89</td>
<td>70</td>
<td>74</td>
<td>25 (± 1.0)</td>
<td>22</td>
<td>372,000</td>
</tr>
<tr>
<td>40:60</td>
<td>80</td>
<td>54</td>
<td>66</td>
<td>29 (± 1.0)</td>
<td>26</td>
<td>172,000</td>
</tr>
<tr>
<td>50:50</td>
<td>67</td>
<td>41</td>
<td>52</td>
<td>32 (± 1.0)</td>
<td>31</td>
<td>77,000</td>
</tr>
<tr>
<td>70:30*</td>
<td>47</td>
<td>27</td>
<td>31</td>
<td>-</td>
<td>40</td>
<td>8,000</td>
</tr>
<tr>
<td>90:10†</td>
<td>11</td>
<td>-</td>
<td>6</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

There is a clear trend for the monomer conversion, yield and \( M_{(peak)} \) to decrease with increasing VPA:AA in the feed, while the mol% VPA increases. These observations can be attributed to the low reactivity of the VPA monomer. From these results it was concluded that this method was suitable for producing PVPA-co-AA up to 40 mol% VPA, but no higher; alternative approaches were explored to reach polymers of higher mol% VPA.

Adding both the AA and AAPH over a long period of time biases the system in favour of the VPA, but it does not reach the levels of conversion associated with Bingöl’s method, which is performed over a shorter time period with minimal solvent.\(^{84}\)

Table 14. Results from experiments varying the feed ratio when just AA was added gradually.

<table>
<thead>
<tr>
<th>Feed Ratio (VPA:AA)</th>
<th>Total Conversion (%)</th>
<th>VPA Conversion (%)</th>
<th>Yield (%)</th>
<th>mol% VPA (EA)</th>
<th>mol% VPA (NMR)</th>
<th>( M_{(peak)} ) (g mol(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Commercial</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>30 (± 1.0)</td>
<td>34</td>
<td>11,000</td>
</tr>
<tr>
<td>40:60</td>
<td>79</td>
<td>57</td>
<td>66</td>
<td>32 (± 1.0)</td>
<td>27</td>
<td>474,000</td>
</tr>
<tr>
<td>50:50</td>
<td>66</td>
<td>53</td>
<td>61</td>
<td>34 (± 1.0)</td>
<td>32</td>
<td>318,000</td>
</tr>
<tr>
<td>70:30</td>
<td>60</td>
<td>43</td>
<td>50</td>
<td>56 (± 1.3)</td>
<td>52</td>
<td>20,000</td>
</tr>
<tr>
<td>90:10</td>
<td>38</td>
<td>33</td>
<td>30</td>
<td>86 (± 1.6)</td>
<td>78</td>
<td>9,000</td>
</tr>
<tr>
<td>100:0</td>
<td>60</td>
<td>56</td>
<td>45</td>
<td>100 (± 1.7)</td>
<td>100</td>
<td>9,000</td>
</tr>
</tbody>
</table>

Therefore the method was altered to have all of the initiator in the reaction vessel from the beginning, and adding the AA over a shorter time period before leaving the reaction to stir (Table
This would give the VPA the most exposure to the radical flux, while still keeping it at a higher concentration than the AA. The results follow the same trends as seen previously, with increasing VPA in the feed lowering the monomer conversions, yield and molecular weight while increasing the mol% VPA in the copolymer (Fig. 31). However, when compared to the previous attempts the results are much better, with higher VPA conversion for all the samples, especially for feed ratios over 70 mol% VPA. This results in copolymers with compositions of over 50 mol% VPA, which were previously unobtainable.

![Figure 31. A summary of data for the 3 methods of PVPA-co-AA synthesis.](image)

It should be noted that the copolymers produced have a very high polydispersity and reach very high molecular weight. In many cases this places at least some of the GPC trace beyond the range of the calibration curve, preventing accurate determination of $M_n$. Therefore the $M_{\text{peak}}$ value (i.e. the molecular weight that is most abundant as a fraction of mass) and direct comparison of the GPC traces were used to make comparisons between the polymers. In all cases, the molar masses of the polymers synthesised here are equal to or higher than that of the commercial polymer (Fig. 32).
3.4 Conclusions and Further Work

The straightforward free radical polymerisation of AA and VPA with AAPH as initiator is not suitable for the production of PVPA-co-AA as VPA is too unreactive compared to AA. Following the suggestion of a patent, gradual addition of the AA and AAPH was attempted, which gave some improvements, but was unsuitable for obtaining high VPA content copolymers. A third method, adding just the AA gradually, could be used to synthesise PVPA-co-AA with higher VPA contents.

In most cases the molecular weights of the polymers were much higher than the commercial polymer. This was addressed by further work in the Budd group by Rebecca Dey who used 1-octanethiol as a chain transfer agent to limit the masses of low VPA content copolymers. This meant that controlled experiments could be performed directly comparing PVPA-co-AA of different compositions, which was done for the chelation of calcium ions from solution (Fig. 33).
It was found that copolymers of approximately 30 mol% VPA were the most efficient at taking up calcium, implying that this may be the best composition for encouraging the mineralisation of tissue scaffolds.

Furthermore, cell assays performed by Dr Qi Guang Wang in the Faculty of Medical and Human Sciences (as yet unpublished) found that 30 mol% VPA copolymers in solution were the best for a range of biomarkers related to bone formation (alkaline phosphatase, mineralisation and collagen).
4 Electrospinning PVPA-co-AA

4.1 Introduction

Much of the work in this thesis, and the over-arching project, is devoted to the development of a bone tissue scaffold consisting of a structural component of PCL and a bioactive component of PVPA-co-AA. The work in this chapter concerns the electrospinning of PVPA-co-AA, followed by crosslinking in order to make the fibres water-stable, thereby making the PVPA-co-AA structural as well as bioactive.

There have been a few such transformations using PAA and a variety of polyols, heating to achieve crosslinking by ester formation. Therefore the applicability of this approach was explored for PVPA-co-AA and the resulting fibres characterised.

4.1.1 Aims and objectives

The aim of this chapter is to fabricate water-stable PVPA-co-AA nanofibres. This will be achieved by electrospinning copolymer solutions under various conditions to determine which produces the best quality nanofibres, and then crosslinking said nanofibres by the inclusion of ethylene glycol and esterification. The water stability of the nanofibres will then be tested.

4.2 Experimental

4.2.1 Materials

Methanol (≥99.8%, Sigma-Aldrich), ethylene glycol (anhydrous, 99.8%, Sigma-Aldrich), sulfuric acid (>95%, S.G. 1.83, Sigma-Aldrich) were used as supplied. PVPA-co-AA was synthesised by gradual addition of AA to VPA and AAPH, as described in Section 3.2.3 (34 mol% VPA, $M_w = 281,000$ g mol$^{-1}$).

4.2.2 Instrumentation

The electrospinning apparatus consisted of a syringe pump (Cole-Parmer two-syringe infusion pump) with a high voltage supply (Glassman FC Series) and a grounded aluminium foil collector in a fume hood fitted with an interlock.

Scanning Electron Microscopy (SEM) was performed using a Phenom G2 Pro instrument. Samples for SEM were carbon-coated to a depth of 20 nm prior to imaging using a Quorum Q150T Turbo-Pumped Carbon Coater. Fibre diameters were obtained using ImageJ.

ATR-FTIR was performed as described in Section 2.2.2.
4.2.3 Procedures

Electrospinning PVPA-co-AA
PVPA-co-AA was dissolved in sufficient methanol and water (4/1 v/v) to give an 18 wt% solution. Ethylene glycol was then added at 10 wt% (relative to the dry mass of PVPA-co-AA used), followed by 1 M H₂SO₄ (50 μL per mL of solution). A 21G needle was used at 10 kV with a 20 cm working distance and a flow rate of 0.2 mL h⁻¹. The resulting fibre mats were air-dried at ambient temperature over 24 h.

Crosslinking PVPA-co-AA
The fibre mats were placed in a vacuum oven at 110°C for 2 h to crosslink the PVPA-co-AA.

4.3 Results and Discussion
Electrospinning requires viscous polymer solutions, therefore the commercial PVPA-co-AA could not be used due to its low molecular weight; its $M_w$ is 31,000 g mol⁻¹ when polymers typically used for electrospinning are of at least 100,000 g mol⁻¹. Accordingly, a high molecular weight PVPA-co-AA was synthesised by gradual addition of AA, as described in Section 3.2.3. The resulting polymer was 34 mol% VPA and had an $M_w$ of 281,000 g mol⁻¹, as determined by aqueous GPC. Sufficient copolymer (> 6 g) was synthesised to allow many attempts at electrospinning, varying the conditions, with one batch of PVPA-co-AA for consistency between the experiments.

4.3.1 Electrospinning of PVPA-co-AA
Previous attempts at electrospinning PAA used polymers of high molecular weight, $M_v$ of 450,000 g mol⁻¹, and were most successful at concentrations of 2-8 wt%. The PVPA-co-AA that was to be spun was of a lower molecular weight, and so initial experiments were performed at 10 wt% in methanol (Table 15).

<table>
<thead>
<tr>
<th>Voltage (kV)</th>
<th>10</th>
<th>12.5</th>
<th>15</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.75</td>
<td>319 (±114)</td>
<td>297 (±109)</td>
<td>X</td>
</tr>
<tr>
<td>1.00</td>
<td>307 (±98)</td>
<td>312 (±102)</td>
<td>X</td>
</tr>
<tr>
<td>1.25</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
</tbody>
</table>

Table 15. Success of electrospinning and diameters (in nm) of the resulting nanofibres. An 'X' indicates that fibres could not be spun under these conditions. All of the successful attempts resulted in nanofibres of essentially the same diameter.
The flow rate and voltage were initially based on literature procedures and adjusted until a stable jet was formed. Small quantities of solution were electrospun and the resulting fibres were carbon-coated and imaged by SEM.

Higher flow rates resulted in dripping from the needle, while higher voltages led to spraying and the total depletion of the droplet, resulting in an unstable jet. Accordingly, these conditions were not suitable for electrospinning. The slower flow rates and lower voltages however appeared to electrospin well and all gave nanofibres with diameters of approximately 300 nm. However, the nanofibres suffered from beading, as can be seen from the SEM images (see Fig. 34).

The formation of beads during electrospinning usually indicates that the viscosity of the solution is too low; therefore further attempts were made using higher concentrations of the PVPA-co-AA.
However, increasing the copolymer concentration in methanol led to turbid solutions, implying that the copolymer was not fully dissolved. Consequently a small proportion of water was included with the methanol to improve the solubility. Solutions of 12.5, 15 and 20 wt% in methanol and water (4/1, v/v) were electrospun, using the previous conditions as a starting point (see Table 16). The resulting fibres were carbon-coated and imaged by SEM (see Fig. 35).

Table 16. Qualitative results for the electrospinning of PVPA-co-AA from 4/1 (v/v) methanol/water solutions. Beaded means that the resulting nanofibres are beaded, yarn means that the nanofibres attempt to bridge back from the collector to the needle tip. As can be seen, none of the conditions below were suitable for electrospinning smooth nanofibres.

<table>
<thead>
<tr>
<th>Concentration (wt%)</th>
<th>Flow rate (mL hour⁻¹)</th>
<th>Voltage (kV)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>10</td>
</tr>
<tr>
<td>12.5</td>
<td>1</td>
<td>Beaded</td>
</tr>
<tr>
<td>15</td>
<td>0.8</td>
<td>Beaded</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>-</td>
</tr>
<tr>
<td>20</td>
<td>0.8</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>-</td>
</tr>
</tbody>
</table>

The lower concentrations exhibited beading, regardless of the spinning conditions used, indicating that these solutions were not viscous enough for successful electrospinning. However, at 20 wt% the copolymer nanofibres begin forming yarns that stand clear of the collector; this appeared to become more prevalent as thicker fibre mats were deposited.

This phenomenon is known to occur for ionically charged polymers and is a function of their low electrical resistance. The polymer yarn initially stands proud of the collector and then touches the spinning jet, grounding the electrical charge and encouraging further deposition, causing the yarn to continue to grow towards the needle. This is not necessarily problematic; but it does shorten the distance between the needle tip and where the polymers are collected, resulting in the collection of solvent-rich fibres which amalgamate. In extreme cases, as occurred for the PVPA-co-AA here, the fibres form a bridge between the needle and the collector, preventing any further electrospinning from taking place.
Figure 35. SEM images of PVPA-co-AA nanofibres spun from 12.5 and 15 wt% methanol/water (4/1, v/v) solutions at x 500 magnification. All of the nanofibres exhibit significant bead defects.

Solutions with PVPA-co-AA concentrations of 15 wt% or below were found to be insufficiently viscous, while those of 20 wt% were vulnerable to forming yarn structures which hampered electrospinning. That this phenomenon did not occur significantly at lower concentrations indicated that a minimum rate of polymer deposition is required to grow the yarns before they can fall back onto the collector. Therefore further attempts were made at electrospinning using an intermediate concentration, 18 wt%, and a much slower flow rate, 0.2 mL h⁻¹. Formation of yarns was reduced, but still occurred over time. It was found that increasing the air flow rate of the fume hood from 0.6 m³ s⁻¹ to 2 m³ s⁻¹ prevented the formation of the yarns by pressing the deposited fibres onto the collector. The nanofibres spun under these conditions were imaged by SEM (see Fig. 36) and were found to be smooth with very few defects and an average diameter of 877 nm (± 333 nm). The diameter was significantly larger than for the previous attempts as the concentration of the solution, and hence its viscosity, was much higher.

The nanofibres were found to be smooth, with very few defects. These conditions were subsequently used to electrospin further fibres which also contained ethylene glycol in various concentrations, as well as a small quantity of sulfuric acid. These reagents were included to crosslink the PVPA-co-AA. The ethylene glycol can form two ester linkages, linking polymer chains within the nanofibres; the sulfuric acid is included to catalyse the reaction. The addition of these
components to the electrospinning solution was found to have no significant effect on the quality
or size of the resulting nanofibres.

Figure 36. SEM images of PVPA-co-AA nanofibres spun from 18 wt% methanol/water (4/1, v/v) solutions at x 2000
magnification. Smooth nanofibres with few defects are obtained under these conditions at all loadings of ethylene
glycol.

4.3.2 Crosslinking and Water Stability of PVPA-co-AA Nanofibres

Having successfully formed PVPA-co-AA nanofibres doped with ethylene glycol by electrospinning,
the next step was to crosslink the copolymer in the nanofibres. The required esterification was
achieved by simply heating the nanofibres to a high temperature (110°C) under vacuum (see
Scheme 2). The ethylene glycol and acidic groups condense to form esters and the resulting water
is driven off by the elevated temperature and low pressure, pushing the reaction forward.

Scheme 2. The crosslinking of PVPA-co-AA by esterification with ethylene glycol. Though the carboxylic ester is
shown, the phosphonate ester could also be formed.

Samples of the nanofibres before and after the heat treatment were examined by ATR-FTIR (see
Fig. 37) for evidence of the incorporation of ethylene glycol into the nanofibres, or that the
esterification reaction had occurred. The spectra showed no significant difference between the
PVPA-co-AA nanofibres before and after heat treatment, or indeed from pristine PVPA-co-AA.
Figure 37. ATR-FTIR spectra for the PVPA-co-AA nanofibres with a 10 wt% loading of ethylene glycol as spun and after heat treatment, with pure PVPA-co-AA for reference. There are no discernible differences between the spectra. This is not an entirely unexpected result as others have failed to detect such esterification reactions using this technique. An alternative strategy to establish whether crosslinking has occurred is to observe any differences in solution behaviour between the nanofibres with different loadings of ethylene glycol. Therefore the heat-treated nanofibres were immersed in water for 24 hours and observed for any changes (see Fig. 38).

Figure 38. a) Samples of the ethylene glycol containing PVPA-co-AA nanofibres after being heat-treated (left) and after being immersed in water for 24 hours (right); the 0 wt% sample dissolved completely, while the others remained cohesive mats. b) The swollen PVPA-co-AA nanofibres with 5 wt% ethylene glycol before and after drying. c) The swollen PVPA-co-AA nanofibres with 10 wt% ethylene glycol before and after drying.

After 24 hours the nanofibres without any ethylene glycol had dissolved entirely as expected, demonstrating that heat treatment alone is insufficient to stabilise PVPA-co-AA nanofibres. The sample with a 5 wt% ethylene glycol loading was fully transparent and had swollen greatly; on removal from the water the sample was found to be highly fragile, tearing when manipulated with
tweezers. The 10 wt% nanofibres appeared to be slightly turbid, and had retained trapped pockets of air; this sample was less swollen and was more robust, being transferred to a petri dish without sustaining any noticeable damage. That the PVPA-co-AA samples with ethylene glycol were insoluble in water demonstrates that the copolymer is crosslinked and that the esterification reaction was successful under the conditions used.

Once in glass petri dishes the samples were placed in a vacuum oven at 90°C and allowed to dry for 24 hours. The 5 wt% sample formed a thin layer of material that adhered strongly to the glass and could not be removed without damaging the sample; conversely the 10 wt% sample formed a thicker layer, which was robust enough to be removed and imaged by SEM (see Fig. 39).

![SEM images of PVPA-co-AA nanofibres after heat treatment and washing, where applicable, at x 2000 magnification. The quality and size of the nanofibres appear to be unaffected by the crosslinking of the PVPA-co-AA or by the action of water.](image)

The heat treatment of the nanofibre mats appears to have little effect on the quality of the nanofibres, which remain smooth with few defects. This implies that the high temperature (110°C) used to drive the esterification and crosslinking of the PVPA-co-AA, does not cause any significant softening, melting or degradation of the copolymer. The sample with an ethylene glycol loading of 10 wt% was suitable for SEM imaging after being hydrated and dried (Fig. 39).
Again, the quality of the nanofibres appears to be unaffected. However, the porosity of the nanofibre mats appears to be reduced. Presumably, when the nanofibres are hydrated they swell, become more flexible, and are able to move relative to one another; once the nanofibres dry out again, these new positions are retained, condensing the overall structure without affecting the individual nanofibres.

Table 17. Average diameters of PVPA-co-AA nanofibres spun from 18 wt% methanol/water (4/1, v/v) solutions with various loadings of ethylene glycol. The diameters are all within the margin of error and do not vary in a consistent manner, implying that the various treatments have no significant effect on the nanofibre diameters.

<table>
<thead>
<tr>
<th>Ethylene glycol</th>
<th>Average nanofibre diameters (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>As spun</td>
</tr>
<tr>
<td>0 wt%</td>
<td>877 (± 333)</td>
</tr>
<tr>
<td>5 wt%</td>
<td>858 (± 305)</td>
</tr>
<tr>
<td>10 wt%</td>
<td>889 (± 243)</td>
</tr>
</tbody>
</table>

The observation that the heat treatment and hydration of the crosslinked PVPA-co-AA has little effect on the quality of the nanofibres is supported by the average fibre diameters (see Table 17), which were found to be unaffected by the loading of ethylene glycol, the heat treatment and crosslinking, or the hydration and drying process, remaining close to 850 nm. It should be noted that these diameters are significantly larger than those of nanofibres electrospun from 10 wt% solutions (300 nm), which can be attributed to the higher viscosity of the 18 wt% solutions.

Figure 40. Environmental SEM images of partially hydrated, crosslinked PVPA-co-AA nanofibres at magnifications of x 1000 (left) and x 3000 (right). Even at the highest available pressure of water vapour (1 Torr) the samples dehydrated rapidly.

Attempts were made to image and analyse the nanofibres in the hydrated state using environmental SEM (see Fig. 40), where the higher pressure and elevated humidity of the sample
chamber could keep the sample hydrated, and negate the need for a conductive coating. Unfortunately, even under the highest available pressure of water vapour (1 Torr) the samples dehydrated rapidly, preventing any quantitative analysis. However, it can be seen that the fibrous structure is retained, though the nanofibres have swollen and eliminated the porosity of the structure, even when only partially hydrated.

4.4 Conclusions

The aim of the work in this chapter was to fabricate water-stable nanofibres of PVPA-co-AA, in order to demonstrate that the copolymer can be used as both the bioactive and structural component of a material suitable for investigation as a bone tissue scaffold. This aim has been achieved; using high molecular weight PVPA-co-AA (M_w of 281,000 g mol^{-1}) smooth nanofibres with diameters of 850 (± 300) nm were successfully electrospun. Ethylene glycol was incorporated into the nanofibres, and had no deleterious effects on their quality or size. The PVPA-co-AA was subsequently crosslinked by thermal esterification with the ethylene glycol, as demonstrated by the fact that the fibres were water-stable once heat-treated; in addition, a 10 wt% loading of ethylene glycol was found to be optimal as the resulting fibres were mechanically stable even when hydrated. In the hydrated state the porosity of the structure appears to be greatly reduced, but this is restored once the fibres are dried.
5 Synthesis of PCL-\textit{b}-PAA

5.1 Introduction

Previous work has produced bone tissue scaffolds by dipping PCL nanofibers in aqueous PVPA-co-AH solutions to coat the scaffolds in the bioactive polymer.\textsuperscript{5} This method is effective and has led to promising results in previous work, but it has some disadvantages: it is difficult to control the PVPA-co-AH content of the final scaffold and a coating may not be the best method of using the copolymer. It may be better to include the PVPA-co-AH in the bulk of the scaffold, in order to prevent, or at least slow, the loss of the copolymer from the scaffold.

PCL and PVPA-co-AH have no mutual solvents and so cannot be spun together under normal electrospinning conditions. However, they can be spun together as an emulsion: emulsion electrospinning. But this requires the use of surfactants to form the emulsion; amphiphilic block copolymers can be used as surfactants or compatibilisers for polymer solutions, especially if the blocks can be tailored to match the polymers concerned. Therefore a block copolymer of PCL and PAA was needed to act as a compatibiliser.

Making block copolymers of PCL and PAA is not straightforward as one is a polyester and the other is a polyacrylate, each of which requires a different type of polymerisation. One approach is to make one polymer then grow the second from it. In this case the PCL could be synthesised by ROP, a group capable of growing a radical on demand then appended to one end and then grow the PAA block using living radical polymerisation.

5.1.1 Atom Transfer Radical Polymerisation

One method that has been used for the creation of block copolymers is ATRP, which can generate radicals from secondary or tertiary halogens; moieties containing such groups can be appended to polymer chains in a number of ways. For example, PCL can be easily modified by esterification of the terminal alcohol.\textsuperscript{128}

ATRP is a controlled polymerisation technique based on atom transfer addition reactions such as the Kharasch addition reaction (see Fig. 41).\textsuperscript{129, 130} A free radical is generated from a haloalkane with a transition metal catalyst (generally a Cu complex with nitrogen-based, multidentate ligands) and the radical generated must be stabilised (i.e. it must be a secondary or tertiary radical). This radical then adds onto the alkene to generate a second, less stable, radical which recombines with the halogen.\textsuperscript{130}
It is possible for the second radical to react further, but this possibility is minimised by selecting substrates to minimise $K_1$ and maximise $K_2$, hence the importance of the second radical being less stable. In this way the concentration of free radicals is kept low, reducing the possibility of side reactions. However, if the second radical has more stability, then the reaction can be repeated (see Fig. 42), leading to multiple additions: polymerisation.

As with the addition reactions, Cu catalysts are the most common, though there are examples that use other metals (e.g. Fe, Ru, Mo). A wide variety of nitrogen-based ligands and haloalkane initiators have been developed. The ligands and initiators used for a polymerisation must be selected carefully as they can have large effects on the rate constants, and hence the extent to which the polymerisation is a controlled polymerisation. Other factors are the temperature, solvent (the transfer of the halogen to and from the metal centre is an inner sphere electron process, and is therefore influenced by the polarity of the solvent), and the monomer.
ATRP has been used successfully over a wide range of conditions for a variety of monomers, and a wide range of ATRP-based approaches has been developed.\textsuperscript{131}

While a powerful and versatile technique, ATRP does have limitations and drawbacks. Many of the transition metals used as catalysts are cytotoxic and removing them entirely from the finished polymer is not a trivial task, making it less suited for the development of biomaterials. Additionally, simple ATRP cannot be used for acidic monomers: it is thought that the low pH of such systems interferes with the catalyst, either by protonating the basic ligands or binding to the metal centre directly.\textsuperscript{131}

The ATRP synthesis of PCL-\textit{b}-PAA has been reported in the literature (see Scheme 3).\textsuperscript{134-136} PCL is synthesised by ROP of εCL, which is then reacted with an acyl bromide to insert the tertiary bromide via the terminal alcohol. AA cannot be added directly as the acidic monomer is incompatible with ATRP, therefore an acrylate ester is used, in this case the tert-butyl acrylate (tBA). The acrylate is then polymerized by ATRP. The final step is to cleave the acrylate ester to unmask the PAA; hence the selection of tBA, as it can be hydrolysed selectively leaving the PCL unaffected.\textsuperscript{137}

\begin{center}
\textbf{Scheme 3. Proposed reaction scheme for the synthesis of PCL-\textit{b}-PAA via ATRP.}\textsuperscript{128}
\end{center}
5.1.2 Nitroxide Mediated Polymerisation

Nitroxide mediated polymerisation (NMP) is an example of stable free radical polymerisation. The principle at the heart of NMP is the equilibrium between thermal dissociation and recombination of a nitroxide radical with the propagating polymer chain (see Fig. 43).

![Figure 43. A generalised scheme for NMP. The alkoxyamine bond undergoes thermolysis, generating a persistent nitroxide radical and a reactive carbon-centred radical which propagates. Like ATRP, having a low concentration of the reactive radicals reduces termination reactions.](image)

Nitroxides are well known persistent radicals that are stabilised due to delocalisation of the lone electron over both the N and O atoms (see Fig. 44). This stability is further enhanced by the use of bulky R groups around the N atom, these sterically limit the access of reactive molecules to the radical and prevent abstraction of a β hydrogen; which would generate a far more reactive, and short lived, carbon-centred radical.139

![Figure 44. a) Delocalisation over the N and O leads to a persistent radical; b) TEMPO is a well-known stable nitroxide, note that there is a methyl group in each β position.](image)

The first NMP experiments used conventional radical initiators to provide the reactive radicals to start the polymerisation and can be thought of as normal radical polymerisations performed in the presence of nitroxides.140 More recently, alkoxyamines (see Fig. 45) have been employed as ‘unimers’, a single molecule that has the nitroxide covalently bonded to an alkane; on exposure to
heat the C-O bond undergoes thermolysis, revealing the stabilised nitroxide radical and a reactive carbon-centred radical.$^{141}$

![Figure 45. Thermolysis of alkoxyamine ‘unimer’ generates a stabilised nitroxide and a reactive carbon-centred radical.](image)

Initial experiments with TEMPO found that it was only suitable for styrenes, and the reactions required very high temperatures and long reaction times; since then a selection of nitroxides and alkoxyamines have been developed, modifying the rate of dissociation and stabilities of the resultant radicals. With such a broad arsenal NMP is suitable for the synthesis of many polymers under a range of conditions.$^{142}$

NMP works for a wide range of monomers. It has been used to make complex polymers and the chemistry is readily tuneable by a number of parameters, such as temperature and solvent. In addition, the nitroxide is amenable to a wide range of chemistry, allowing for post-polymerisation modification and more complex polymer architectures.$^{138}$ Additionally, the initiators for NMP can be tailored to include groups suitable for other chemistries, for instance alcohols to initiate ROP, creating ‘double headed’ initiators that can begin both types of polymerisation.$^{143}$

### 5.1.3 Aims and objectives

To synthesise and characterise PCL-b-PAA and demonstrate that it is a block copolymer. In the first instance this will be attempted by a four-step route of ROP of εCL, end-group modification, ATRP of tBA and finally selective ester hydrolysis; this will be preceded by a test reaction for ATRP with tBA.

The second approach is to perform the ROP of εCL and NMP of tBA simultaneously, followed by selective hydrolysis of the tert-butyl ester. Again, this will be preceded by a test reaction to determine if tBA is a suitable monomer for NMP.

A secondary aim will be to see if these methods can be extended to allow the creation of PCL-b-PVPA by the polymerisation of DMVP.
5.2 Experimental

5.2.1 Materials
tert-Butylamine (99.5%, Aldrich), pivaldehyde (96%, Alfa), calcium hydride (CaH₂, 99.9%, Aldrich), diethylphosphite (98%, Aldrich), 3-chloroperoxybenzoic acid (mCPBA, 77%, Aldrich), pyridine (anhydrous, 99.8%, Sigma-Aldrich), ethylene glycol (anhydrous, 99.8%, Sigma-Aldrich), 2-bromo-2-methylpropanoyl bromide (98%, Aldrich), N,N,N',N''-pentamethyldiethylenetriamine (PMDETA, 99%, Aldrich), 1,1,4,7,10,10-hexamethyltriethylenetetramine (HMTETA, 97%, Sigma-Aldrich), 2,2'-bipyridyl (Bipy, ≥99%, Sigma-Aldrich), copper (99.7%, 3 μm powder, Sigma-Aldrich), trifluoroacetic acid (TFA, 99%, Sigma-Aldrich), sodium hydroxide (NaOH, ≥98%, Sigma-Aldrich), CDCl₃ (99.8% D atom %, Sigma-Aldrich) and DMSO-d₆ (≥99.96 atom % D, Sigma-Aldrich) were used as supplied. Copper(I) bromide (98%, Sigma-Aldrich) was purified by stirring with acetic acid for 24 h followed by washing with ethanol and storage under vacuum. tert-Butyl acrylate (tBA, 98%, Aldrich) was passed through a basic alumina column and dried over activated 3 Å molecular sieves. ε-Caprolactone (εCL, 99%, Alfa), tetrahydrofuran (THF, 99.9%, Fisher), dichloromethane (DCM, ≥99.5%, Sigma-Aldrich), dimethylformamide (DMF, ≥99.9%, Sigma-Aldrich) and toluene (≥99.5%, Sigma-Aldrich) were dried in turn over activated 3 Å molecular sieves for 48 h before use. Sn(Oct)₂ (95%, Aldrich) was dried under vacuum for 24 h prior to use.

5.2.2 Instrumentation
THF GPC was performed with a Viscotek GPCmax VE2001 instrument equipped with a PL 2MB500A column set, at 35°C using THF as the eluent with a series of polystyrene calibrants. The injection volume was 100 μL and the flow rate was 1 mL min⁻¹. Samples were prepared by dissolving in THF to a concentration of 1 mg mL⁻¹ and passing through a 0.45 μm Millipore Millex PTFE filter.

Mass spectroscopy was performed using a Waters SQD2, with electrospray ionisation.

Dynamic Light Scattering (DLS) was performed using a Malvern Zetasizer Nano Z instrument. Solutions had a concentration of 0.25 mg mL⁻¹, were passed through a 0.22 μm pore size filter and placed in disposable plastic cuvettes.

NMR, ATR-FTIR, elemental analysis and aqueous GPC were performed as described in Section 2.2.2.
5.2.3 Reactions and Procedures

Synthesis of ATRP Initiator

Butan-1-ol (1.5 mL, 1.22 g, 16.4 mmol) and pyridine (1.46 mL, 1.43 g, 18.0 mmol) were dissolved in dry DCM (20 mL) and cooled to 0°C. 2-Bromo-2-methylpropanoyl bromide (2.27 mL, 4.15 g, 18.0 mmol) was then added dropwise. The reaction was allowed to warm to room temperature and stirred for 18 h. The insoluble salts were removed by filtration and the filtrate washed with saturated NaHCO₃ (2 × 40 mL). The organic layer was evaporated to dryness under reduced pressure before being stirred with CaH₂ for 1 h and the product collected by vacuum distillation. The product was afforded as a clear oil (2.78 g, 76% yield).

¹H NMR (400 MHz, CDCl₃) δ= 4.19 (t, J = 6.6 Hz, 2H), 4.23 - 4.15 (m, 2H), 1.94 (s, 6H), 1.78 - 1.57 (m, 2H), 1.49 - 1.38 (m, 2H), 0.96 (t, J = 7.3 Hz, 3H)

MS (70eV): m/z (%): 245 (50) [M⁺Br]+Na⁺], 247 (50) [M布拉]+Na⁺]

Typical ATRP Synthesis of PtBA

ATRP initiator (42.4 mg, 0.19 mmol), copper(I) bromide (33 mg, 0.23 mmol), tBA (5.71 mL, 5 g, 39 mmol), PMDETA (0.10 mL, 81 mg, 0.47 mmol) and dry toluene (3.94 mL) were placed in a 2-necked round-bottomed flask and degassed by three freeze-pump-thaw (FPT) cycles. The reaction vessel was then placed in an oil bath preheated to 100°C and stirred under N₂ for 24 h. The reaction was then terminated by exposing to air. The reaction mixture was dissolved in THF and passed through a basic alumina column and then evaporated to dryness, yielding an off-white polymer (2.95 g, 59% yield).

¹H NMR (CDCl₃, 400 MHz): δ= 2.06-2.27 (m, 1H), 1.66-1.84 (m, 0.5H), 1.42-1.56 (m, 1.5H), 1.37 (br. s., 9H)
Synthesis of PCL

\[
\text{\( \varepsilon \)-CL (27.8 mL, 30.0 g, 263 mmol), butan-1-ol (0.16 mL, 130 mg, 1.75 mmol) and Sn(Oct)$_2$ (0.28 mL, 355 mg, 0.876 mmol) were placed in a dry 2-necked round-bottomed flask and dissolved in dry DMF (30 mL) under N$_2$. The flask was fitted with a water condenser and lowered into a 120°C oil bath. The reaction was left to stir for 24 h before cooling to room temperature. The reaction mixture was precipitated in cold methanol (1.5 L) and the polymer collected by filtration and washed with methanol (3 \times 200 mL). The resultant solid was left to dry under vacuum for 24 h giving a white polymer (28.56 g, 95% yield). The NMR spectrum corresponds to the literature.}^{128}
\]

\(^1^\text{H} \text{ NMR (CDCl}_3, 400 \text{ MHz): } \delta = 4.06 (t, J = 6.7 \text{ Hz, } 2\text{H}), 3.64 (t, J = 6.6 \text{ Hz, } \*\text{H}), 2.30 (t, J = 7.4 \text{ Hz, } 2\text{H}), 1.56-1.73 (m, 4\text{H}), 1.33-1.44 (m, 2\text{H})

Synthesis of PCL macroinitiator

\[
\text{PCL (28 g, 1.867 mmol) and pyridine (1.51 mL, 1.48 g, 18.67 mmol) were dissolved in dry THF (50 mL) and cooled to 0°C. 2-Bromo-2-methylpropanoyl bromide (2.31 mL, 4.29 g, 18.67 mmol) was then added dropwise. The reaction was allowed to warm to room temperature and stirred for 18 h. The reaction mixture was precipitated in cold methanol (2 L) and the polymer collected by filtration and washed with methanol (3 \times 200 mL). The resultant solid was left to dry under vacuum for 24 h, giving a white polymer (4.39 g, 97% yield). The NMR spectrum corresponds to the literature.}^{128}
\]

\(^1^\text{H} \text{ NMR (CDCl}_3, 400 \text{ MHz): } \delta = 4.06 (t, J = 6.7 \text{ Hz, } 2\text{H}), 2.31 (t, J = 7.4 \text{ Hz, } 2\text{H}), 1.93 (s, 0.08\text{H}), 1.59-1.72 (m, 4\text{H}), 1.34-1.44 (m, 2\text{H})
**Typical ATRP Synthesis of PCL-b-PtBA**

PCL macroinitiator (10 g, 0.714 mmol), copper(I) bromide (123 mg, 0.857 mmol), tBA (20.93 mL, 18.31 g, 143 mmol), PMDETA (0.36 mL, 297 mg, 1.714 mmol) and dry toluene (25 mL) were placed in a 2-necked round-bottomed flask and degassed by three cycles of FPT. The reaction vessel was then placed in an oil bath preheated to 100°C and stirred under N₂ for 24 h. The reaction was then terminated by exposing to air. The reaction mixture was dissolved in THF and passed through a basic alumina column. The polymer solution was then concentrated under reduced pressure and precipitated in cold hexane (1.5 L). The solid was collected by filtration, washed with hexane (3 × 250 mL) and left to dry under vacuum for 24 h. An off-white polymer was obtained (16.54 g, 58%). The NMR spectra corresponds to the literature.\(^{128}\)

\(^1\)H NMR (CDCl₃, 400 MHz): \(\delta = 4.04\) (t, \(J = 6.7\) Hz, 2H, CCH₂O in PCL), 2.29 (t, \(J = 7.4\) Hz, 2H, O₂CCH₂ in PCL), 2.13-2.24 (m, 2H, CH in PtBA), 1.73-1.88 (m, 1H, meso CH₂ in PtBA), 1.57-1.69 (m, 4H, CH₂ in PCL), 1.48-1.56 (m, 2H, racemo CH₂ in PtBA), 1.42 (br. s., 20H, CH₃ and meso CH₂ in PtBA), 1.30-1.37 ppm (m, 2H, CH₂ in PCL)

**tert-Butyl Hydrolysis of PCL-b-PtBA**

TFA, DCM
PCL-b-PtBA (3.7 g, 0.106 mmol [20.97 mmol of tert-butyl groups]) was dissolved in dry DCM (100 mL) and cooled to 0°C under N$_2$. TFA (24 mL, 35.9 g, 314.8 mmol) was added gradually. The reaction was left to stir at room temperature for 48 h and then precipitated into cold hexane (2 L). The solid was collected by filtration, washed with hexane (4 × 200 mL) and dried under vacuum. This solid was then ground into a powder and washed with acetonitrile (3 × 300 mL) and dried under vacuum for 24 h. An off-white solid was obtained (1.863 g, 74% yield). The NMR spectra corresponds to the literature.\textsuperscript{128}

$^1$H NMR (DMSO-d$_6$, 400 MHz): $\delta$ = 11.92-12.33 (m, 1H, COOH in PAA), 3.98 (t, $J$ = 6.4 Hz, 2H, CCH$_2$O in PCL), 2.24-2.31 (m, 2H, O$_2$CCH$_2$ in PCL), 2.14-2.24 (m, 2H, CH in PAA), 1.68-1.83 (m, 2H, meso CH$_2$ in PAA), 1.36-1.62 (m, 6H, CH$_2$ in PCL and racemo CH$_2$ in PAA), 1.22-1.34 ppm (m, 2H, CH$_2$ in PCL).

**Synthesis of the diethyl (1-(tert-butylamino)-2,2-dimethylpropyl)phosphonate**

\begin{align*}
&\text{tert-Butylamine (1.829g, 25 mmol) and pivaldehyde (2.153 g, 25 mmol) were stirred in a round-bottomed flask at 40°C under N$_2$. After one hour, diethyl phosphite (6.905 g, 50 mmol) was added and the reaction stirred for a further 24 h. The reaction mixture was diluted with diethyl ether (50 mL) and extracted with 5% HCl solution (50 mL). The aqueous layer was neutralised with NaHCO$_3$ and extracted with ether (2 × 50 mL), the organic layers were dried over MgSO$_4$ and evaporated under reduced pressure. The resulting oil was purified by column chromatography using ethyl acetate and hexane (1:1) as the eluent. The pure product was obtained as a clear oil (4.599 g, 66% yield). The NMR spectra correspond to the literature.}\textsuperscript{144}
\end{align*}

$^1$H NMR (CDCl$_3$, 400 MHz): $\delta$ = 3.98-4.13 (m, 4H), 2.70 (d, $J$ = 17.9 Hz, 1H), 1.25-1.32 (m, 6H), 1.07 (s, 9H), 1.01 ppm (s, 9H)

$^{13}$C NMR (CDCl$_3$, 101 MHz): $\delta$ = 61.1, 60.0, 58.7, 50.8, 35.1, 30.6, 27.8, 16.4 ppm

$^{31}$P{$^1$H} NMR (CDCl$_3$, 162 MHz): $\delta$ = 30.50 ppm (s., 1P)

MS (70eV): m/z (%): 280 (30) [M+H$^+$], 142 (100) [C$_9$H$_{20}$N$^+$]
Synthesis of the N-tert-butyl-N-1-diethylphosphono-(2,2-dimethylpropyl) (SG1)

Diethyl (1-(tert-butylamino)-2,2-dimethyl(propyl)phosphonate (10.0 g, 35.8 mmol) was dissolved in DCM (50 mL) and cooled to 0°C. mCPBA (8.65 g, 50.1 mmol) was dissolved in DCM (250 mL) and added dropwise. The reaction mixture was warmed to room temperature and stirred for 18 h; the reaction mixture went orange in colour in this time. The reaction was washed sequentially with saturated aqueous NaHCO$_3$ (200 mL), H$_2$O (200 mL), 5% HCl solution (200 mL), H$_2$O (200 mL), saturated aqueous NaHCO$_3$ (200 mL) and H$_2$O (200 mL). The organic layer was then dried over MgSO$_4$ and evaporated to dryness to give an orange oil. This was purified by column chromatography using ethyl acetate and hexane (1:2) as the eluent. The pure product was obtained as a distinctive orange oil (6.83 g, 65% yield).

MS (70eV): m/z (%): 295 (90) [M+H$^+$], 158 (65) [C$_9$H$_{13}$NO+H$^+$]

Synthesis of 2-hydroxyethyl-2-bromo-2-methylpropanoate

Pyridine (3.16 g, 3.24 mL, 40 mmol) was dissolved in ethylene glycol (19.86 g, 17.85 mL, 320 mmol) and cooled to 0°C. 2-Bromo-2-methylpropanoyl bromide (9.196 g, 5.03 mL, 40 mmol) was then added dropwise and the reaction solution was stirred for 6 h. The reaction mixture was then diluted with DCM (100 mL) and washed with H$_2$O (2 × 100 mL), then saturated aqueous NaHCO$_3$ (2 × 100 mL) and H$_2$O (100 mL). The organic layer was then dried over MgSO$_4$ and evaporated to dryness. The resulting clear oil was purified by column chromatography using ethyl acetate and hexane (1:1) as the eluent. The pure product was obtained as a clear oil (6.153 g, 77% yield). The NMR spectra correspond with the literature.\textsuperscript{145}

$^1$H NMR (CDCl$_3$, 400 MHz): δ = 4.28 (t, $J = 4.5$ Hz, 2H), 3.84 (t, $J = 4.5$ Hz, 2H), 2.46 (br. s., 1H), 1.93 ppm (s, 6H)
Synthesis of Dual Initiator (DI) by Nitroxide Coupling

SG1 (2.94 g, 10 mmol), 2-hydroxyethyl-2-bromo-2-methylpropanoate (4.22 g, 20 mmol) and PMDETA (6.93 g, 40 mmol) were dissolved in toluene (20 mL) and degassed by one cycle of FPT and then refrozen. Copper (0.636 g, 10 mmol) and copper(I) bromide (2.87 g, 20 mmol) were added and the reaction mixture degassed by two further cycles of FPT. The reaction was then stirred under N₂ at room temperature for 20 h. The mixture was then passed through a neutral alumina column with ethyl acetate and evaporated to dryness. The resulting yellow oil was purified by column chromatography using ethyl acetate and hexane (1:1) as the eluent. The pure product was obtained as a clear oil (0.958 g, 23% yield), which solidified on storage in the fridge. The NMR spectra correspond to the literature.¹⁴⁶

¹³C NMR (CDCl₃, 101 MHz): δ = 171.8, 67.3, 60.6, 55.8, 30.6 ppm

MS (70eV): m/z (%): 210 (100) [M⁺]

¹³C NMR (CDCl₃, 101 MHz): δ = 175.3, 83.3, 70.6, 68.9, 62.3, 62.3, 60.4, 58.9, 35.8, 29.0, 28.9, 27.4, 22.9, 16.6, 16.1 ppm

³¹P{¹H} NMR (CDCl₃, 162 MHz): δ = 26.12 ppm (s, 1P)

MS (70eV): m/z (%): 426 (55) [M+H⁺], 873 (100) [2M+Na⁺]
**Typical Nitroxide Mediated Polymerisation of tBA**

![Chemical structure of tBA](image)

typ BA (6.72 g, 52.4 mmol) and dual initiator (56 mg, 0.131 mmol) were placed in a round-bottomed flask and degassed by 3 cycles of FPT. The reaction vessel was then placed in an oil bath pre-set to 115°C and stirred under N₂ for 24 h. The resulting polymer was dissolved in THF, precipitated in methanol and water (1:1, 800 mL) and dried under vacuum to afford a white solid (6.172 g, 91% yield).

^1H NMR (CDCl₃, 400 MHz): δ = 2.12-2.36 (m, 1H), 1.74-1.93 (m, 1H), 1.49-1.63 (m, 1H), 1.44 ppm (br. s., 9H)

**Typical Simultaneous Polymerisation of tBA and εCL**

To a dry 2-necked flask was added dual initiator (112 mg, 0.262 mmol), SG1 (0.5 mg, 0.00262 mmol), Sn(Oct)₂ (54 mg, 0.132 mmol), εCL (7.87 g, 68.91 mmol), tBA (2.62 g, 20.4 mmol) and toluene (4.1 mL). The reaction mixture was degassed by three cycles of FPT. The reaction vessel was then placed in an oil bath pre-set to 110°C and stirred under N₂ for 24 h. 0.5 mL samples were taken to follow the progress of the reaction. Once complete, the reaction mixture was cooled to room temperature and exposed to air. The resulting intractable gel was dissolved in THF and then evaporated to dryness. The resulting solid was then dissolved in THF (100 mL) and precipitated in...
methanol and water (1:1, 800 mL). The precipitate was dried under vacuum, affording a white solid (7.389 g, 93% yield).

$^1$H NMR (CDCl$_3$, 400 MHz): $\delta = 4.05$ (t, $J = 6.7$ Hz, 2H, CCH$_2$O in PCL), 3.64 (t, $J = 6.6$ Hz, <1H), 2.30 (t, $J = 7.6$ Hz, 2H, O$_2$CH$_2$ in PCL), 2.07-2.26 (m, 1H, CH in PtBA), 1.73-1.90 (m, 1H, meso CH$_2$ in PtBA), 1.57-1.71 (m, 4H, CH$_2$ in PCL), 1.50-1.56 (m, 1H, racemo CH$_2$ in PtBA), 1.43 ppm (br. s., 15H, CH$_3$ and meso CH$_2$ in PtBA), 1.32-1.40 ppm (m, 3H, CH$_2$ in PCL)

Typical PCL Hydrolysis

PCL-b-PtBA (792 mg, 1.54 mmol of εCL in PCL chains) was placed in a round-bottomed flask and dissolved in THF (40 mL). Water (5 mL) was then added followed by NaOH (247 mg, 6.17 mmol) and the reaction solution stirred at room temperature for 7 days. The reaction mixture was then neutralised with 5% aqueous HCl and THF was evaporated under reduced pressure, the solid collected by filtration and washed with further water. The precipitate was dried under vacuum, affording an off-white solid (221 mg, 36% yield).

$^1$H NMR (CDCl$_3$, 400 MHz): $\delta = 2.23$ (m, 1H), 1.83 (m, 1H), 1.50-1.68 (m, 1H), 1.44 ppm (br. s., 9H)

Preparation of Micellar Solutions

50 mg of PCL-b-PAA was dissolved in 5 mL of THF and added dropwise to 50 mL of water with stirring. The solution was left at ambient temperature until all the THF had evaporated. The solutions were diluted 4-fold with water. The pH was measured using a Hanni Instruments HI2211 pH probe and adjusted by the addition of 1 M HCl and 1M NaOH. The samples were then analysed by DLS.
5.3 Results and Discussion

5.3.1 Synthesis of PCL-b-PAA via ATRP

An initial test reaction was performed to establish the effectiveness of ATRP for the polymerisation of tBA (see Scheme 4). A suitable initiator was prepared by reacting 2-bromo-2-methylpropanoyl bromide with butanol in the presence of pyridine; the reaction was effective, with a yield of 76% after purification. This initiator was chosen for its similarity to the macroinitiator used for the PCL-b-PAA in Scheme 3 (i.e. a dimethyl bromide adjacent to an ester). The ATRP was performed as reported previously\textsuperscript{128} and afforded PtBA.

\begin{align*}
\text{HO-CH}_2 & \quad \rightarrow \quad \text{Br-CH}_2-\text{COO} \quad \text{Br} \quad \text{a) DCM, pyridine, 2-bromo-2-methylpropanoyl bromide, 0\textdegree C, 18 h;} \\
\text{Br-CH}_2-\text{COO} & \quad \rightarrow \quad \text{Br-CH}_2-\text{COO} \quad \text{Br} \quad \text{b) toluene, CuBr, PMDETA, tBA, 100\textdegree C, 24 h;} \\
\text{Br-CH}_2-\text{COO} \quad \text{Br} & \quad \rightarrow \quad \text{Br-CH}_2-\text{COO} \quad \text{OH} \quad \text{c) DCM, TFA, 48 h.}
\end{align*}

Scheme 4. Reaction scheme for the test synthesis of PAA by ATRP of tBA followed by selective hydrolysis of the tert-butyl ester. a) DCM, pyridine, 2-bromo-2-methylpropanoyl bromide, 0\textdegree C, 18 h; b) toluene, CuBr, PMDETA, tBA, 100\textdegree C, 24 h; c) DCM, TFA, 48 h.

The reaction was successful, as was the purification by filtering through an alumina column. It is vital that the final polymer contains minimal residual copper ions as they are known to be cytotoxic. The copper was adsorbed to the top few millimetres of the alumina bed, giving a blue band. The PtBA afforded was off-white in colour and ICP-OES determined that the copper content of the final polymer was <0.3 wt%. The polymerisation had a moderate yield of 59% and produced PtBA with $M_n$ of 13,500 g mol\textsuperscript{-1} and $M_w$ of 20,000 g mol\textsuperscript{-1}. 

96
Figure 46. $^1$H NMR spectrum of PAA. The peak at 1.32 ppm is for residual tert-butyl groups while the peak at 2.29 ppm corresponds to the methine protons. The peak at 1.66 ppm is for the methylene protons in racemo diads, the protons are in equivalent environments and so have the same chemical shift, the peaks at 1.83 and 1.51 ppm correspond to the methylene protons for the meso diad, which are inequivalent.

The hydrolysis reaction was performed using TFA in DCM. After 48 hours, the reaction was stopped but was found to have only gone to 98% completion. It was decided that for future syntheses it would be necessary to increase the equivalents and/or concentration of TFA to enable fast, quantitative hydrolysis of the tert-butyl esters. This test reaction also highlighted the potential difficulties in removing residual TFA, but demonstrated that $^{19}$F NMR was a suitable method for identifying traces of this acid. Examining the diads of the PAA using $^1$H NMR it was found that the PAA was atactic with 1.15 meso to 1 racemo diads (see Fig. 46).$^{111,147}$

The synthesis of PCL-b-PAA via the ATRP route began with the ROP of PCL using Sn(Oct)$_2$ as a catalyst and n-butanol as an initiator. After work-up, the $^1$H NMR spectrum (Fig. 47) indicated that all the monomer had been removed and that the product was PCL.

Figure 47. $^1$H NMR spectrum of PCL synthesised by ROP with Sn(Oct)$_2$.

Additionally, the $^1$H NMR spectrum could be used to estimate the $M_n$ of the polymer via end-group analysis. The ‘e’ protons appear at 4.06 ppm for most of the polymer chain, but are shifted
to 3.64 ppm for the final repeat unit as they are adjacent to an alcohol rather than an ester. The degree of polymerisation is given by the ratio of alcohol to ester groups (i.e. the ratio of terminal to chain ‘e’ protons). In this way the $M_n$ was calculated. THF GPC was also used to determine the apparent $M_n$, $M_w$ and polydispersity of the polymer (see Table 1).

Figure 48. $^1$H NMR spectrum for the PCL macroinitiator.

This PCL was then reacted with excess acyl bromide to yield a PCL macroinitiator. The reaction was judged to have gone to completion by the migration of the terminal ‘e’ proton peak in the $^1$H NMR spectrum (see Fig. 48) to 4.18 ppm, and the appearance of a peak at 1.93 ppm corresponding to the new methyl groups (‘f’ protons). Again, these groups were suitable for end-group analysis and further information was gained by THF GPC (see Table 1).

Figure 49. $^1$H NMR spectrum for PCL-b-PtBA produced by ATRP.

The conditions for the ATRP of tBuA with the PCL macroinitiator were kept the same as for the test reaction, and this polymerisation was successful. It is not possible to estimate the $M_n$ using end-group analysis for the copolymer as the peaks for the PtBA overlap with the peaks of the f protons of the PCL macroinitiator (see Fig. 49). However, the molar ratio of PCL to PtBA can be calculated by comparison of the integral for proton ‘e’ with the integrals for protons ‘a’ and ‘h’,
allowing for an estimation of $M_n$ if the degree of polymerisation of the PCL block is assumed to be the same as for the PCL macroinitiator.

![Figure 50. Contrasting mechanisms for the hydrolysis of typical esters (above) and tert-butyl esters (below) under acidic conditions. In the absence of suitable nucleophiles typical esters are stable, while the tert-butyl ester is cleaved.](image)

The tert-butyl group can be hydrolysed selectively in the presence of other esters (i.e. the PCL chain) as the mechanism differs from that of regular ester hydrolysis (see Fig. 50). Rather than the carbonyl being attacked by nucleophiles, tert-butyl hydrolysis proceeds by the loss of a tertiary carbocation. As the mechanism is completely different and does not require the presence of a nucleophile, the reaction is highly selective as long as conditions are anhydrous.

The estimates of $M_n$ were used to calculate the number of moles of tert-butyl groups present, which was used as the basis for calculating the equivalents of TFA required for hydrolysis. Bearing in mind the fact that the test hydrolysis did not go to completion with 5 equivalents of TFA, and the inaccuracy of the $M_n$ estimates, the number of equivalents of TFA was raised to 15. The reaction was successful, but removing the final traces of TFA proved problematic.

![Figure 51. FTIR of PCL-b-PAANa (blue) and PCL-b-PAA (red). Note the disappearance of the peak at 1570 cm$^{-1}$, which corresponds to carboxylate salts, and the appearance of carboxylic acid O-H stretches for the polymer purified by washing compared to the polymer obtained by extraction with NaHCO$_3$.](image)
The original intention was to dissolve the polymer in DCM and extract out the TFA with saturated NaHCO₃ solution. Unfortunately this produced an emulsion which separated slowly, resulting in low yields of material which proved to be insoluble in DMSO. FTIR studies of the solid eventually obtained (Fig. 51) lacked a carboxylic acid O-H stretch but had an unexpected peak at 1570 cm⁻¹, which is indicative of the C=O stretch of an anionic carboxylate salt. Therefore it seems likely that the material obtained from this method was the sodium salt: PCL-b-PANa.

Subsequently washing was attempted with n-hexane and acetone, both of which were found to be ineffective, as well as methanol which removed the TFA but suffered from very low yields. The most effective solution was to wash the solid gained from the precipitation in n-hexane with acetonitrile. Samples did not fully dissolve in CDCl₃ and ¹H NMR spectra run in this solvent only showed peaks corresponding to PCL. ¹H NMR spectra run in DMSO-d₆ however indicated the presence of both PCL and PAA. This indicates interesting solvation behaviour of the copolymer due to it having such radically different components; only the PCL block dissolves in CDCl₃, so only these peaks are evident (see Fig. 52). Moreover, the CDCl₃ ¹H NMR is free of ‘e’ protons at 3.64 ppm, which indicates that the esters in the PCL blocks have not undergone hydrolysis during the selective hydrolysis step as there is no terminal alcohol present.

The molecular weights for the PCL-b-PAA cannot be determined using ¹H NMR as it suffers the same problems with peak overlap as PCL-b-PtBA, with the additional difficulty of the NMR needing to be run in a different solvent. PCL-b-PAA is soluble in THF which is the eluent for the GPC; however it is eluted extremely quickly from the column, indicating that it is either being rejected from the column media by unfavourable interactions or forming aggregates of large hydrodynamic volume. Therefore the molar mass of the PCL-b-PAA cannot be determined using these methods.
Table 18. Yields and molar masses (g mol\(^{-1}\)) for the polymerisations above. * Indicates that the measurement is an estimate based in part on data from previous samples.

<table>
<thead>
<tr>
<th>Polymer</th>
<th>Yield (%)</th>
<th>Molar masses (g mol(^{-1}))</th>
<th>THF GPC</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>M(_n)</td>
<td>M(_n^*)</td>
</tr>
<tr>
<td>PCL</td>
<td>95</td>
<td>17,100</td>
<td>14,600</td>
</tr>
<tr>
<td>PCL macroinitiator</td>
<td>97</td>
<td>17,100</td>
<td>14,000</td>
</tr>
<tr>
<td>PCL-b-PtBA</td>
<td>58</td>
<td>42,700</td>
<td>34,000*</td>
</tr>
</tbody>
</table>

The ROP of εCL proceeded well, reaching a high yield and the resulting PCL had a similar molecular weight to what was expected (see Table 18). The polydispersity is quite high, but this is to be expected due to transesterification side-reactions that occur during the polymerisation. The conversion of the free alcohol chain end was nearly quantitative, with no trace of the unmodified PCL being detected by \(^1\)H NMR. Any loss can be attributed to the work-up by precipitation, which would remove the lowest mass PCL (i.e. any oligomeric chains) thus explaining the increase in molar mass and lowering of polydispersity indicated by GPC. The yield of the ATRP step was modest at 58%, but comparable to other reports.\(^{134}\) This is due to incomplete conversion of the \(t\)BA monomer, which is reflected in the molar mass of the PCL-b-PtBA being lower than expected. Selective hydrolysis of the \(t\)ert-butyl ester to obtain the final PCL-b-PAA proceeded well, with no \(t\)ert-butyl groups being detected by \(^1\)H NMR and no evidence of any damage to the PCL chain. However, no data could be obtained on the molar mass of the final polymer due to the overlap of peaks in its \(^1\)H NMR spectrum and its incompatibility with the GPC protocol. Elemental analysis of the PCL-b-PAA found that trace copper was still evident in the copolymer, which is not ideal for biological applications as copper is known to be cytotoxic.

To conclude this section, the four-step ATRP synthesis of PCL-b-PAA is effective, but time-consuming and leaves traces of copper which are non-trivial to remove. This makes this route less attractive for biological applications, and necessitates an alternative approach.

5.3.2 Synthesis of PCL-b-PAA via NMP

NMP is an alternative method for controlled free radical polymerisation, and has been used with success for the polymerisation of \(t\)BA.\(^{148-150}\) Of particular interest is work by Chagneux and Trimaille where a double-headed initiator approach was used to synthesise PCL-b-P(nBA) by both sequential and simultaneous polymerisations.\(^{151}\)
The ability to perform both reactions simultaneously is a great benefit of this approach, as it allows a range of PCL-b-PAA copolymers to be synthesised in short order. The synthesis of the dual initiator (DI, Fig. 53) began with synthesis of \textit{N}-tert-butyl-N-1-diethylphosphono-(2,2-dimethylpropyl), a nitroxide commonly known as SG1.\textsuperscript{144}

SG1 has been found to be highly active for polymerisations and has a high degree of stability. The rationale behind including a phosphonate is that the steric bulk of the group increases the rate of dissociation of the corresponding alkoxyamines, hence increasing the rate of propagation of the polymer chain. They are stable, despite having a hydrogen atom in the β position; the \textit{tert}\textendash butyl groups, in combination with the phosphonate, force the molecule to adopt a conformation where the orbitals of the C-H bond are orthogonal to the free radical (Fig. 54), helping to prevent any reaction.

The nitroxide precursor, diethyl (1-(\textit{tert}-butylamino)-2,2-dimethylpropyl)phosphonate, is formed by stirring \textit{tert}-butylamine with pivaldehyde in bulk, forming the imine intermediate which is then
attacked by the phosphite. This precursor is readily oxidised by mCPBA in DCM to give SG1, which is easily purified by column chromatography to give an orange oil.\textsuperscript{144}

Reacting an acyl bromide with a large excess of anhydrous ethylene glycol gave 2-hydroxyethyl-2-bromo-2-methylpropanoate. This tertiary bromide that then undergoes a free radical addition reaction; a copper catalyst abstracts the bromide leaving a free radical which is then trapped by the SG1 to leave the alkoxyamine.\textsuperscript{142}

The copper is removed by passing through an alumina column and the product purified by column chromatography to give a clear oil, which solidifies upon refrigeration. The first three reactions (see Scheme 5) have moderately good yields, while the coupling reaction has a poor yield. This can be attributed to homocoupling of the radicals during the course of the reaction and thermal degradation of the DI at room temperature, necessitating cold storage.

![Scheme 5](attachment:image.png)

Scheme 5. Scheme for the synthesis of the dual initiator. Reaction conditions: a) 40°C, 24 h; b) pyridine, 0°C to room temperature, 24 h; c) mCPBA, DCM, 0°C to room temperature, 24 h; d) Cu, CuBr, PMDETA, 0°C, 24 h.\textsuperscript{144, 151}

A successful test polymerisation was performed, giving PtBA with $M_n$ of 55,100 g mol\textsuperscript{-1} (as determined by THF GPC) in 91% yield. Subsequently, simultaneous polymerisations of tBA and εCL
were performed and were again successful. A range of PCL-b-PtBA polymers were synthesised by simultaneous ROP and NMP and characterised by $^1$H NMR and THF GPC.

Figure 55. $^1$H NMR spectrum for PCL-b-PtBA 1. The terminal e protons are visible, allowing for $M_n$ to be calculated from the spectra.

$^1$H NMR studies (see Fig. 55) confirmed that the expected polymers were synthesised and that all residual solvent and monomers had been removed successfully; additionally, the NMR was used to determine the DP of the PCL block and relative length of the tBA block by end-group analysis. This is possible for the PCL-b-PtBA synthesised by NMP as the alcohol end-group remains free in this method (unlike for the ATRP method), keeping this proton signal adjacent to the terminal alcohol distinct from other signals.

Table 19. Results for the synthesis of PCL-b-PtBA by simultaneous NMP and ROP.

<table>
<thead>
<tr>
<th>Polymer</th>
<th>Block $M_n$ (g mol$^{-1}$) (tBA:εCL)</th>
<th>Degree of Polymerization (tBA:εCL)</th>
<th>Monomer conversion (%)</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Targeted</td>
<td>Found ($^1$H NMR)</td>
<td>Targeted</td>
<td>Found ($^1$H NMR)</td>
</tr>
<tr>
<td>PtBA</td>
<td></td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>PCL-b-PtBA 1</td>
<td>30,000:10,000</td>
<td>25,000:7,300</td>
<td>230:90</td>
<td>200:60</td>
</tr>
<tr>
<td>PCL-b-PtBA 2</td>
<td>30,000:30,000</td>
<td>22,000:22,800</td>
<td>230:260</td>
<td>170:200</td>
</tr>
<tr>
<td>PCL-b-PtBA 3</td>
<td>10,000:30,000</td>
<td>5,700:18,200</td>
<td>80:260</td>
<td>50:160</td>
</tr>
<tr>
<td>PCL-b-PtBA 4</td>
<td>60,000:60,000</td>
<td>28,500:35,700</td>
<td>470:530</td>
<td>220:310</td>
</tr>
<tr>
<td>PCL-b-PtBA 5</td>
<td>30,000:60,000</td>
<td>10,900:31,700</td>
<td>230:530</td>
<td>90:280</td>
</tr>
<tr>
<td>PCL-b-PtBA 6</td>
<td>10,000:60,000</td>
<td>5,700:25,400</td>
<td>80:530</td>
<td>50:220</td>
</tr>
</tbody>
</table>

Overall, the polymerisations proceeded well, with high monomer conversions and yields (see Table 19). The molar ratio of εCL to tBA in the block copolymers corresponds well to the targeted ratio. However, the calculated molar masses are less than would be expected from the high conversion rates and yields. It may be that the calculated $M_n$ is being suppressed by the presence
of oligomeric PCL formed by trace water in the reaction, which would also explain why the
difference between the theoretical and calculated molar masses increases with PCL content.

Figure 56. $^1$H NMR spectrum of the reaction mixture for the simultaneous ROP of εCL and NMP of tBA.

In addition to analysis of the final polymer, samples were taken for $^1$H NMR throughout the
polymerisations in order to track the progress of the reactions (see Fig. 56). From these spectra it
is possible to calculate the conversion of the εCL, $C_{\text{εCL}}$, through the integrals corresponding to the
B, B’ and B” protons:

$$C_{\text{εCL}} = \frac{B' + B''}{B + B' + B''}$$

It is also possible to calculate the conversion of the tBA, $C_{\text{tBA}}$, by calculating the loss of the vinyl
protons, A, as time progresses. This can be done by normalising the signal strength between the
spectra by using the sum of the B, B’ and B” protons, which will be constant throughout the
reaction:

$$C_{\text{tBA}} = 1 - \frac{A}{\frac{B + B' + B''}{A_0} + B_0' + B_0''}$$

Using these data, graphs of εCL and tBA conversion versus time can be plotted (Fig. 57). High εCL
conversions are achieved for all of the examples within 24 hours. However, the polymerisation
begins slowly, before accelerating which implies that there is a slow initiation process. This can be
attributed to inefficient transfer of heat between the oil bath and the reaction mixture, or slow
reaction between the alcohol initiator and the Sn(Oct)$_2$ catalyst to form the reactive centre.$^{73}$ The
polymerisation of tBA is faster than that of εCL but reaches similarly high conversions over the
course of the reaction.
Figure 57. εCL conversion (top) and tBA conversion (middle) versus time and of ln([tBA]₀/[tBA]) versus time (bottom) for the simultaneous polymerisations.

These data, in combination with information about the reaction conditions, can be used to plot a graph of ln([tBA]₀/[tBA]) versus time. The linear plot implies that the polymerisation is well controlled. To make this observation clearer a further experiment was run reproducing the conditions for PCL-b-PtBA 2 but collecting samples at more time points (see Fig. 58).
Figure 58. εCL conversion (top) and tBA conversion (middle) versus time plots and corresponding ln([tBA]₀/[tBA]) versus time plots (bottom) for a simultaneous polymerisation with an increased number of time points.

Including these additional time points clarifies the situation, showing that the εCL conversion lags initially and that the semilogarithmic plot for tBA is linear and hence that the concentration of the propagating species is constant, implying that the NMP of tBA is well-controlled.

In addition to ¹H NMR, the PCL-b-PtBA copolymers were analysed by THF GPC (see Table 20). There is poor correlation between the \( M_n \) values calculated from the ¹H NMR spectra and those determined by THF GPC. This is because the GPC determines the molecular weight based on the size of the polymer chains in solution. The addition of a repeat unit to a polymer chain will increase its molar mass and its hydrodynamic volume by a proportional amount; however, here two different repeat units are being added, each with different masses and volumes. This complicates the analysis, as the different examples of PCL-b-PtBA differ in the ratio of their block lengths as well as by molar mass. However, the individual results can still be considered.
Table 20. The molar masses of the PCL-\(b\)-PtBA block copolymers as determined by \(^1\)H NMR and THF GPC, also included are the molar masses of the corresponding PtBA homopolymers.

<table>
<thead>
<tr>
<th>Polymer</th>
<th>(^1)H NMR (g mol(^{-1})) (tBA:εCL)</th>
<th>THF GPC (g mol(^{-1}))</th>
<th>As Synthesised</th>
<th>PtBA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(M_n)</td>
<td>(M_w)</td>
<td>PdI</td>
<td>(M_n)</td>
</tr>
<tr>
<td>PtBA</td>
<td>-</td>
<td>55,100</td>
<td>100,100</td>
<td>1.8</td>
</tr>
<tr>
<td>PCL-(b)-PtBA 1</td>
<td>25,000:7,300</td>
<td>26,800</td>
<td>51,500</td>
<td>1.9</td>
</tr>
<tr>
<td>PCL-(b)-PtBA 2</td>
<td>22,000:22,800</td>
<td>37,400</td>
<td>71,300</td>
<td>1.9</td>
</tr>
<tr>
<td>PCL-(b)-PtBA 3</td>
<td>5,700:18,200</td>
<td>25,700</td>
<td>44,700</td>
<td>1.7</td>
</tr>
<tr>
<td>PCL-(b)-PtBA 4</td>
<td>28,500:35,700</td>
<td>41,200</td>
<td>80,000</td>
<td>1.9</td>
</tr>
<tr>
<td>PCL-(b)-PtBA 5</td>
<td>10,900:31,700</td>
<td>42,400</td>
<td>92,000</td>
<td>2.2</td>
</tr>
<tr>
<td>PCL-(b)-PtBA 6</td>
<td>5,700:25,400</td>
<td>38,500</td>
<td>59,700</td>
<td>1.6</td>
</tr>
</tbody>
</table>

The block copolymers have relatively high polydispersities, ranging from 1.6 to 2.2. This can be attributed to the ROP of εCL by Sn(Oct)$_2$ which always results in a fairly broad polydispersity due to transesterification reactions, as discussed previously.

Samples of the PCL-\(b\)-PtBA copolymers were stirred in a THF/water mixture with 0.2 M NaOH. Under these basic conditions the PCL is hydrolysed but the tert-butyl esters remain intact, resulting in isolation of the PtBA block. \(^1\)H NMR of the resulting polymer demonstrated that there was no PCL remaining (see Fig. 59), and that there were no acid peaks present, showing that the PtBA groups had not been hydrolysed.

The polydispersities of the polyacrylate blocks varied from 1.5 to 1.9 (see Table 20), in every case equal to or below that of the block copolymer, showing that the PCL blocks are more polydisperse. However, the polydispersities are still relatively high for the PtBA produced by NMP.
(see Table 20), showing that the polymerisation is poorly controlled, counter to the implications from the NMR experiments.

![Figure 60. GPC trace showing the effects of hydrolysing the PCL segment on the molecular weight of PCL-b-PtBA 6. Blue shows the whole polymer, red shows after hydrolysis of the PCL section. Inset: molecular weight distributions of the same.](image)

The GPC traces from the block copolymers (see Fig. 60) were largely monomodal, implying that a block copolymer had been synthesised rather than a mixture of homopolymers. However, it should be noted that the PCL-b-PtBA samples tend to have a long tail of lower molecular weight material, again implying the presence of short PCL blocks. If there were a mixture of homopolymers of the same molecular weight, then destruction of the PCL would not alter the GPC trace; conversely, if there is a block copolymer then the removal of the PCL would shift the peak to lower molecular weight. This is observed when comparing the GPC traces of PCL-b-PtBA 6 and its corresponding PtBA, demonstrating that a block copolymer has been synthesised successfully.

Having proven that the PCL-b-PtBA samples were truly block copolymers, they were then subjected to selective hydrolysis of the tert-butyl esters in anhydrous acidic conditions using TFA, as for the ATRP method. This gave a selection of PCL-b-PAA copolymers with varying chain and block lengths.

Though there is convincing evidence that the PCL-b-PtBA was a block copolymer, there was no direct evidence that the resulting PCL-b-PAA was a block copolymer. One of the most convincing ways to prove that a copolymer is a block copolymer is to check that it behaves like one. To this end, each of the amphiphilic copolymers were used to prepare aqueous micellar solutions by cosolvent evaporation using THF and were subsequently analysed by DLS (see Table 21). If they
are truly block copolymers then micelles should be formed, if they are not then the result will be a solution of PAA, with the PCL removed via filtration during the sample preparation.

Table 21. DLS data for the PCL-b-PAA block copolymers. Maximum chain lengths are calculated from the molar masses of the PCL-b-PtBA precursors, using a C-C bond length of 0.153 nm and a C-C-C bond angle of 109.5°.

<table>
<thead>
<tr>
<th>Sample Name</th>
<th>Intensity Mean (d.nm)</th>
<th>Polydispersity Index</th>
<th>Maximum calculated chain lengths (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCL-b-PAA 1</td>
<td>152</td>
<td>0.079</td>
<td>56 11 67</td>
</tr>
<tr>
<td>PCL-b-PAA 2</td>
<td>153</td>
<td>0.041</td>
<td>175 56 230</td>
</tr>
<tr>
<td>PCL-b-PAA 3</td>
<td>153</td>
<td>0.057</td>
<td>140 49 188</td>
</tr>
<tr>
<td>PCL-b-PAA 4</td>
<td>232</td>
<td>0.061</td>
<td>274 43 317</td>
</tr>
<tr>
<td>PCL-b-PAA 5</td>
<td>181</td>
<td>0.052</td>
<td>243 11 254</td>
</tr>
<tr>
<td>PCL-b-PAA 6</td>
<td>161</td>
<td>0.067</td>
<td>195 21 216</td>
</tr>
</tbody>
</table>

All six of the PCL-b-PAA copolymers formed micelles of low polydispersity and of the correct order of magnitude for polymeric micelles, thereby demonstrating that the PCL-b-PAA synthesised by simultaneous ROP and NMP are amphiphilic block copolymers.

From the graphs (Fig. 61), the micelle size correlates well with the maximum length of the block copolymer and very well with that of the PCL block, but poorly with the length of the PAA block.
Moreover, there seems to be a minimum particle size for these polymers at approximately 150 nm, with all three of the smallest PCL-b-PAA examples being this size.

As the PAA block contains carboxylic acids, the block copolymer micelles should also exhibit pH responsiveness. This was tested by adjusting the pH of micellar solutions of PCL-b-PAA 2 with HCl and NaOH and observing the particle size by DLS (see Fig. 62).

![Figure 62. A plot showing the pH responsive behaviour of PCL-b-PAA 2 in terms of hydrodynamic diameter (blue) and polydispersity (red).](image)

The unadulterated micellar solution has a pH of 4.6 and a diameter of 153 nm. With increasing pH the micelles grow in size, reach a plateau and then begin to shrink. It is suggested that this is due to the carboxylic acids becoming increasingly deprotonated, and hence charged, which increases inter-chain repulsion and particle size. This then reaches a maximum possible size as the chains are fully extended. Eventually, the charge repulsion becomes too great and the micelles dissociate to form smaller particles with an accompanying reduction in charge density. With decreasing pH the particle size initially decreases, before increasing again. This is due to the carboxylic acids becoming increasingly protonated, reducing inter-chain repulsion and particle size, before eventually beginning to aggregate, registering as an increase in particle size, as the PAA’s affinity for water reduces. This interpretation is reflected in the increase of polydispersity towards low or high pH when micelle aggregation or disassociation occur respectively. These observations match what is known to occur for PCL-b-PAA block copolymers, confirming that this synthetic route is successful. For example, Li et al found that the size of their PCL-b-PAA micelles increased from 143 nm to 213 nm on increasing the pH from 3.6 to 5.6, and then decreased to 147 nm as the pH increased further to 8.134
In summary, the simultaneous ROP of εCL and NMP of tBA was found to be an efficient and effective method for the synthesis of PCL-b-PAA. Additionally, it did not use copper or other potentially cytotoxic catalysts during the polymerisations, making the resulting copolymer more suitable for biological applications.

5.3.3 Attempted Synthesis of PCL-b-PVPA by ATRP

![Scheme 6. Scheme for the ATRP of DMVP.](image)

While the PCL-b-PAA could be useful as a compatibiliser to fabricate PCL/PVPA-co-AA composite nanofibres, a more elegant solution would be to synthesise PCL-b-PVPA (see Scheme 6). If covalently bonded to the PCL, the PVPA will be completely retained on the scaffold. It would be useful if the approach used for PCL-b-PAA could be adapted for PCL-b-PVPA, using esters of PVPA and selectively hydrolysing them with TMSBr.93 However, the literature is conflicted on whether phosphonates (particularly vinyl phosphonates) are suitable substrates for ATRP.153,154 To this end preliminary experiments were performed attempting to synthesise poly(dimethyl vinylphosphonate) (PDMVP) using the same reaction conditions as for the PtBA, but with a selection of ligands and at a range of temperatures (see Table 22).

<table>
<thead>
<tr>
<th>Conditions</th>
<th>Monomer conversion (%)</th>
<th>Yield (%)</th>
<th>$M_n$ (g mol$^{-1}$)</th>
<th>$M_w$ (g mol$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>200eq, PMDETA, 100°C, 24 h</td>
<td>0</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>100eq, PMDETA, 120°C, 48 h</td>
<td>0</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>200eq, HMTETA, 100°C, 48 h</td>
<td>0</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>200eq, Bipy, 80°C, 48 h</td>
<td>21</td>
<td>15</td>
<td>7,000</td>
<td>27,600</td>
</tr>
<tr>
<td>200eq, Bipy, 100°C, 48 h</td>
<td>17</td>
<td>7</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>200eq, Bipy, 120°C, 48 h</td>
<td>32</td>
<td>7</td>
<td>3,600 (bimodal)</td>
<td>23,300</td>
</tr>
</tbody>
</table>

The first experiment was performed at 100°C over 24 hours with PMDETA as the ligand; no product was evident. All later experiments were run over 48 hours, the rationale being that as VPA is much less reactive than AA, DMVP is likely to be less reactive than tBA and so the reaction would be slower. The experiment was duly repeated over this time scale and at a higher
temperature, again producing no product. In both of these experiments the reaction solution was initially green/blue in colour and became brown, returning to green on exposure to air; additionally, a brown solid was observed in the reaction vessel. These observations imply that the phosphonate monomer is interacting with the metal catalyst in an undesirable manner: either it is displacing the ligand and affecting the rate constants for the equilibrium reaction or the ligand/metal complex is insoluble in the reaction medium, or a combination of the two factors.

To investigate further, reactions were performed with 1,1,4,7,10,10-hexamethyltriethylene-tetramine (HMTETA) and 2,2'-bipyridyl (Bipy) at 100°C over 48 hours (see Fig. 63). These two ligands were selected as they are at opposite ends of the reactivity scale for ATRP ligands. The HMTETA polymerisation failed, while the Bipy achieved a low conversion of 17% and a yield of just 7%. Attempts were made to optimise the reaction, repeating it at 80°C and 120°C, but the conversions and yields remained too low to be of practical use.

In all cases brown solids were observed to precipitate out of the reaction mixture and there were unusual colour changes, implying both solubility problems and that the monomer interacts with the catalytic complex. It is interesting to note that the only successful polymerisation used the least active ligand and was most successful at the lowest temperature. This implies that $K_{\text{atrp}}$ is too far to the right (i.e. the reactive radicals are formed too quickly compared to the reactivity of the monomers), leading to a high initial concentration of radicals and subsequent recombination, rather than polymerisation. To conclude this section, ATRP is not a suitable technique for the synthesis of PDMVP and hence not a viable route to PCL-$b$-PVPA.

5.3.4 Attempted Synthesis of PCL-$b$-PVPA by NMP

It was decided to examine the NMP strategy with DMVP to see if PCL-$b$-PVPA was a realistic prospect. ATRP did not work because the monomer interacted with the copper catalyst, interfering with the key radical forming process. This is not the case in NMP, where the radical is
produced by thermolysis. The initial test reaction proceeded with 32% yield which was significantly higher than from the ATRP reaction, and achieved in half the reaction time.

![Graph](image)

**Figure 64.** Aqueous GPC trace for PDMVP synthesised by NMP. The polymer peak is multimodal, and significant monomer remains.

GPC analysis was attempted in both THF and chloroform, but the polymer proved to be insoluble in either solvent. Accordingly, the polymer was dissolved in aqueous phosphate buffer and run on the same system as the PVPA-co-AA polymers. This incompatibility with organic solvents was not found with the low molecular weight PDMVP synthesised by ATRP in Section 5.3.3 - its greater solubility in THF again implies the oligomeric nature of that material.

The GPC trace (Fig. 64) is multimodal, the first with $M_{\text{peak}} = 18,200 \text{ g mol}^{-1}$ and for the second $M_{\text{peak}} = 1,000 \text{ g mol}^{-1}$; there is also an additional peak before the solvent peak, consistent with residual monomer. One interpretation of this spectrum is that there is polymer being synthesised, but also a significant proportion of oligomers. This could be due to either chain transfer to the monomer or internal transfer to the methyl group followed by hydrolysis, both of which would result in the formation of shorter chains.

A copolymerisation with $\varepsilon$CL was attempted; if successful then optimisation of the polymerisation might be worthwhile. Initial observations were not promising: the reaction mixtures became cloudy in both toluene and DMF, implying solubility problems with the monomers. Unfortunately, the copolymerisation failed and all that was observed by $^1$H NMR after work-up was PDMVP, obtained in poor yields (see Fig. 65). $^1$H NMR analysis of the reaction mixture showed the presence of PCL and PDMVP, at 15% and 17% monomer conversion respectively. That only PDMVP was observed after work-up implies that both polymerisations are occurring, but form only homopolymers, which are then phase separating during the work-up. Additionally, both the $\varepsilon$CL and DMVP polymerisations have low conversions, 15 % and 17% respectively, implying that
these polymerisations are incompatible under these conditions; this is likely due to solubility issues.

![Figure 65. $^1$H NMR spectra of PDMVP (top) and reaction mixture (bottom), conversion of both DMVP and εCL is evident from peaks at 3.82 and 4.08 ppm respectively, but only PDMVP is seen after work-up.]

To conclude this section, it has been found that NMP of DMVP, while superior to ATRP, is still too inefficient in terms of monomer conversion to be an acceptable method of producing PDMVP. It has also been found that the NMP polymerisation of DMVP is incompatible with the ROP of εCL and was not successful for the production of the copolymer.

### 5.4 Conclusions

ATRP and NMP based approaches were both used to successfully create PCL-b-PAA via PCL-b-PtBA. The ATRP approach had acceptable yields and molar masses, but concerns remain over the possible retention of trace amounts of copper. The NMP approach has no such problems and is more efficient, only requiring two steps from monomer to product, resulting in faster synthesis and greater yields. Once synthesised, the PCL-b-PAA copolymers were used to make micelles which were examined by DLS; their behaviour was found to be consistent with literature reports, confirming that the synthesis of PCL-b-PAA was successful.

Attempts were made to synthesise PDMVP with a view to producing PCL-b-PVPA diblocks. However, neither ATRP nor NMP were found to be suitable for the polymerisation of PDMVP. This is speculated to be due to the low reactivity of the DMVP monomer and likely chain transfer reactions.
6 Emulsion Electrospinning of PCL and PVPA-co-AA

6.1 Introduction

In previous work PCL/PVPA-co-AA scaffolds were fabricated by dip-coating PCL nanofibres in aqueous PVPA-co-AA solutions. It would be preferable to include the PVPA-co-AA in the nanofibres to improve control over the composition, simplify the production of scaffolds and to prevent or slow the release of the PVPA-co-AA into the aqueous media. However, the polymers are incompatible, so an emulsion suitable for electrospinning needs to be made; for this reason PCL-b-PAA has been synthesised for the emulsion electrospinning of PCL/PVPA-co-AA composite nanofibres.

6.1.1 Emulsion Electrospinning

Emulsion electrospinning is a variant of electrospinning where, rather than a simple polymer solution, an emulsion is spun. This allows for composite fibres of otherwise incompatible polymers to be spun, for more complex architectures, such as core-shell fibres, or for the inclusion of water-soluble moieties such as proteins or drugs. This technique is of great interest for tissue engineering and controlled drug release applications.

![Diagram](image)

Figure 66. During electrospinning the emulsion is stretched along the axis of the nanofibre, resulting in long compartments of the discontinuous phase in a fibre of the continuous phase.

The apparatus and basic mechanism is the same as for normal electrospinning: a Taylor cone develops and a jet is formed, which goes through a whipping region with stretching and solvent evaporation to leave an elongated nanofibre. The difference is that the emulsion is separated into distinct phases, which remain separated and stretch axially; leaving elongated compartments within the fibres (see Fig. 66). Depending on the system these compartments can be hollow, filled with another polymer or contain some payload such as drug molecules. Additionally, depending on the system used the compartment can run the length of the fibre, creating a nanotube, or can be in shorter sections.
6.1.2 Aims and Objectives

The aim of the work in this chapter was to create composite PCL/PVPA-co-AA nanofibres by emulsion electrospinning using the previously synthesised PCL-b-PAA, to characterise the fibres and to compare their PVPA-co-AA release behaviour with dip-coated fibres and fibres spun from an emulsion formed using a surfactant.

6.2 Experimental

6.2.1 Materials

Polycaprolactone (PCL, \( M_n = 80,000 \text{ g mol}^{-1} \), Sigma-Aldrich), Span® 80 (Sigma-Aldrich), ethanol (≥99.8%, Sigma-Aldrich) and chloroform (≥99.5%, Sigma-Aldrich) were used as supplied. Commercial PVPA-co-AA (40 wt% aqueous solution, First Scientific Dental GmbH) was purified by dialysis prior to use. PCL-b-PAA was synthesised as described in Section 5.2.3.

6.2.2 Instrumentation

Solution viscosities were measured using a Brookfield DV-II+ Pro Viscometer with a ULA-EY UL adapter at 25°C. Readings were taken at 0.5 revolutions per minute and are reported as the average of three readings.

SEM and electrospinning apparatus were as described for Section 4.2.2.

ATR-FTIR spectroscopy was performed as described in Section 2.2.2.

6.2.3 Methods

Emulsion Electrospinning of PCL/PVPA-co-AA with PCL-b-PAA

PCL, PVPA-co-AA and PCL-b-PAA were dissolved in sufficient ethanol/chloroform (1/1 v/v) to give solutions of 11.25 wt%. A 21G needle was used at 11 kV with a 20 cm working distance and a flow rate of 0.03 mL min\(^{-1}\). The resulting nanofibre mats were air-dried at ambient temperature over 72 h.

PCL Fibre Dissolution

50 mg of the nanofibre mats were immersed in chloroform (3 mL) for 1 h and the undissolved material was collected by centrifugation (5000 rpm for 10 min). The resulting pellets were washed with further chloroform (10 mL) and dried under vacuum at 40°C for 2 h. The dried pellets were analysed by ATR-FTIR spectroscopy.
**Emulsion Electrospinning for PVPA-co-AA Release Experiments**

PCL, PVPA-co-AA and PCL-b-PAA/Span® 80 were dissolved in sufficient ethanol/chloroform (3/2 v/v) to give solutions of 15 wt%. A 21G needle was used to spin the solutions at 11 kV with a 20 cm working distance and a flow rate of 0.03 mL min⁻¹. The resulting nanofibre mats were air-dried at ambient temperature over 72 h; 1 cm diameter discs were cut from the mats for the release experiments. The PCL nanofibres were placed in an aqueous 15 wt% PVPA-co-AA solution for 24 h and then dried under vacuum. Half of the discs were used as is and the remainder were heated to 50°C for 24 h.

**PVPA-co-AA Release Experiments**

Samples of each of the fibre mats were weighed and immersed in 20 mL water. At time points of 2 and 7 days the samples were removed, rinsed and dried. The fibre mats were then reweighed and submitted for elemental analysis.

### 6.3 Results and Discussion

From the range of block copolymers synthesised, PCL-b-PAA 4 was chosen as the surfactant for the emulsion electrospinning in this chapter as it was the largest copolymer with each block being approximately the same molecular weight and was therefore thought to be the most capable of stabilizing the two phases of the emulsions. Although a range of PVPA-co-AA copolymers have been synthesised, the emulsion electrospinning was performed using the commercial PVPA-co-AA as it was readily available in large quantities, and was used in previous experiments examining the release of PVPA-co-AA from the scaffolds.³

#### 6.3.1 Emulsion Electrospinning of PCL and PVPA-co-AA

Before emulsion electrospinning could be performed, an emulsion of the PCL and PVPA-co-AA was required. PCL is soluble in chloroform and has been spun successfully from 10 wt% solutions in the past.⁶⁴ Ethanol was selected as the solvent for the PVPA-co-AA as it dissolves the copolymer well, is miscible with chloroform, has a similar boiling point to chloroform and is significantly less toxic than methanol. It was found that though each polymer was soluble in ethanol/chloroform 2/1 (v/v) solution, phase separation occurred within a few minutes of mixing (see Table 23). The addition of PCL-b-PAA stabilised the solutions sufficiently for electrospinning, maintaining the emulsion over 24 hours.

As the PCL-b-PAA is an additive to the system, it should be present in as a low a quantity as possible. Accordingly, the 5% loading (w/w of dry polymer mass) was selected for further
experiments as it was the lowest loading to stabilise the solutions at the higher concentration of polymer.

Table 23. A summary of emulsion stabilities; the solutions are highly concentrated (up to 28 wt%) to be representative of solutions used for electrospinning. At high concentrations only the highest block copolymer loadings were stable, once diluted by a factor of two all the emulsions were found to be stable over 24 hours.

<table>
<thead>
<tr>
<th>Wt% of Polymers in 2/1 ethanol/chloroform</th>
<th>Stable Emulsion over 24 hours</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCL</td>
<td>PCL-b-PAA</td>
</tr>
<tr>
<td>20</td>
<td>5.7</td>
</tr>
<tr>
<td>20</td>
<td>2.5</td>
</tr>
<tr>
<td>20</td>
<td>1.2</td>
</tr>
<tr>
<td>20</td>
<td>0.6</td>
</tr>
<tr>
<td>20</td>
<td>0.2</td>
</tr>
<tr>
<td>PVPA-co-AA</td>
<td>2.5</td>
</tr>
<tr>
<td></td>
<td>2.4</td>
</tr>
<tr>
<td></td>
<td>2.3</td>
</tr>
<tr>
<td></td>
<td>2.2</td>
</tr>
<tr>
<td></td>
<td>2.2</td>
</tr>
<tr>
<td>As made</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>-</td>
</tr>
<tr>
<td>After dilution</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>+</td>
</tr>
</tbody>
</table>

Polymer solutions were made for initial electrospinning experiments, varying the PVPA-co-AA concentration from 0 to 20% (w/w of dry polymer mass) at increments of 5%, with a total polymer concentration of 15 wt% in ethanol/chloroform 2/1 (v/v). The PVPA-co-AA loading of the solutions was varied to see if this would affect the resulting fibres and to determine if there was a limit to the incorporation of the copolymer into the fibres. These initial solutions were not suitable for electrospinning as the needle tip fouled immediately, leading to dripping and spraying rather than fibres. This was attributed to the fast evaporation of the chloroform causing the PCL to precipitate before it could be ejected from the needle tip, thus blocking the needle. The solutions were diluted with chloroform to change the ethanol/chloroform ratio to 1/1 (v/v) and the total polymer concentration to 11.25 wt%.

Table 24. Compositions of the electrospinning solutions, their viscosities and the average diameters of the resultant nanofibres.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Composition (w/w)</th>
<th>Viscosity (cP)</th>
<th>Average Fibre Diameter (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PCL:PCL-b-PAA:PVPA-co-AA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>95 : 5 : 0</td>
<td>407</td>
<td>810 (± 310)</td>
</tr>
<tr>
<td>B</td>
<td>90 : 5 : 5</td>
<td>342</td>
<td>826 (± 353)</td>
</tr>
<tr>
<td>C</td>
<td>85 : 5 : 10</td>
<td>324</td>
<td>742 (± 321)</td>
</tr>
<tr>
<td>D</td>
<td>80 : 5 : 15</td>
<td>287</td>
<td>661 (± 290)</td>
</tr>
<tr>
<td>E</td>
<td>75 : 5 : 20</td>
<td>228</td>
<td>637 (± 288)</td>
</tr>
</tbody>
</table>

Needle fouling was reduced, but remained an issue with the needle tip needing to be cleaned at regular intervals. Despite this problem, all 5 solutions were successfully electrospun to give nanofibre mats (see Table 24). The mats were carbon-coated and examined by SEM to determine
their quality and dimensions (see Fig. 67). The nanofibres are randomly aligned and are mainly smooth, with occasional bead defects, and have an unusually large polydispersity. These observations can be attributed to the needle fouling resulting in inconsistent spinning conditions at the needle tip. The average fibre diameters varied from 800 nm down to 600 nm, decreasing as the proportion of PVPA-co-AA increases. Although the difference is within the standard deviation of each of the samples, the trend is consistent between the samples with the higher PVPA-co-AA content fibres always having a lower average diameter, suggesting that this is a real effect. This can be explained by considering the molecular weights of the two polymers: the $M_n$ of PCL is 80,000 g mol$^{-1}$ while the $M_n$ of PVPA-co-AA is only 8,000 g mol$^{-1}$. Therefore increasing the proportion of the PVPA-co-AA in the solution decreases its viscosity, which in turn causes the fibre diameter to decrease as they are strongly correlated.$^{58}$ In addition, PVPA-co-AA is a polyelectrolyte and increasing its concentration will increase the conductivity of the solution. This will contribute to the observed reduction in nanofibre diameter as a more highly charged jet will be more strongly affected by the bending instability and hence be more elongated.$^{158}$

![SEM images of the composite nanofibres A-E taken at x 5000 magnification](image)

Figure 67. SEM images of the composite nanofibres A-E taken at x 5000 magnification; the nanofibres are mainly smooth, with the occasional slight bead defect and have a wide range of diameters.

The nanofibre mats were then analysed by FTIR to determine what functional groups were present, and therefore whether both of the polymers had been incorporated successfully (see Fig. 68). It was expected that sample A would match PCL, with the PVPA-co-AA signals appearing and
becoming more prevalent as its concentration increases in samples B-E. Sample A did match PCL; however, none of the remaining samples displayed any absorptions representative of PVPA-co-AA. The fact that no bands corresponding to PVPA-co-AA can be observed can be attributed to the relatively small amount of the copolymer present and that the most significant band (i.e. the C=O stretch of the carboxylic acids) coincides with, and is obscured by, the strongest band in the PCL spectrum (i.e. the C=O stretch of the esters).

![ATR-FTIR spectra of the electrospun nanofibre mats A-E, with spectra of pure PCL and PVPA-co-AA for comparison. All of the samples appear to contain PCL alone, with no evidence for PVPA-co-AA.](image)

To determine if PVPA-co-AA was present, the nanofibre mats were immersed in chloroform to dissolve the PCL. Removing the PCL would increase the concentration of the PVPA-co-AA and remove the obscuring PCL bands, both of which would make the copolymer easier to detect. Sample A dissolved entirely as it contained no PVPA-co-AA, while sample B did not return sufficient material to analyse by ATR-FTIR. The residual solids from samples C-E were isolated by centrifugation and reanalysed (see Fig. 69). All three samples returned bands corresponding to PVPA-co-AA, in addition to some residual PCL, meaning that the PVPA-co-AA was successfully incorporated into the PCL nanofibres by emulsion electrospinning with PCL-b-PAA.

Having confirmed the presence of PVPA-co-AA, the next step was to quantify how much was present. Accordingly, samples were submitted for elemental analysis in order to determine how much phosphorus, and therefore how much PVPA-co-AA, was present in each of the samples (see Table 25).
In each case the quantity of PVPA-co-AA detected was lower than expected, indicating that some of the copolymer is lost during the electrospinning process. A probable explanation is that the material that builds up when the needle fouls is rich in PVPA-co-AA, lowering the amount incorporated into the final composite nanofibres. It can also be seen that samples D and E, despite having different quantities of PVPA-co-AA in the solutions, contain the same proportion of the copolymer in the resulting scaffolds; this suggests that there is a maximum value for the amount of PVPA-co-AA that can be incorporated using this method. Despite this, the nanofibres produced consistently contained 50-70% of the expected quantity of PVPA-co-AA, meaning that specific loadings can be targeted up to a maximum of 10% using this methodology.

Table 25. A summary of the expected and found wt% of phosphorus in each sample, the corresponding percentage of PVPA-co-AA and the percentage of the PVPA-co-AA in the solutions that was incorporated into the nanofibres.

<table>
<thead>
<tr>
<th>Samples</th>
<th>Expected</th>
<th>Found</th>
<th>Percentage of PVPA-co-AA incorporated</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PVPA-co-AA (%)</td>
<td>wt% P</td>
<td>PVPA-co-AA (%)</td>
</tr>
<tr>
<td>A</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>B</td>
<td>5</td>
<td>0.61</td>
<td>2.7</td>
</tr>
<tr>
<td>C</td>
<td>10</td>
<td>1.23</td>
<td>6.8</td>
</tr>
<tr>
<td>D</td>
<td>15</td>
<td>1.84</td>
<td>10.3</td>
</tr>
<tr>
<td>E</td>
<td>20</td>
<td>2.46</td>
<td>10.3</td>
</tr>
</tbody>
</table>

In summary, PCL-b-PAA was found to act as a suitable surfactant for the emulsion electrospinning of PVPA-co-AA and PCL, the resulting nanofibres were of good quality and were proven to contain both polymers; furthermore, the incorporation of the copolymer into the nanofibres is predictable, allowing for specific loadings of the bioactive polymer to be targeted.
6.3.2 Electrospinning for PVPA-co-AA Release Experiments

One of the reasons for pursuing emulsion electrospinning as a method for nanofibre production was to see if it would alter the rate of PVPA-co-AA release from the scaffold. Therefore a series of nanofibre mats were produced by dip-coating electrospun PCL in PVPA-co-AA,\(^3\) emulsion electrospinning with Span\(^8\) 80 (see Fig. 70), a non-ionic surfactant that has been used for electrospinning previously,\(^{159}\) and emulsion electrospinning with PCL-b-PAA.

![Figure 70. Structure of Span\(^8\) 80; it is the ester of sorbitan, a derivative of sorbitol, and mixture of fatty acids, predominantly oleic acid.](image)

Initial biological tests performed by Dr Qi Guang Wang (as yet unpublished) indicated that nanofibres with PVPA-co-AA loadings of 5-10 wt% gave the best performance in terms of cell proliferation and expression of alkaline phosphatase and collagen; therefore the scaffolds targeted this range of copolymer loading. These scaffolds were electrospun under the same conditions, but the solutions were modified slightly. The original attempts used total polymer concentrations of 15 wt% in 2/1 ethanol/chloroform (v/v) but suffered from severe needle fouling and blocking, hence the solutions were diluted to 11.25 wt% in 1/1 ethanol/chloroform (v/v) which alleviated these problems. As a compromise between these conditions, concentrations of 15 wt% were used in 3/2 ethanol/chloroform (v/v); the solutions could be electrospun well, but again suffered from minor needle fouling problems (see Table 26).

<table>
<thead>
<tr>
<th>Sample</th>
<th>Compatibiliser Used</th>
<th>Composition (%)</th>
<th>Average Fibre Diameter (μm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCL-b-PAA</td>
<td>PCL-b-PAA</td>
<td>85:5:10</td>
<td>1.76 (± 0.93)</td>
</tr>
<tr>
<td>Span(^8) 80</td>
<td>Span(^8) 80</td>
<td>85:5:10</td>
<td>1.88 (± 0.91)</td>
</tr>
<tr>
<td>PCL</td>
<td>-</td>
<td>100:0:0</td>
<td>3.25 (± 1.66)</td>
</tr>
<tr>
<td>Dip-coated PCL</td>
<td>-</td>
<td>-</td>
<td>4.07 (± 1.92)</td>
</tr>
<tr>
<td>Heat-treated PCL</td>
<td>-</td>
<td>-</td>
<td>3.86 (± 1.30)</td>
</tr>
</tbody>
</table>

The fibres formed were again predominantly smooth with minor beading defects caused by the needle fouling. However, the fibre diameters were significantly higher than for the previous
examples, being 1.76 and 1.88 μm for the solutions stabilised by PCL-b-PAA and Span® 80 respectively; this can be attributed to the higher concentration used for these fibres. The diameter of the pure PCL fibres was higher still at 3.25 μm, this is due to their consisting solely of the high molar mass PCL, making the solutions more viscous.

Figure 71. SEM images of the composite nanofibres taken at x 1000 magnification; as for samples A-E earlier the fibres are mainly smooth, with the occasional slight bead defect and have a wide range of diameters. Thin films of PVPA-co-AA can be seen draped over the PCL fibres after dip-coating.

In addition, the fibres spun from solutions containing PVPA-co-AA will have a higher conductivity, and hence will tend to have lower diameters. It can be seen that the dip-coating process appreciably increases the fibre diameters to 4.07 μm, as would be expected as a coating of PVPA-co-AA has been added to it. However, examining the SEM images (see Fig. 71), indicates that the copolymer has been added in a localised manner, with areas being covered in thin films of PVPA-co-AA.

This localised deposition of PVPA-co-AA can be explained by the dip-coating methodology. Once the scaffolds are withdrawn from the solution the water begins to evaporate slowly, pulling the dissolved PVPA-co-AA with it, increasing its concentration. Only when the solutions are saturated does deposition of the copolymer begin. Therefore no PVPA-co-AA was deposited in the areas from which the water had retreated before becoming saturated, while in areas where the water
was saturated the copolymer has been deposited in films. This explains the tendency of the films to be suspended between fibres, where water droplets would naturally sit.

Heat-treating the dip-coated fibres had little effect on the fibre diameter and the samples appear unchanged, leading to the conclusion that heat treatment of the dip-coated fibres has no appreciable effect on their morphology.

Table 27. Data for the PVPA-co-AA release experiments. Samples were weighed before immersion and after drying, PVPA-co-AA content was calculated from the phosphorous content determined by elemental analysis and is accurate to 2%.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Day 0</th>
<th>Day 2</th>
<th>Day 7</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mass Remaining (%)</td>
<td>PVPA-co-AA Loading (%)</td>
<td>Mass Remaining (%)</td>
</tr>
<tr>
<td>PVPA-co-AA</td>
<td>100</td>
<td>7</td>
<td>91</td>
</tr>
<tr>
<td>Span® 80</td>
<td>100</td>
<td>7</td>
<td>91</td>
</tr>
<tr>
<td>Dip-coated PCL</td>
<td>100</td>
<td>30</td>
<td>62</td>
</tr>
<tr>
<td>Heat-treated PCL</td>
<td>100</td>
<td>32</td>
<td>63</td>
</tr>
</tbody>
</table>

Samples of each of the fibres were immersed in water in order to compare the rate of PVPA-co-AA loss. The samples were analysed in terms of mass loss and the wt% of phosphorus as determined by elemental analysis (see Table 27); initial measurements of pristine samples were also taken.

The fibres can be placed into two groups with essentially the same characteristics: fibres from the PVPA-co-AA and Span® 80 stabilised solutions, and dip-coated and heat-treated PCL samples. The obvious difference for the initial samples is that the dip-coated PCL fibres have a PVPA-co-AA loading of 30%, far higher than the fibres produced by emulsion electrospinning at 7%. It should also be noted that the 7% loading of PVPA-co-AA for the composite fibres is consistent with the results for emulsion electrospinning from a 10 wt% solution presented earlier in this chapter. Furthermore, this shows that the loss of PVPA-co-AA in the electrospinning process is not due to the PCL-b-PAA as the Span® 80 stabilised solutions suffer equally (see Fig. 72).
Figure 72. Data for the PVPA-co-AA release experiments, showing how the wt% of PVPA-co-AA (top) and the mass of the fibres (bottom) change over time while being immersed in water.

For all of the samples, the PVPA-co-AA is lost almost entirely by the first time point, with all of the fibres reaching a loading of 2% after two days. After seven days the PVPA-co-AA loading had fallen to 1% or below as only a trace amount (<0.3 wt%) of phosphorus was detected; this indicates that although most of the polymer is lost quickly, a small portion is better attached to the scaffold. Again, it should be noted that the fibres electrospun with PCL-b-PAA are equal in performance to those electrospun with Span® 80.

Importantly, the polymer persists on the scaffold for at least two days, which is long enough to promote cell attachment, which typically takes 30 minutes to a few hours to occur.\textsuperscript{160, 161} This means that PVPA-co-AA will still influence the adhesion of the cells as it will still be present on the fibres when cell attachment occurs.

After being immersed in water samples of each of the fibres were dried, carbon-coated and imaged by SEM (see Fig. 73). All visible traces of the PVPA-co-AA on both the dip-coated and heat-treated PCL fibres were removed after two days immersion, restoring the PCL to its original condition, reflecting the elemental analysis data. The composite fibres however, appear to be unchanged by their immersion in water, despite losing 9% of their mass due to the dissolution of the PVPA-co-AA component.
Figure 73. SEM images of the fibres used for the release experiment after 0, 2 and 7 days immersion in water, taken at x1000 magnification. The composite fibres do not appear to be altered, while the dip-coated samples quickly lose their PVPA-co- AA coating.

Analysis of the fibre diameters (see Table 28) reinforce the perception that the dip-coated PCL fibres have been restored to their original condition following the loss of the PVPA-co-AA, with
thinner fibres being obtained. The observed diameters of the dip-coated and heat-treated fibres, 3.21 and 3.10 μm, correspond well with that found for the PCL fibres as they were spun (3.25 μm). The composite fibres however, show the opposite trend, increasing in diameter following the loss of the PVPA-co-AA. This unexpected result could be due to the PVPA-co-AA being present within the original fibres; initially on exposure to water the PVPA-co-AA swells, distorting and increasing the diameter of the fibres, before dissolving into the water. The PCL is completely insoluble in water, and so has no mechanism to form thinner fibres, hence the new, larger diameter is retained.

Table 28. The variation of average diameters (μm) for the various samples as fabricated, after two days in water and after seven days in water.

<table>
<thead>
<tr>
<th>Nanofibre Sample</th>
<th>Average Fibre Diameter (μm)</th>
<th>Day 0</th>
<th>Day 2</th>
<th>Day 7</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCL-b-PAA</td>
<td></td>
<td>1.76 (± 0.93)</td>
<td>2.31 (± 1.07)</td>
<td>2.40 (± 0.96)</td>
</tr>
<tr>
<td>Span® 80</td>
<td></td>
<td>1.88 (± 0.91)</td>
<td>2.35 (± 1.03)</td>
<td>2.32 (± 0.86)</td>
</tr>
<tr>
<td>Dip-coated PCL</td>
<td></td>
<td>4.07 (± 1.92)</td>
<td>3.12 (± 1.10)</td>
<td>3.21 (± 1.15)</td>
</tr>
<tr>
<td>Heat-treated PCL</td>
<td></td>
<td>3.86 (± 1.30)</td>
<td>3.04 (± 0.95)</td>
<td>3.10 (± 1.08)</td>
</tr>
</tbody>
</table>

In summary, composite fibres of PCL and PVPA-co-AA were spun using PCL-b-PAA and Span® 80. The block copolymer was found to be suitable for emulsion electrospinning, matching the performance of the commercial surfactant and dip-coating approaches for the release of PVPA-co-AA from the fibre.

6.4 Conclusions

PCL-b-PAA was successfully used to stabilise solutions of PVPA-co-AA and PCL, which were in turn used to produce PVPA-co-PAA/PCL composite nanofibres with predictable compositions. The fibres were highly polydisperse with average nanofibre diameters of 600-800 nm, increasing with solution viscosity, which was further demonstrated by the more concentrated solutions used later having greater diameters. The fibres were largely smooth, but there were occasional large bead defects which were attributed to the needle fouling. The PVPA-co-AA loading of the nanofibres was found to be 50-70% of the solution used, appearing to reach a maximum PVPA-co-AA loading of 10%; again, the losses were attributed to the needle fouling.

Fibres electrospun from 15 wt% solutions stabilised with PCL-b-PAA had a larger diameter of 1.76 μm, but had the same PVPA-co-AA loading as for the smaller fibres at 7% from a 10 wt% solution. The composite fibres were comparable to those from solutions stabilised with Span® 80 in terms of their fibre diameter, composition and release of PVPA-co-AA. This shows that the PCL-b-PAA is
an effective agent for enabling the emulsion electrospinning of PCL and PVPA-co-AA. Furthermore, the release behaviour of the fibres from emulsion electrospinning matches that of the dip-coated fibres, indicating that they should also be suitable for testing as materials for bone tissue scaffolds.
7 Towards Phosphonated PCL

7.1 Introduction

This project has been geared towards the development of phosphonated polymers for application as bone tissue scaffolds; most of the work involved has been focussed on developing a previous system that has used a biodegradable, structural component of PCL and a bioactive component of PVPA-co-AA. An alternative approach is to combine the structural and bioactive parts into one component that would be biodegradable and contain bioactive phosphonic acid groups: phosphonated PCL (see Fig. 74).

![Chemical structure of phosphonated PCL](image)

Figure 74. An example of a PCL based polymer containing a phosphonic acid group, which could fulfil both the structural role of PCL and the bioactive role of PVPA-co-AA used in previous work.

The inclusion of phosphonic acid groups on the PCL backbone could potentially enhance its performance as a bone tissue scaffold by the chelation of Ca^{2+} and encouragement of mineralisation, inhibition of osteocytes and decreasing its hydrophobicity, promoting cell attachment. However, it would be a new chemical entity and its cytotoxicity profile, and that of its metabolites, are unknown and would need to be explored in detail.

![Generalised scheme for synthesis of phosphonated PCL](image)

Figure 75. Generalised scheme for the synthesis of a phosphonated PCL from a phosphonated εCL monomer.
Pursuing this option involves the synthesis of εCL monomers containing phosphonate esters, polymerisation of those monomers and finally selectively hydrolysing the phosphonate esters to reveal the phosphonic acids (see Fig. 75).

Figure 76. Structure of diethyl (2-oxooxepan-3-yl)phosphonate and the resulting polymer. A six membered ring transition state would make the polyester more prone to hydrolysis.

εCL is a seven-membered lactone, therefore a range of possible monomers could be pursued placing the phosphonate on different ring positions. Perhaps the most synthetically accessible position is adjacent to the carbonyl; however, this would place the acid group next to the ester in the final polymer (see Fig. 76). It is known that grafting phosphonic acids to PCL increases its rate of degradation, holding the acidic protons so close to the ester could further increase its vulnerability to nucleophilic attack and hence increasing the slow rate of degradation, which is one of the qualities that makes PCL particularly attractive for bone tissue scaffolds.

Figure 77. Structure of diethyl (7-oxooxepan-4-yl)phosphonate and the corresponding polymer.

Another attractive option is diethyl (7-oxooxepan-4-yl)phosphonate, which would avoid holding a labile proton so near to the carbonyl, and hence not suffer from such accelerated degradation (see Fig. 77). For this reason, efforts were first directed towards diethyl (7-oxooxepan-4-yl)phosphonate.
7.1.1 Aims and Objectives
The aim of this chapter is to synthesise phosphonated εCL monomers suitable for creating PCL with a phosphonate group appended directly to the polymer backbone. This will be attempted by placing the phosphonate group in the γ position through a bromide intermediate in the first instance, or in the α position through enolate chemistry.

7.2 Experimental

7.2.1 Materials
Ether (≥99.7%, Sigma-Aldrich), dichloromethane (DCM, ≥99.5%, Sigma-Aldrich), ethyl acetate (≥99.5%, Sigma-Aldrich) and hexane (≥98.5%, Sigma-Aldrich) were used as supplied; if anhydrous solvent was required, the solvent was dried over activated 3 Å molecular sieves for 48 hours before use. 1,4-Cyclohexanediol (99%, Sigma-Aldrich), neutral alumina (58 Å mesh, Sigma-Aldrich), hydrogen bromide solution (48 wt% in water, Sigma-Aldrich), pyridinium chlorochromate (PCC, 98%, Sigma-Aldrich), 3-chloroperoxybenzoic acid (mCPBA, 77%, Aldrich), ε-caprolactone (εCL, 99%, Alfa-Aesar), diethyl chlorophosphite (95%, Sigma-Aldrich), lithium diisopropylamide (LDA, 2 M in THF, Sigma-Aldrich) and CDCl₃ (99.8% D atom %, Sigma-Aldrich) were used as supplied.

7.2.2 Instrumentation
GC-MS was performed using an Agilent 7890C GC with an Agilent 5975C Mass Selective Detector; samples were analysed using electron ionisation (EI).

Reverse phase preparatory HPLC was performed on an Agilent 1260 Infinity LC system fitted with an ACE 10 C18-AR column (10 μm particle size, 250 mm length by 21.2 mm internal diameter), using acetonitrile and water (1/1 v/v) with a flow rate of 15 mL min⁻¹ and an injection volume of 200 μL and concentration of 100 mg mL⁻¹.

Centrifugation was performed using a Sigma 1-14K Refrigerated Microfuge.

NMR was performed as described in Section 2.2.2.

Mass spectroscopy was performed as described in Section 5.2.2.
7.2.3 Reactions

**Synthesis of 1,4-Epoxycyclohexane**

\[
\text{OH} \quad \text{Alumina} \quad 240^\circ C \quad \text{OH}
\]

1,4-Cyclohexanediol (50 g, 431 mmol) and neutral alumina (50 g) were placed in a round-bottomed flask and a still head attached. The flask was then heated to 240°C and the distillate collected at 100-135°C. The distillate separated into two layers, which were separated and the aqueous layer was extracted with ether (3 × 100 mL). The organic layers were combined, dried over MgSO\(_4\), filtered and the ether removed by distillation at 40°C to leave the product as a clear oil (24.69 g, 58% yield). The NMR spectrum corresponds to the literature.\(^{163}\)

\(^1\)H NMR (CDCl\(_3\), 400MHz): δ = 4.46-4.53 (m, 2H), 1.57-1.69 (m, 4H), 1.27-1.46 ppm (m, 4H)

\(^{13}\)C NMR (CDCl\(_3\), 101 MHz): δ = 76.2, 30.2 ppm

GC-MS: m/z: 120 [M+Na\(^+\)]

**Synthesis of trans-4-Bromocyclohexanol**

\[
\text{OH} \quad \text{HBr} \quad \text{Br}
\]

1,4-Epoxycyclohexane (24.6 g, 250 mmol) was mixed with hydrogen bromide (48 wt% in water, 40 mL) and left stirring at room temperature for 6 days. The resulting mixture was extracted with ether (3 × 300 mL), the organic fractions dried over MgSO\(_4\) and evaporated to dryness under reduced pressure. The brown oil obtained was purified by vacuum distillation at 20 mbar and
distillate was collected at 120°C, producing the product as a white solid (21.82 g, 49% yield, plus 17 g of impure distillate). The NMR spectrum corresponds to the literature.\textsuperscript{164}

\textsuperscript{1}H NMR (CDCl\textsubscript{3}, 400 MHz): \( \delta = 4.05-4.25 \) (m, 1H), 3.74-3.89 (m, 1H), 2.26-2.43 (m, 2H), 2.20-2.25 (m, 1H), 1.96-2.10 (m, 2H), 1.73-1.92 (m, 2H), 1.36-1.54 ppm (m, 2H)

GC-MS: \( m/z: 180 \ [M^{81}Br]^+ \), 178 \( [M^{79}Br]^+ \)

\textit{Synthesis of 4-Bromocyclohexanone}

\begin{center}
\begin{tikzpicture}
\node (A) at (0,0) {OH};
\node (B) at (2,0) {PCC};
\node (C) at (4,0) {DCM};
\node (D) at (6,0) {Br};
\node (E) at (8,0) {Br};
\node (F) at (10,0) {O};
\draw[->] (A) -- (B);
\draw[->] (B) -- (C);
\draw[->] (C) -- (D);
\draw[->] (D) -- (E);
\draw[->] (E) -- (F);
\end{tikzpicture}
\end{center}

\textit{trans-4-Bromocyclohexanol} (17 g, 95.0 mmol) was dissolved in DCM (170 mL) and PCC added (34 g, 157.7 mmol). The reaction was stirred overnight at room temperature, after which the reaction mixture was filtered and evaporated to dryness. Ether (200 mL) was added to the resulting brown oil and the precipitate filtered off, the ether was washed with H\textsubscript{2}O (3 \( \times \) 300 mL), dried over MgSO\textsubscript{4} and evaporated to dryness. The product was obtained as a pale yellow oil (12.13 g, 72% yield). The NMR spectrum corresponds to the literature.\textsuperscript{164}

\textsuperscript{1}H NMR (CDCl\textsubscript{3}, 400 MHz): \( \delta = 4.51-4.66 \) (m, 1H), 2.58-2.82 (m, 2H), 2.22-2.44 ppm (m, 6H)

\textsuperscript{13}C NMR (CDCl\textsubscript{3}, 101 MHz): \( \delta = 208.7, 49.3, 38.5, 35.7 \) ppm

GC-MS: \( m/z: 178 \ [M^{81}Br]^+ \), 176 \( [M^{79}Br]^+ \)

\textit{Synthesis of \( \gamma \)-Bromo-\( \varepsilon \)-caprolactone}

\begin{center}
\begin{tikzpicture}
\node (A) at (0,0) {Br};
\node (B) at (2,0) {O};
\node (C) at (4,0) {Br};
\node (D) at (6,0) {O};
\node (E) at (8,0) {mCPBA};
\node (F) at (10,0) {DCM};
\draw[->] (A) -- (E);
\draw[->] (E) -- (B);
\draw[->] (B) -- (C);
\draw[->] (C) -- (D);
\end{tikzpicture}
\end{center}

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4-Bromocyclohexanone (9 g, 50.8 mmol) was dissolved in DCM (150 mL) and cooled to 0°C. mCPBA (20 g, 115.9 mmol) was dissolved in DCM (100 mL) and added dropwise, the reaction was then allowed to warm to room temperature with stirring over 5 h. The precipitate was filtered off and the filtrate washed with Na$_2$S$_2$O$_3$ (saturated aqueous solution, 3 x 200 mL), NaHCO$_3$ (saturated aqueous solution, 3 x 200 mL) and H$_2$O (200 mL). The organic layer was then dried over MgSO$_4$ and evaporated to dryness. The crude material was purified by column chromatography using ethyl acetate and hexane (1:2) as eluent. The product was obtained as a clear oil which gradually solidified to a white solid (6.26 g, 64% yield). The NMR spectrum corresponds to the literature.$^{164}$

$^1$H NMR (CDCl$_3$, 400 MHz): $\delta$ = 4.45-4.70 (m, 2H), 4.11-4.26 (m, 1H), 3.08 (m, 1H), 2.49-2.71 (m, 1H), 2.15-2.44 ppm (m, 4H)

$^{13}$C NMR (CDCl$_3$, 101 MHz): $\delta$ = 174.7, 64.7, 51.7, 38.7, 32.2, 30.6 ppm

GC-MS: $m/z$: 194 [M$^{81}$Br$^+$], 192 [M$^{79}$Br$^+$]

**Synthesis of Diethyl (2-oxooxepan-3-yl)phosphonate**

LDA (2 M in THF, 25 mL, 50 mmol) was dissolved in ether (25 mL) in a dry 2-necked flask under N$_2$ and cooled to -78°C. εCL (5.18 g, 5 mL, 45.5 mmol) was added dropwise and the reaction mixture stirred at -78°C for 1 h. Diethyl chorophosphite (7.82 g, 7.2 mL, 50 mmol) was added dropwise and the reaction was allowed to warm to room temperature over 3 h before being quenched with acetic acid (1 M in ether, 55 mL). The precipitate was filtered off and the filtrate was washed with water (1 × 50 mL) and NaHCO$_3$ (saturated aqueous solution, 1 × 50 mL). The aqueous layers were extracted with chloroform (1 × 50 mL). The organic layers were combined, dried over MgSO$_4$ and concentrated under vacuum before being stirred under air overnight. Initial purification was performed by column chromatography using ethyl acetate and hexane (1:1), but was unsuccessful. A small amount of product was isolated by reverse phase preparatory HPLC using water and acetonitrile as eluent, giving a white solid. The spectra correspond with the literature.$^{165}$
$^1$H NMR (CDCl$_3$, 400 MHz): $\delta = 4.33-4.45$ (m, 1H), 4.06-4.26 (m, 5H), 3.06-3.20 (ddd, $J = 21.2, 10.6, 2.0$ Hz, 1H), 2.15-2.29 (m, 1H), 1.85-2.09 (m, 2H), 1.67-1.82 (m, 2H), 1.52-1.65 (m, 1H), 1.27 ppm (td, $J = 7.1, 3.6$ Hz, 6H)

$^{31}$P($^1$H) NMR (CDCl$_3$, 162 MHz): $\delta = 22.37$ ppm (s, 1P)

MS (70eV): $m/z$ (%): 251 (95) [$M+H^+$], 273 (100) [$M+Na^+$], 523 (80) [$2M+Na^+$]

### 7.3 Results and discussion

#### 7.3.1 Attempted Synthesis of Diethyl (7-oxooxepan-4-yl)phosphonate

When planning how to synthesise diethyl (7-oxooxepan-4-yl)phosphonate a survey of the literature revealed a promising intermediate: γ-bromo-ε-caprolactone. This bromide had been synthesised previously (see Scheme 7) and copolymerised with εCL, with a view to making crosslinked or functionalised PCL. This bromide therefore has a known synthesis and could be a suitable substrate for the Michaelis-Arbuzov rearrangement, which is commonly used to replace halogens with phosphonates.

![Scheme 7. Synthesis of γ-bromo-ε-caprolactone. Reagents and conditions: a) Alumina, 240°C; b) HBr, 6 days; c) PCC, 18 hours; d) mCPBA, 0°C to room temperature, 5 hours.](image)

The suggested synthesis begins with the 1,4-epoxycyclohexane, which can be purchased, but only in relatively small quantities. Therefore it was prepared by the dehydration of 1,4-cyclohexanediol, which is readily available, with activated alumina at high temperature. The reaction was performed without solvent as the diol is molten at the temperatures used; as the product forms it is removed by evaporation and collected as a distillate. The reaction proceeds in a straightforward manner to give 1,4-epoxycyclohexane in an acceptable yield.
1,4-Epoxycyclohexane was then stirred in concentrated aqueous HBr solution for 6 days to effect the ring-opening bromination reaction (see Fig. 78). The product was isolated by vacuum distillation, the first portion of distillate was pure and solidified to give a white solid on cooling, the second portion however remained a liquid and contained impurities, most likely 1,4-cyclohexanediol which can form as a side-product during the reaction.

![Figure 78. Reaction mechanism for the formation of trans-4-bromocyclohexanol, and the side-product 1,4-cyclohexandiol.](image)

The alcohol is then oxidised with PCC to give 4-bromocyclohexanone in good yield, despite the lengthy work-up required to remove the viscous by-products. The ketone is then converted to the lactone by Baeyer-Villiger oxidation with mCPBA, which proceeds with good yield to produce racemic γ-bromo-ε-caprolactone. Interestingly, the product crystallises from an oil to afford a white solid over the course of several days, drawing itself up from a pool of oil into a single mound of solid.

The final step was to attempt the Michaelis-Arbuzov rearrangement; the reaction was performed following a literature protocol, using neat triethyl phosphite at reflux (160°C) for 24 hours. After removal of the triethyl phosphite by vacuum distillation, the remaining material was examined by NMR. The ¹H NMR spectrum initially looked promising, containing all the peaks of the γ-bromo-ε-caprolactone and the peaks for the diethyl phosphonate in approximately the correct ratios. However, the signal for the γ-proton (see Fig. 79) had not changed; a shift away from 4.6 ppm would be expected for changing the bromide for a phosphonate.
Examination of the $^{31}$P{\textsuperscript{1}H} NMR spectrum showed that the main phosphorus-containing compound was triethyl phosphate with a peak around -1 ppm, along with diethyl phosphonate at 5 and 9.5 ppm (see Fig. 80). However, the minor peaks, at 29 and 33.5 ppm, fall into the range for phosphonates, indicating that some product could be present in small quantities.

GC-MS analysis (see Fig. 81) indicated that the major peak was γ-bromo-ε-caprolactone ($m/z$ of 192 and 194). There were minor peaks for triethyl phosphate ($m/z$ of 182), bromocylohexane ($m/z$ of 162 and 164) and dimethyl (7-oxooxepan-4-yl)phosphonate ($m/z$ of 251), indicating that the reaction proceeded with extremely poor conversion, as low as 14% by GC-MS.
It is known that secondary alkyl bromides are less reactive than primary alkyl bromides as they are less susceptible to $S_N2$ substitution. Therefore the reaction was repeated with trimethyl phosphite as this smaller nucleophile might increase the efficiency of the reaction. However, analysis of the reaction mixture by GC-MS indicated inferior results, with only the original bromide ($m/z$ of 192 and 194) and dimethyl phosphonate ($m/z$ of 111) being observed (see Fig. 82). This can be contributed to the reaction being performed at a lower temperature, as trimethyl phosphite has a lower boiling point than triethyl phosphite (111°C versus 156°C).

To conclude this section, γ-bromo-ε-caprolactone was synthesised successfully with the intention of using it as an intermediate for dimethyl (7-oxooxepan-4-yl)phosphonate. However, the bromide could not be converted to the desired phosphonate via the Michaelis-Arbuzov rearrangement as it is too unreactive. Accordingly, attention turned to other examples of phosphonate-bearing εCL.
7.3.2 Towards Diethyl (2-oxooxepan-3-yl)phosphonate

An alternative approach is to place the phosphonate in the α position on the lactone ring. Though this could potentially result in faster hydrolysis of the final polyester, it is more synthetically accessible through enolate chemistry (see Scheme 8). It is therefore a promising route to phosphonated εCL as it can be synthesised from well-known and readily available reagents in a single step.

Scheme 8. Reaction scheme for the synthesis of diethyl (2-oxooxepan-3-yl)phosphonate. Reagents and conditions: i) LDA in THF, -78°C, 1 hour; ii) diethyl chlorophosphite, -78°C to room temperature, 3 hours; iii) room temperature, 18 hours.

The εCL was added dropwise to the LDA solution to limit self-condensation and ring-opening of the lactone under the basic conditions. The enolate forms and then reacts with the diethyl chlorophosphite to generate the phosphite intermediate, which is oxidised by air under ambient conditions. The reaction proceeds well, but yields a mixture of polar products that are difficult to isolate as they co-elute during column chromatography. Use of reverse-phase preparatory HPLC isolated a sample suitable for analysis.

The $^{31}$P($^1$H) NMR of the sample indicated that it was the correct product (see Fig. 83), matching literature reports and was pure, containing no other phosphorus species. In addition, the mass spectroscopy determined that the product had the correct molecular weight (m/z of 251).
The $^1$H NMR spectra matched what would be expected (see Fig. 84), except for the multiplet centred on 2.0 ppm integrates to three protons rather than the expected two; this can be attributed to residual water from the HPLC. The reaction is therefore, a success; however, preparatory HPLC, though effective, is not a suitable method for the isolation of the bulk quantities of diethyl (2-oxooxepan-3-yl)phosphonate needed for use as a monomer. The system used was capable of acceptable peak resolution with injections up to 200 μL of the 100 mg mL$^{-1}$ solution with a run time of 30 minutes. Assuming a starting purity of 90% it would take over 550 hours to acquire 10 g of pure product, which is prohibitively impractical.

To conclude this section, diethyl (2-oxooxepan-3-yl)phosphonate was successfully synthesised in one step, but could not be isolated in viable quantities for use as a monomer.

7.4 Conclusions and Suggestions for Future Work

Two phosphonated analogues of εCL were considered and attempts were made at their synthesis. γ-Bromo-ε-caprolactone was successfully synthesised over 4 steps, but the Michaelis-Arbuzov rearrangement to introduce the phosphonate group was ineffective as the secondary bromide was too unreactive. Future attempts could try using catalysts to promote the reaction, such as Amberlyst-15 which has had some success for simple secondary bromides, or examine alternative reactions such as the Michaelis-Becker reaction.

The analogue with the phosphonate in the α position was synthesised directly from εCL using enolate chemistry. However, it could not be purified easily, only being isolated by preparatory reverse phase HPLC. As a starting point I would suggest column chromatography with reverse phase silica, as this would allow for better separation of the polar compounds.
Figure 85. Mechanism for the selective hydrolysis of phosphonate esters by trimethylsilyl bromide.

Should the phosphonated monomers be isolated and polymerized, the phosphonate esters could be selectively hydrolysed by trimethylsilyl bromide (see Fig. 85). 92
Conclusions and Recommendations for Future Work

The work in this thesis is focused on developing bone tissue scaffolds based on PCL and PVPA-co-AA, with a specific aim of improving their performance by the application of polymer chemistry. There were three well-defined objectives:

1. To develop methods suitable for the characterization of PVPA-co-AA and defining the commercially-sourced copolymer that had been used previously.
2. To develop an effective synthesis of PVPA-co-AA and produce a range of copolymers covering a wide range of compositions to allow biological testing to determine the best example of PVPA-co-AA.
3. To synthesise PCL-b-PAA and to use it as a compatibiliser for the emulsion electrospinning of PCL and PVPA-co-AA.

The first objective began by determining that the commercial PVPA-co-AA was impure, but could be successfully purified by dialysis. The composition was defined using a quantitative $^{31}$P($^1$H) NMR method in combination with a marker compound (TMP), and the results checked by elemental analysis. Both methods rely on the fact that only one of the comonomers, VPA, contains phosphorus. The molecular weight of the copolymer was determined using aqueous GPC with a 0.1 M NaH$_2$PO$_4$ buffer and calibrated using PAA standards. Under these conditions PVPA and PAA have comparable hydrodynamic diameters and are therefore suitable for measuring their copolymer. The commercial PVPA-co-AA was found to be 34 mol% VPA and have an $M_n$ and $M_w$ of 8,000 and 31,000 g mol$^{-1}$ respectively. These analytical methods were then applied to evaluate further samples of PVPA-co-AA that were synthesised to fulfil the second objective.

An effective synthesis of PVPA-co-AA was developed where the AA was added gradually to the VPA and AAPH initiator in order to account for AA being a far more reactive monomer than VPA. This method allowed access to high VPA content copolymers for biological tests. Work performed by Dr Qi Guang Wang in the Faculty of Medical and Human Sciences (as yet unpublished) found that, in aqueous solution, PVPA-co-AA copolymers with 30 mol% VPA were the best for bone mineralisation.

For the third objective, PCL-b-PAA was successfully synthesised by an ATRP approach according to well-known literature procedure. However, following concerns about the possible cytotoxic effects of residual copper, a new NMP based method was developed. This two-step synthesis used simultaneous ROP of εCL and NMP of tBA from a dual initiator and was shorter, higher-yielding and avoided the use of cytotoxic transition metal catalysts. The resulting PCL-b-PAA was
used to stabilise emulsions of PCL and PVPA-co-AA, which were subsequently electrospun to give smooth composite fibres whose diameters shrunk with decreasing solution viscosity.

Release experiments determined that the loss of PVPA-co-AA from these fibres was comparable to that of fibres spun using Span® 80, a commercial anionic surfactant, and consistent with dip-coated fibres; this demonstrates that the fibres spun using PCL-b-PAA should be suitable for further biological testing.

A further, more open-ended objective was also to:

4. create new phosphonate-containing polymer materials for investigation as bone tissue scaffolds.

The system from previous work used PCL as a structural component and PVPA-co-AA as a phosphonate-bearing, bioactive component to promote bone healing, therefore the two approaches considered were to make the PVPA-co-AA water-stable by crosslinking or to introduce a phosphonate group to PCL.

The former was achieved by electrospinning PVPA-co-AA with ethylene glycol and then crosslinking by thermal esterification, to form water-stable nanofibres of the copolymer. The nanofibres remained fibrous during and after hydration, though once swollen the porosity of the structure appears to be greatly reduced.

For the latter, synthetic attempts were made to synthesise phosphonated εCL, with a view to polymerisation to give a phosphonated PCL analogue; unfortunately attempts to phosphonate the γ position were unsuccessful and, while phosphonation did occur at the α position, the product could not be isolated on a viable scale.

For future work I would recommend attempting to use the dual initiator used for the synthesis of PCL-b-PtBA for the simultaneous polymerisation of further block copolymers using other lactones (such as lactide and glycolide) and vinyl monomers (e.g. other acrylates, methacrylates and styrenes), to determine if the method has broader application for polymer synthesis.

Alternatively, further work could look to evaluate the performance of the composite nanofibres formed by emulsion electrospinning with PCL-b-PAA as candidates for bone tissue scaffolds, and additionally, compare them with composite nanofibres formed using Span® 80 and the original dip-coated nanofibres. Other biological work could evaluate the crosslinked electrospun PVPA-co-AA nanofibres for their application as a tissue scaffold, perhaps in comparison with PVPA-co-AA hydrogels.
A more ambitious line of work would be to continue attempting to synthesise a phosphonated PCL analogue. This would include the suggestions for the isolation of the α-phosphonated εCL in section 7.4, but could be expanded to include more complex syntheses to phosphonate other ring positions or perhaps other polymers suitable for post-polymerisation modification.
9 References


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10 Appendices

10.1 Appendix 1 – Data for PVPA-co-AA samples

10.1.1 Spectroscopic Data

*Commercial PVPA-co-AA (31 mol% VPA, after dialysis)*

$^2$H NMR

![2H NMR graph]

$^{31}$P{$^1$H} NMR

![31P1H NMR graph]
PVPA

$^1$H NMR

PVPA-co-AA (78 mol% VPA)

$^1$H NMR
$^{31}$P($^1$H) NMR

PVPA-co-AA (52 mol% VPA)

$^1$H NMR

$^{31}$P($^1$H) NMR
PVPA-co-AA (32 mol% VPA, prior to purification)

\( ^1H \text{NMR} \)

PVPA-co-AA (27 mol% VPA)

\( ^1H \text{NMR} \)
10.1.2 Aqueous GPC Data

**Commercial PVPA-co-AA (32 mol% VPA)**

![Graph showing refractive index vs elution time for Commercial PVPA-co-AA (32 mol% VPA)]

**PVPA**

![Graph showing refractive index vs elution time for PVPA]
PVPA-co-AA (78 mol% VPA)

PVPA-co-AA (52 mol% VPA)

PVPA-co-AA (32 mol% VPA)
PVPA-co-AA (27 mol% VPA)

10.2 Appendix 2 – Synthesis of PCL-b-PAA

10.2.1 Spectroscopic Data

ATRP Initiator

$^1$H NMR
**P(tBA) from ATRP**

**\(^1\)H NMR**

![NMR Spectrum](image1)

**FTIR**

![FTIR Spectrum](image2)

**PAA from ATRP**

**\(^1\)H NMR**

![NMR Spectrum](image3)
FTIR

Transmittance

Wavenumber / cm$^{-1}$

PCL

$^1$H NMR

FTIR

Transmittance

Wavenumber / cm$^{-1}$
**PCL Macroinitiator**

$^1$H NMR

**FTIR**

**PCL-b-P(tBA) from ATRP**

$^1$H NMR
FTIR

PCL-b-PAA from ATRP

$^2$H NMR (DMSO-d$_6$ in red; CDCl$_3$ in black)
Nitroxide Precursor

$^1$H NMR

$^{13}$C NMR

$^{31}$P$^{1}$H) NMR
2-hydroxyethyl-2-bromo-2-methylpropanoate

$^1$H NMR

$^1$C NMR

Dual initiator

$^1$H NMR
10.2.2 THF GPC Data

P(tBA) from ATRP
**PCL**

![Graph](image1.png)

**PCL macroinitiator**

![Graph](image2.png)

**PCL-b-P(tBA) from ATRP**

![Graph](image3.png)
$P(tBA)$ from NMP

$PCL$-$b$-$P(tBA)$ 1 (blue) and corresponding $P(tBA)$ (red)

$PCL$-$b$-$P(tBA)$ 2 (blue) and corresponding $P(tBA)$ (red)
*PCL-b-P(tBA) 3 (blue) and corresponding P(tBA) (red)*

![Graph 1](image1.png)

*PCL-b-P(tBA) 4 (blue) and corresponding P(tBA) (red)*

![Graph 2](image2.png)

*PCL-b-P(tBA) 5 (blue) and corresponding P(tBA) (red)*

![Graph 3](image3.png)
PCL-b-P(tBA) 6 (blue) and corresponding P(tBA) (red)

10.3 Appendix 3 – Towards Phosphonated PCL

10.3.1 Spectroscopic Data

Epoxy cyclohexane

$^1$H NMR

$^{13}$C NMR
trans-4-Bromohexanol

$^1$H NMR

![trans-4-Bromohexanol NMR spectrum](image)

4-Bromohexanone

$^1$H NMR

![4-Bromohexanone NMR spectrum](image)

$^{13}$C NMR

![4-Bromohexanone C NMR spectrum](image)
γ-Bromo-ε-caprolactone

$^1$H NMR

Diethyl (2-oxooxepan-3-yl)phosphonate

$^1$H NMR
10.3.2 Chromatographic Data

*Diethyl (2-oxooxepan-3-yl)phosphonate*

HPLC
10.4 Appendix 4 – Macromolecules Publication

Synthesis and Characterization of Poly(vinylphosphonic acid-co-acrylic acid) Copolymers for Application in Bone Tissue Scaffolds

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Supporting Information

ABSTRACT: Poly(vinylphosphonic acid-co-acrylic acid) (PVPA-co-AA) has recently been identified as a potential candidate for use in bone tissue engineering. It is hypothesized that the strong binding of PVPA-co-AA to calcium in natural bone inhibits osteoclast activity. The free radical polymerization of acrylic acid (AA) with vinylphosphonic acid (VPA) has been investigated with varying experimental conditions. A range of copolymers were successfully produced and their compositions were determined quantitatively using $^{31}$P NMR spectroscopy. Monomer conversions were calculated using $^{1}$H NMR spectroscopy and a general decrease was found with increasing VPA content. Titration studies demonstrated an increase in the degree of dissociation as a function of VPA in the copolymer. However, a VPA content ca. 30 mol% was found to be the optimum for calcium chelation, suggesting that this composition is the most promising for biomaterials applications. Assessment of cell metabolic activity showed that PVPA-co-AA has no detrimental effect on cells, regardless of copolymer composition.

INTRODUCTION

With an increasingly aging population, there is a need for a synthetic bone graft substitute to meet the growing demand for the repair of bone defects, caused by diseases such as osteoporosis. Tissue engineering is a rapidly expanding field that seeks to meet these demands by developing biological substitutes to restore, maintain or improve tissue function. 1 Traditionally, tissue scaffolds are utilized as a support structure to facilitate the growth of new cells. However, in recent years there has been a shift in focus to more functionalized materials, which act as a mimic of the native extracellular matrix (ECM) and which exhibit osteoconductive and osteoinductive properties.

There are many ways to functionalize a scaffold to resemble the ECM. Above all the material must be biocompatible and biodegradable, with nontoxic breakdown products. 5 It should also aid in mineralization 6 and facilitate protein and cell interactions. 7 This can be achieved by the incorporation of bioactive moieties into the scaffold matrix, including nano-hydroxyapatite (nHAp) 9 as well as numerous growth factors 10 and proteins. 11 Much work has also been carried out on the production of tissue scaffolds for the controlled and localized delivery of a number of therapeutic drugs. 12,13 Furthermore, polymers—both natural and synthetic—are often fabricated onto the surface of tissue scaffolds to increase cell adhesion. 14,15

A novel phosphonate containing polymer, poly(vinylphosphonic acid-co-acrylic acid) (PVPA-co-AA) has recently been identified as a potential candidate for use in bone tissue scaffolds. 16,17 It is hypothesized that this copolymer mimics the action of bisphosphonates (BPs), a class of drugs used to treat osteoporosis. 18 Bisphosphonates are considered to be synthetic analogues of inorganic pyrophosphate, a physiological regulator of calcification and bone resorption. The close proximity of the acid groups and the strong negative charge surrounding PVPA-co-AA is speculated to mimic the structure of bisphosphonates. This backbone structure enables BPs to bind avidly to divalent metal ions such as calcium, and as a result BPs can bind to bone mineral surfaces in vivo. Although the exact mechanism is not fully understood, it is thought that BPs are internalized by resorbing osteoclasts, eventually leading to osteoclast apoptosis. 19

However, there are certain side effects associated with bisphosphonates, including osteonecrosis of the jaw and irritation at the injection site. 20 Therefore, it is proposed that PVPA-co-AA can mimic the action of bisphosphonates, leading to a reduction in osteoclast activity and thus decreased bone
regulate the mineralization process. In a recent study, an electrospun nanofibrous scaffold of partially phosphorylated poly(vinyl alcohol) (PVA) was prepared and studied for matrix mineralization and maturation of human preosteoblasts. This material—whereby phosphate groups were few and dispersed over the length of the macromolecule—was speculated to mimic the structure of noncollagenous phosphoproteins. It was found that mineralization was significantly increased with partial phosphorylation and cell-matrix calcium levels were about two times higher than in PVA nanofibers.

Gemeinhart et al. have produced copolymers of poly-(vinylphosphonic acid-co-acrylamide) (PVPA-co-AA) and they found that 30 mol % was the optimum VPA feed content for enhanced mineralization of the matrix produced by osteoblast cells. It was suggested that this was due to polymer-based calcification. This is consistent with the results from the present work, in which acrylic acid rather than acrylamide was used as the comonomer. PVPA-co-AA, with a VPA content of ca. 30 mol %, results in the highest levels of calcium chelation and is expected to lead to increased matrix mineralization, thus making it a promising candidate for applications in bone tissue scaffolds.

**Assessment of Cell Metabolic Activity.** The fold change in cell metabolic activity was determined as a function of VPA content and the results are presented in Figure 6. Addition of all PVPA-co-AA polymers had no detrimental effect on SaOS-2 cell metabolic activity. No statistical difference was found between each polymer treatment compared to the PBS control at both 0 and 24 h time points. After 72 h, the copolymers with 60–90% VPA displayed a fold change that was similar to that of the control, whereas with 0–30% VPA, there was a slight decrease. This could be due to the increased hydrophilicity and charge density with increasing VPA in the polymer, leading to greater protein interactions. A recent study by Tan et al. evaluated the effect of VPA content on cell viability with a PVPA-co-Am hydrogel and reported that with increasing VPA content, protein uptake and cell adhesion increased, attributing this to the electrostatic interactions induced by the VPA groups with serum proteins.

However, our results show that the difference in cell metabolic activity is not statistically significant, indicating that the PVPA-co-AA is biocompatible and not cytotoxic over this culture period, regardless of copolymer composition.

The cytotoxicity of acrylic acid monomers has been investigated by Kurata et al. They found that, after cultivation for 4 days, cell growth was significantly decreased when exposed to AA at concentrations ranging from 1.0 to 5.0 mM. Therefore, it can be concluded that AA is cytotoxic at concentrations higher than 1 mM.

The 1H NMR spectra of PVPA-co-AA copolymers were recorded after purification and no monomer residues could be detected. This suggests that any residual monomer would be at a concentration below 500 μM. Therefore, the cytotoxic effects of AA and VPA monomers were investigated at concentrations of 1–500 μM and the results are presented in the Supporting Information. The results show that at these low concentrations, AA and VPA monomers have no detrimental effect on SaOS-2 cell metabolic activity over the 72 h culture period.

**CONCLUSIONS**

It was the aim of this work to produce a series of copolymers of VPA and AA to investigate their calcium chelation affinity and biocompatibility. This would give some indication as to the effectiveness of the copolymer for applications in bone tissue engineering.

The reactivity ratios of the monomers were calculated as 4.07 for AA and 0.048 for VPA, confirming the very low reactivity of VPA in the copolymerization system. With this in mind, the synthetic method was altered to allow for the successful production of a range of PVPA-co-AA copolymers, with a degree of control over their composition and molecular weight. Confirmation of the structure of the copolymers was provided by evaluation of their 1H NMR and FT-IR spectra. A new method was developed to elucidate the copolymer composition quantitatively using 31P NMR spectroscopy.

Titration curves of the polymers showed that whereas PAA and PVPA behave as monoprotic acids, PVPA-co-AA exhibits a two-step neutralization curve, although the steps are not very distinct. With increasing VPA content in the copolymer, the degree of dissociation increases. However, the calcium chelation affinity exhibited a maximum with a VPA content of 30 mol %. It is proposed that this copolymer composition offers the best structural mimic of naturally occurring NCIPs. Therefore, it can be concluded that the charge distribution of phosphonate groups plays a significant role in the polymer's ability to chelate calcium ions and thus promote mineralization.

Assessment of cell metabolic activity has shown that PVPA-co-AA has no detrimental effect on cells, regardless of copolymer composition. Therefore, these results suggest that PVPA-co-AA is biocompatible and has the potential to be used in bone tissue scaffolds.

**ASSOCIATED CONTENT**

1 Supporting information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.macro- mol.5b02594.

(1) Synthesis of PVPA-co-AA, (2) characterization of PVPA-co-AA by GPC, elemental analysis, and FT-IR spectroscopy, (3) monomer reactivity ratios of AA and VPA, (4) calcium chelation calibration, (5) theology and (6) ζ potential of PVPA-co-AA:calcium complexes, and (7) assessment of cell metabolic activity for VPA and AA (PDF)
and PVPA-30. The signals which appear at 1300–1050 cm⁻¹, of medium strong intensity, denote the C–O stretch of the same group. The spectrum of PVPA-100 exhibits a band at 1090 cm⁻¹, which represents the P=O stretch, whereas the P=O stretching bands can be seen at 985–905 cm⁻¹. These bands do not appear in the spectrum of PVPA-4 and increase in intensity as the VPA content in the polymer is increased (see Supporting Information). This coincides with a decrease in the C=O band of the carboxylic acid group. Therefore, the FT-IR results provide strong evidence for the successful synthesis of PVPA-co-AA.

**Potentiometric Titration.** The nominal degree of neutralization (α) of the copolymers was calculated by taking into account the analytical composition of the copolymer, revealed by elemental analysis (see Supporting Information).

The change in pH as a function of nominal degree of neutralization is presented in Figure 4. The titration curve of the PVPA homopolymer shows an almost linear increase of pH with α, up to degree of neutralization 0.7. This is followed by a steeper increase in pH with increasing α until the limiting value is reached, which represents the dissociation of one of the hydroxyl groups of the phosphonic acid. These results are in agreement with previous literature on the topic, which have demonstrated that while the VPA monomer shows the two dissociation steps expected, PVPA behaves as a monoprotic acid.³²,³³

As opposed to the homopolymers, the titration curves of the copolymers show two neutralization steps, which may be attributed to contributions of VPA with a pKₐ of 2.89 (step 1) and AA with a pKₐ of 7.74 (step 2). However, these steps are not very distinct and it was shown by Bingöl et al.²² that even in the presence of low molecular weight salt (NaCl), it is not possible to discern the two clear dissociation steps.

As the VPA content in the copolymer is increased, the pH of the solution decreases, at a given α, due to the increased acidity of PVPA when compared with PAA. Whereas PAA is a weak polyelectrolyte, PVPA has intermediate strength. Therefore, copolymers with higher VPA contents will show increased dissociation, and thus a greater negative charge density.

**Calcium Chelation.** The degree of dissociation of a polyelectrolyte is important in terms of its ability to chelate metal ions from the surrounding environment. As discussed previously, PVPA and PAA behave as monoprotic acids. Therefore, requires two acid groups to chelate one Ca²⁺ ion. The exact mechanism of chelation is not known, but it is thought that there exists a combination of electrostatic effects as well as a chemical association of the calcium with the negatively charged group.

Figure 5 shows the calcium chelation capacity of PVPA-co-AA, at pH 7.3 and 9.0, with copolymer compositions ranging from 0 to 100 mol % of VPA. As expected, there is an increase in calcium chelation with increasing pH of the medium. As the pH increases, the acid groups become increasingly deprotonated. This results in intramolecular repulsion and hence an expansion of the polymer chain, leading to more available binding sites for Ca²⁺.

With increasing VPA content, there is an increase in calcium chelation, reaching a maximum at ca. 30 mol % VPA; after which there is a steady decrease up to PVPA-100 (Figure 5). Generally, it would be expected that calcium chelation would increase linearly with increasing VPA content, owing to the increased dissociation of acidic groups. However, it is worth noting that copolymers with high VPA contents (>60%) have much lower molar masses. Therefore, the observed trend in calcium chelation may arise as a result of various competing factors. Furthermore, the distribution of phosphonic acid groups may also play a significant role in the polymer’s ability to chelate calcium ions.

Natural bone contains noncollagenous proteins (NCPs), many of which are phosphorylated to varying degrees. It has been suggested that variations in phosphorylation can help to
Macromolecules

Cytotoxicity Studies. Human osteosarcoma derived osteoblast cells (SaOS-2) were cultured in Dulbecco’s modified Eagle’s medium (DMEM) (Gibco, Paisley), supplemented with 10% fetal calf serum (FCS), antibiotics (100 U mL⁻¹ penicillin, 100 mg mL⁻¹ streptomycin) and 50 μM ascorbic acid. SaOS-2 cells were seeded onto a 24-well plate at a density of 20,000 cells per cm². After 24 h, initial metabolic activity was measured using the Alamar Blue assay, before 500 μg mL⁻¹ of the PVP-co-AA solution was added to the culture medium. Phosphate buffered saline (PBS) was used as a control and a number of PVP-co-AA solutions were tested, with VPA contents ranging from 0 to 100 mol%. Cell metabolic activity was then measured at 24 and 72 h time points using the Alamar Blue assay. The resulting fluorescence was read on a plate reader (FLS800, Biotek, UK) with an excitation wavelength of 540 nm and an emission wavelength of 600 nm. The fold change in metabolic activity was calculated by comparing the cell metabolic activity at 24 and 72 h, to the initial time point (prior to addition of polymer). The statistical difference between each treatment and the PBS control was compared using the one-way ANOVA analysis.

RESULTS AND DISCUSSION

Synthesis and Characterization of PVP-co-AA. The free radical polymerization of AA and VPA (Scheme 1) has been carried out in water, with various feed compositions, to produce PVP-co-AA. AAPH was used as a water-compatible initiator. This method was adapted from a patent by Dirsch et al.

Monomer conversions were determined by taking ¹H NMR spectra prior to purification, as described above. Figure 1a–c shows the ¹H NMR spectra for PVP-0, PVP-100, and PVP-30, the latter is given as an example since all the copolymers exhibit the same features. The methylene (CH₂) and methine (CH) protons of the polymer backbone—which show signals at 1.3–2.1 and 2.2–2.8 ppm, respectively—are shifted upfield slightly in the spectrum of PVP-100 due to the shielding effect of the phosphonate from the VPA side group. The –OH signal of the acrylic acid group can be seen at 3.66 in poly(acrylic acid) (PVP-0) and 3.62 in the copolymer (PVP-30), again shifted upfield slightly.

The effects of temperature and initiator concentration on yield, monomer conversion and average molar masses were investigated (Table 1). With regards to temperature, the yield and monomer conversion were highest when the copolymerization was carried out at 90 °C. The monomer conversion was found to increase with increasing initiator concentration. One possible explanation for this is that higher initiator concentrations result in shorter chain lengths and thus lower molar masses, as evidenced in Table 1. Therefore, the viscosity of the medium is decreased, which allows for a greater rate of monomer diffusion and hence an increase in polymerization rate and monomer conversion.

The highest initiator concentration (1 mol%) gave rise to a broad molar mass distribution. This could arise as a result of the semicontinuous addition of initiator and acrylic acid monomer. Therefore, a reaction temperature of 90 °C and an initiator concentration of 0.1 mol% were found to be the optimum conditions to produce PVP-co-AA with a high yield and monomer conversion and a high average molar mass.

The ³¹P NMR spectra of PVP-100 and PVP-30 (before and after purification) are shown in Figure 2(a–c). The characteristic polymer peak (O=P(OH)₃) is observed at 30.62 ppm, whereas the VPA monomer impurity can be seen at 15.96 ppm (Figure 2c). By comparing the polymer integral with that of the TIP marker, the mol% VPA in the copolymer can be calculated using the equations outlined above. Thus, we have successfully developed a quantitative method to calculate the VPA:AA mole ratio using ³¹P NMR spectroscopy. The reliability of this method was tested by comparison with elemental analysis data (Table 2).

As the VPA content in the feed was increased, the monomer conversion and percentage yield of the copolymers showed a general decrease (Table 2). This is attributed to the low reactivity of VPA during copolymerization. This can be explained by the polymerization mechanism proposed by Bingöl et al. The reaction proceeds via cyclopolymerization of the vinylphosphonic acid anhydride, by intra- or intermolecular propagation to form two different radicals. It has been suggested that this mechanism leads to a competition between the propagating radicals, which lowers the reactivity of VPA. The extent to which VPA anhydride forms has been found to depend on many factors including solvent, temperature and the fraction of VPA in the feed. Therefore, the reverse relationship between conversion and VPA content may indicate that the contribution of the anhydride becomes more significant as the VPA content in the feed increases.

When copolymers with specific properties are required, product homogeneity is important and composition drift must be eliminated. The batch wise addition of AA to the VPA polymerization allowed for a more even distribution of monomers. Where the VPA content in the feed was below 50%, chain transfer agent was introduced into the polymerization to restrict the molecular weight. Using this method, a range of copolymer compositions were produced with consistent molecular weights up to a VPA content of 59 mol% (Table 2). At higher VPA contents, high molecular weight polymers could not be obtained. The highest achievable molecular weight for VPA homopolymer was 29100 g mol⁻¹, which is comparable to that of Bingöl et al.

Further investigation into the structure of PVP-co-AA was achieved by evaluation of the FT-IR spectrum of the copolymer with respect to the corresponding homopolymers, shown in Figure 3. The FT-IR spectra for all of the polymers exhibited bands of medium intensity at 3300–2500 and 1500–1375 cm⁻¹, representing the methylene C–H stretch and bend, respectively. The =O–H stretch appears as a broad band between 3000 and 2300 cm⁻¹ in all of the spectra. However, this band is shifted to a lower wavenumber in PVP-100. The P–O–H bend can be seen in the spectrum of PVP-100 at 1647 cm⁻¹.

The strong IR band at 1696 cm⁻¹ was attributed to the C=O stretch of the carboxylic acid, found in the spectra of PVP-0.
approximately 30 cm cellulose tubing. The resulting polymer was dried at 55 °C under vacuum to afford a white solid (1.40 g, 38%).

**Synthesis of PVPA-co-AA for the Evaluation of Reactivity Ratios.** VPA (0.50 g, 4.6 mmol) was dissolved in deionized water (7.0 cm³) and added to a 2-neck round bottomed flask equipped with a reflux condenser. The apparatus was purged with N₂ prior to the polymerization. The solution was heated to 90 °C and left for 30 min. AA (0.49 g, 6.9 mmol) was dissolved in H₂O (10 cm³) and AAPH (3.1 mg, 0.02 mmol) was dissolved in H₂O (0.2 cm³). These were then added to the reaction flask, and the reaction was left for 30 min. The product was purified by dialysis for 24 h, using approximately 30 cm of cellulose tubing. The resulting polymer was dried at 55 °C under vacuum to afford a white solid (0.66 g, 67%).

**Methods.** ¹H NMR spectra were recorded in solution (5 wt % in D₂O) on a Bruker AV400 spectrometer operating at 400.13 MHz. The ¹H NMR spectra were referenced to sodium 3-(trimethylsilyl)-propionate-2,3,3-d₃ (TMS, δ = 0 ppm). Monomer conversions were determined by taking ¹H NMR spectra of the reaction mixtures prior to purification. The methylene (CH₂) and methyl (CH₃) protons of the polymer backbone show signals at 1.3–2.1 and 2.2–2.8 ppm, respectively, and the vinyl protons (CH=CH₂), which occur due to unreacted monomer, appear at 5.9–6.2 ppm (Figure 1d). By comparing the relative intensities of these signals, the degree of overall monomer conversion was estimated using eq 1:

$$\text{monomer conversion (%) } = \frac{I_{CH₂} + I_{CH₃}}{I_{CH=CH₂} + I_{CH₂} + I_{CH₃}} \times 100$$

where $I_{CH₂}$, $I_{CH₃}$, and $I_{CH=CH₂}$ are the integrals under the CH₂, CH₃, and CH=CH₂ peaks, respectively.

³¹P NMR spectra were recorded in solution (5 wt % in D₂O) on a Bruker AV500 spectrometer operating at 202.48 MHz. The ³¹P NMR spectra were referenced to trimethyl phosphate (TMP) (δ(PPP) = 28 ppm). 1.5 wt % TMP was added to the solution prior to the experiment. The mole fraction of VPA in the copolymer was determined, first by calculating the moles per integral of TMP using eq 2:

$$n_{TMP} = \frac{m_{TMP}}{M_{TMP} \times \text{TPM}}$$

where $n_{TPM}$ is number of moles, $m_{TMP}$ is mass and $M_{TMP}$ is molar mass of TMP. TMP is the integral under the TMP peak at 2.8 ppm, taken from the ³¹P NMR spectrum (Figure 1c). This was then related to the moles of VPA, $n_{VPA}$, using eq 3:

$$n_{VPA} = n_{TMP} \times \int VPA$$

Here $\int VPA$ is the integral under the polymer peak at 29–32 ppm. The number of moles of AA, $n_{AA}$, was determined by taking into account the total mass of polymer, $M_{TPM}$, using eq 4:

$$n_{AA} = \left(\frac{(m_{VPA+AA} - n_{VPA} \times M_{VPA})}{M_{AA}}\right)$$

Here $M_{TPM}$ and $M_{AA}$ are the molar masses of VPA and AA, respectively. Finally, the mol % VPA in the copolymer was calculated using eq 5:

$$\text{mol % VPA} = \frac{n_{VPA}}{n_{VPA} + n_{AA}} \times 100$$

Fourier transform infrared (FT-IR) spectra were recorded using a Thermo Scientific Nicolet iS5 spectrometer with an iDS diamond attenuated total reflectance (ATR) attachment over a wavenumber range of 4000–600 cm⁻¹ and a resolution of 4 cm⁻¹. The spectra were obtained from 16 scans.

Elemental analyses were carried out using inductively coupled plasma mass spectrometry (ICP-MS), by the School of Chemistry Microanalysis Service, University of Manchester.

GPC measurements were performed with a Waters S15 HPLC pump, TSK gel columns (5000 and 6000 Å pore size) and an ELSD-7515A refractive index detector. 0.1 M aqueous sodium hydrogen phosphate (Na₂HPO₄) was used as a mobile phase at a flow rate of 0.5 mL/min⁻¹. A set of PAA-WS standards with $M_w$ between 1300 and 11 × 10⁶ g mol⁻¹ were used for calibration with ethylene glycol as an internal standard. Solutions of 1 g L⁻¹ PVPA-co-AA in Na₂HPO₄ were measured at 35 °C. Software was developed in house using LabVIEW.

Potentiometric titrations were carried out using a HANNA pH meter (HI2211) and electrode (HI33111) at 25 °C. Polymer solutions (1 mg mL⁻¹) were prepared in deionized water and titrated with a 0.1 M standard solution of NaOH.

**Calcium Chelation Measurements.** The amount of Ca²⁺ chelated by the polymer was measured using a calcium-selective electrode. A range of PVPA-co-AA copolymers (5 mg mL⁻¹) were dissolved in 0.1 M NaCl solutions in deionized water and neutralized to the required pH (7.5 or 9.0) using 0.1 M NaOH. CaCl₂ was added to the polymer solutions (0.1 M) which were then stirred for 30 min. The calcium-selective electrode was then immersed into the polymer solutions to measure the free Ca²⁺ concentration. A calibration curve was produced prior to the measurement using a range of CaCl₂ standard solutions (0.0001, 0.0001, 0.01, 0.1, and 1 M). The samples were then quantified by means of the calibration curve ($R^2 > 0.999$) (see Supporting Information). The amount of polymer-bound calcium was calculated from the total amount of Ca²⁺ added and the amount of free Ca²⁺ measured.

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Table 1. Effect of Temperature and Initiator (AAAP) Concentration on Yield, Monomer Conversion, Composition of Copolymer, Weight-Average Molar Mass ($M_w$), Number-Average Molar Mass ($M_n$), and Polydispersity ($M_w/M_n$) for Copolymerization of VPA with AA*.

<table>
<thead>
<tr>
<th>$T$ (°C)</th>
<th>AAP concentration (mol %)</th>
<th>yield (%)</th>
<th>monomer conversion (%)</th>
<th>VPA in copolymer (mol %)</th>
<th>$M_w$ (g mol$^{-1}$)</th>
<th>$M_n$ (g mol$^{-1}$)</th>
<th>$M_w/M_n$</th>
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<td>100</td>
<td>0.1</td>
<td>73</td>
<td>84</td>
<td>20</td>
<td>259800</td>
<td>29600</td>
<td>8.77</td>
</tr>
<tr>
<td>90</td>
<td>0.3</td>
<td>84</td>
<td>92</td>
<td>22</td>
<td>195400</td>
<td>20500</td>
<td>7.57</td>
</tr>
<tr>
<td>90</td>
<td>1.0</td>
<td>93</td>
<td>96</td>
<td>16</td>
<td>159400</td>
<td>12400</td>
<td>12.9</td>
</tr>
</tbody>
</table>

*In each case, the monomer feed composition was 30 mol % VPA.

Table 2. Effect of Monomer Feed Ratio and Chain Transfer Agent (CTA) on Yield, Monomer Conversion, Composition (VPA Content in Copolymer As Determined by $^{31}$P NMR and Elemental Analysis), Weight-Average Molar Mass ($M_w$), Number-Average Molar Mass ($M_n$), and polydispersity ($M_w/M_n$) for the Copolymerization of VPA with AA.

<table>
<thead>
<tr>
<th>Sample code</th>
<th>monomer feed ratio (VPA:AA)</th>
<th>CTA (mol %)</th>
<th>yield (%)</th>
<th>monomer conversion (%)</th>
<th>VPA ($^{31}$P NMR) (mol %)</th>
<th>VPA (mol %) (E)</th>
<th>$M_w$ (g mol$^{-1}$)</th>
<th>$M_n$ (g mol$^{-1}$)</th>
<th>$M_w/M_n$</th>
</tr>
</thead>
<tbody>
<tr>
<td>PVPA-0</td>
<td>0:100</td>
<td>0.5</td>
<td>83</td>
<td>96</td>
<td>0</td>
<td>189000</td>
<td>18000</td>
<td>6.03</td>
<td></td>
</tr>
<tr>
<td>PVPA-20</td>
<td>0:200</td>
<td>0.5</td>
<td>75</td>
<td>90</td>
<td>16</td>
<td>185000</td>
<td>22600</td>
<td>8.21</td>
<td></td>
</tr>
<tr>
<td>PVPA-30</td>
<td>0:300</td>
<td>0.5</td>
<td>79</td>
<td>96</td>
<td>27</td>
<td>194000</td>
<td>24200</td>
<td>8.07</td>
<td></td>
</tr>
<tr>
<td>PVPA-40</td>
<td>0:400</td>
<td>0.5</td>
<td>81</td>
<td>96</td>
<td>41</td>
<td>156000</td>
<td>21100</td>
<td>6.74</td>
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</tr>
<tr>
<td>PVPA-60</td>
<td>0:600</td>
<td>0</td>
<td>38</td>
<td>80</td>
<td>59</td>
<td>182700</td>
<td>13800</td>
<td>4.31</td>
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<tr>
<td>PVPA-80</td>
<td>0:800</td>
<td>0</td>
<td>35</td>
<td>87</td>
<td>78</td>
<td>159000</td>
<td>6500</td>
<td>2.43</td>
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<tr>
<td>PVPA-100</td>
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<td>11</td>
<td>57</td>
<td>100</td>
<td>291000</td>
<td>9200</td>
<td>3.15</td>
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</tr>
</tbody>
</table>

resorption, while, at the same time, eliminating the adverse side effects of BP drugs.

Bassi et al.17–18 have produced scaffolds composed of PCL nanofibers functionalized with PVPA-co-AA by immersion of the PCL scaffold, postfabrication, into a solution of the copolymer. Their results confirm an increase in bone fill percentage in the presence of the PCL/PVPA scaffold, compared with PCL alone.17 When PVPA-co-AA was incorporated into the scaffolds, there was a significant increase in hydroxyapatite formation and hence mineralization, proven by the increase in calcium and phosphorus levels. Furthermore, the PCL/PVPA scaffold was shown to increase osteoblast proliferation as well as significantly decreasing osteoclast viability, with comparable results to Alendronate, a commercially available bisphosphonate.21

Despite the recent interest in PVPA-co-AA for applications in tissue engineering, there has been relatively little research into the synthesis of the copolymer. This is due to the difficult polymerization of vinylphosphonic acid (VPA), which has been demonstrated by Bingol et al.22 The free radical polymerization proceeds via cyclopolymerization of VPA anhydride, which leads to a very slow rate of reaction. This has previously been observed for VPA copolymerization with monomers such as styrene,23 vinylpyrrolidone,24 and methyl acrylate.25 Therefore, when the VPA content in the feed is high, it is very difficult to produce high molecular weight polymers.

Although the reactivity of acrylic acid (AA) and VPA may be very different, the hydrodynamic properties of their polymers have been shown to be remarkably similar under given solution conditions.26 The main difference between the polymers is the ease of dissociation of the acid groups in an aqueous medium. Whereas PAA can be characterized as a weak polyelectrolyte, which means that it is only partially dissociated at intermediate pH, PVPA has been described as medium-strong and is therefore less affected by changes in the pH of the medium.27

It is the aim of this work to explore the potential of PVPA-co-AA for biomaterials applications. For this purpose, copolymers of VPA and AA were synthesized via free radical polymerization to obtain a range of copolymer compositions. The ability of the copolymer to affect osteoclast apoptosis is thought to be a result of its calcium chelation affinity, which is itself a product of the degree of dissociation of acidic groups under given solution conditions. Therefore, this study will probe the structure of the copolymer and its calcium chelation capacity with regards to changes in pH and copolymer composition.

**Experimental Section**

Materials. All chemicals were used without further purification unless otherwise stated. Vinylphosphonic acid (VPA), (97%) was purchased from TCI Ltd. Acrylic acid (AA), (99%), 2,3-epoxy-2-methylpropionanilide dihydrochloride (AAPH), (97%), 1-octanethiol, (≥98.5%), trimethyl phosphate (TMP), (97%), and deuterium oxide (D$_2$O), (99.9%) were all purchased from Sigma-Aldrich Ltd. Viscosity cellulose tubing (Scientific Laboratory Supplies) was used for dialysis with a molecular weight cut off (MWCO) of 12000–14000 g mol$^{-1}$ and an inflated diameter of 17.5 mm. Sodium hydroxide (NaOH), sodium chloride (NaCl), and calcium chloride (CaCl$_2$) were supplied by Fisher Scientific Ltd.

**Synthesis of PVPA-co-AA.** The following method details the synthesis of PVPA-40. Further details of the experimental conditions and procedures for all compositions are presented in Tables 1 and 2. 1-Octanethiol was used as a chain transfer agent (CTA) to control the polymer molar masses. For the homopolymerizations of AA and VPA, the initiator, AAPH, was added at the start of the reaction with the monomer. The polymerization of AA was completed in 3 h, whereas for VPA the reaction took 24 h to reach completion.

VPA (2.06 g, 19.0 mmol) was dissolved in deionized water (1.5 cm$^3$) and added to a 2-neck round bottom flask equipped with a reflux condenser. The apparatus was purged with N$_2$ before the polymerization. The solution was heated to 90 °C and left for 30 min. AA (3.06 g, 28.5 mmol) was dissolved in H$_2$O (1.6 cm$^3$), AAPH (12.9 mm, 0.048 mmol) was dissolved in H$_2$O (1.6 cm$^3$), and 1-octanethiol (13.9 mm, 0.010 mmol) was dissolved in H$_2$O (2.0 cm$^3$). These were added to the reaction flask in equal portions, every 30 min, over the course of 6 h. After the last addition, the reaction was left for a further 18 h. The product was purified by dialysis for 24 h, using

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**Author Contributions**

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

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**Notes**

The authors declare no competing financial interest.

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