Syntheses and Electrospinning of Poly(Amino Acid Ester) Phosphazene Biomaterials for Tissue Engineering Applications

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A thesis submitted in partial fulfillment of the requirements for the Doctor of Philosophy degree in Chemical and Biochemical Engineering
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Abstract

Despite advances made in the past decades to design biomaterials for tissue engineering, challenges remain. This thesis investigated the potential of electrospun poly(α-amino acid ester) phosphazenes (PαAPz) as novel biomaterials for vascular tissue engineering applications. As a class of biodegradable biomaterials, PαAPz provides biocompatibility and tunable properties and has gained attention as promising candidates for scaffolds in regenerative medicine, but their synthesis procedure is cumbersome due to the strict anhydrous environment and specialized equipment required for the thermal ring-opening reaction to produce the intermediate poly(dichlorophosphazene) (PDCP) product.

The research begins with the successful synthesis of PDCP using relatively simpler techniques using recrystallization and flame sealing with or without argon gas. The macromolecular substitution reaction to produce the final PαAPz was simplified using a one-step approach instead of the two-step conventional process. The PαAPz were tailored for vascular tissue engineering, focusing on the selection of α-amino acids (L-alanine, L-phenylalanine, and L-methionine) for their electrospinnability, biodegradability, and stem cell interaction properties. Following successful synthesis, electrospinning process parameters, such as polymer concentration, solvent selection, and electrospinning conditions, are systematically varied to fabricate beads-free fibrous mats with fiber diameters of 200nm to 700nm. Surface degradation studies showed PαAPz from L-phenylalanine degraded faster than those based on L-alanine, L-phenylalanine, and L-methionine. Atomic force microscopy (AFM) was used to evaluate the fiber mechanical characteristics and calculate its Young’s modulus, revealing it to closely mimic the stiffness of a natural extracellular matrix (ECM).

Mesenchymal stem cells derived from human induced pluripotent stem cells (iPSC), bone marrow-derived mesenchymal stem cells (BM-MSC) and primary human coronary artery smooth muscle cells (SMC) attached and well-spread on the fibers. Differentiation of iMSC to SMC was characterized by increased transcriptional levels of early to late-stage smooth
muscle marker proteins on electrospun fibrous mats. Evaluation of mesenchymal multipotent 10T1/2 cell and mesenchymal stem cell (MSC) behavior on the scaffolds demonstrated significant cell viability, proliferation, and successful MSC differentiation into smooth muscle cells. Expression of collagen and elastin by MSCs on the fiber mats highlighted potential ECM formation during scaffold degradation. In addition, PαAPz from L-methionine served as a reactive oxygen species (ROS) scavenger, thus protecting cells from stress. In order to expand the utility of the synthesized PαAPz to bone tissue engineering, the effect of their degradation products of osteogenic differentiation of stem cells was studied. It was observed that the late-stage degradation product, such as phosphoric acid, can significantly influence the osteogenic differentiation of MSCs.

The data collectively presented in this thesis demonstrated the potential of PαAPz in vascular tissue engineering, showcasing their potential in functional tissue formation, MSC differentiation, and protection against oxidative stress.

Keywords

Biodegradable poly(organophosphazenes), human mesenchymal stem cells; vascular smooth muscle cells; vascular tissue engineering; electrospinning; atomic force microscopy (AFM); ROS scavenger; aligned fiber
Summary for Lay Audience

Every year, cardiovascular diseases (CVDs) are claiming the lives of 20.5 million people globally. Among these CVDs, coronary artery disease is the leading cause of death. Bypass grafting is currently one of the most common interventions. Many patients lack suitable donor sites, and donor grafts can trigger immune reactions due to foreign body responses. At the same time, the current synthetic graft has poor behavior in medium/small arteries. To address these issues, scientists are turning to tissue engineering, a field where engineering principles meet biological sciences. This approach involves building organs or tissues using a patient’s own cells and supportive scaffolds, much like constructing a building with steel and bricks. Using the patient’s own cells eliminates the problems of immune rejection and donor shortages. But building such a scaffold and maturing cells on this scaffold remains a problem.

This study focuses on finding a suitable synthetic material for creating scaffolds in vascular tissue engineering. Exploration was conducted on a polymer named poly [(α-amino ester) phosphazenes] (PαAPz), whose degradation products are mainly composed of buffering components. These buffering degradation products are believed to be beneficial to vascular cells, suggesting such material can have strong potential in vascular tissue engineering. After synthesizing this polymer, it is made into a fiber mat with a similar structure to mask cloth but a much smaller fiber diameter. This mat simulates the three-dimensional structure of cell growth within the body. Subsequently, various measurements were conducted to assess the properties of this mat, test its ability to support cell growth and differentiation, and evaluate its potential for vascular tissue engineering applications.
In conclusion, this research introduced and investigated the potential of natural α- amino acid-based phosphazene for the application of vascular tissue engineering.
Co-Authorship Statement

The work contained within this thesis was a collaborative effort. Individual contributions are detailed below:

Chapter 1: Meng Wang – author; Dr. Kibret Mequanint – guidance and chapter revision.

Chapter 2: Meng Wang – author; Dr. Kibret Mequanint – guidance and chapter revision.

Chapter 3: Meng Wang – scaffold fabrication and characterization, cell culture experimental setup, author; Dr. Shigang Lin – qPCR and cell staining data collection; Dr. Kibret Mequanint – guidance and manuscript revision.

Chapter 4: Meng Wang – experimental setup, data collection, author; Dr. Kibret Mequanint – guidance and manuscript revision.

Chapter 5: Meng Wang – experimental setup, data collection, author; Dr. Kibret Mequanint – guidance and chapter revision.

Chapter 6: Meng Wang – author; Dr. Kibret Mequanint – guidance and chapter revision.
This work is dedicated to my parents,

Daqing Wang and Yi Qin
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<th>Description</th>
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<tbody>
<tr>
<td>3D</td>
<td>Three dimensional</td>
</tr>
<tr>
<td>8-Phe-4</td>
<td>PEA derived from sebacic acid, L-phenylalanine, and 1,4-butanediol</td>
</tr>
<tr>
<td>Acta2</td>
<td>Smooth muscle alpha-actin</td>
</tr>
<tr>
<td>Alpl</td>
<td>Alkaline phosphatase</td>
</tr>
<tr>
<td>AFM</td>
<td>Atomic force microscope</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>Col1</td>
<td>Collagen type I</td>
</tr>
<tr>
<td>DAPI</td>
<td>4′,6-diamidino-2-phenylindole</td>
</tr>
<tr>
<td>CF</td>
<td>Chloroform</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified Eagle’s medium</td>
</tr>
<tr>
<td>DMF</td>
<td>N,N-dimethylformamide</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethyl sulfoxide</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
</tr>
<tr>
<td>EDX</td>
<td>Energy-dispersive X-ray spectroscopy</td>
</tr>
<tr>
<td>EtOH</td>
<td>Ethanol</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>FTIR</td>
<td>Fourier transform infrared</td>
</tr>
<tr>
<td>HBSS</td>
<td>Hanks’ balanced salt solution</td>
</tr>
<tr>
<td>HCl</td>
<td>Hydrochloric acid</td>
</tr>
<tr>
<td>MEK</td>
<td>Methyl ethyl ketone</td>
</tr>
</tbody>
</table>
mRNA  Messenger RNA
MHC   Myosin heavy chain
MSC   Mesenchymal stem cell
Mw    Weight average molecular weight
NMR   Nuclear magnetic resonance
NEt$_3$  Tri-ethylamine
OCN   Osteocalcin
OPN   Osteopontin
PuAPz Poly [(α-amino acids) phazenes]
PuAPz-A Poly [bis (ethyl alanato) phosphazene]
PuAPz-P Poly [bis (ethyl phenylalanato) phosphazene]
PuAPz-M Poly [bis (ethyl methionato) phosphazene]
Pi    Inorganic phosphate
PBS   Phosphate-buffered saline
PCL   Poly(caprolactone)
PEA   Poly(ester amide)
PFA   Paraformaldehyde
Phe   L-Phenylalanine
qPCR  Quantitative real-time polymerase chain reaction
RNA   Ribonucleic acid
RT    Room temperature
Runx2 Runt-related transcription factor
SBF   Simulated body fluid
SD    Standard deviation
<table>
<thead>
<tr>
<th>Acronym</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>SEM</td>
<td>Scanning electron microscopy</td>
</tr>
<tr>
<td>SMC</td>
<td>Smooth muscle cells</td>
</tr>
<tr>
<td>SMTN</td>
<td>Smoothelin</td>
</tr>
<tr>
<td>TCP</td>
<td>Tissue culture plate</td>
</tr>
<tr>
<td>THF</td>
<td>Tetrahydrofuran</td>
</tr>
<tr>
<td>VSMCs</td>
<td>Vascular smooth muscle cells</td>
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</tbody>
</table>
Chapter 1

1 Introduction

1.1 Overview

Cardiovascular diseases, including coronary artery disease (CAD) are a major cause of death globally, accounting for around 20.5 million in 2021. CAD is caused by the narrowing or blockage of blood vessels supplying nutrients to the muscle of the heart. The two most common coronary interventions are angioplasty (a minimally invasive intervention) and bypass grafting (an invasive intervention). For bypass grafting, autologous arterial and venous transplantation is the optimal choice. However, 20% of patients requiring coronary artery bypass surgery have limited availability of autologous graft, and synthetic vascular grafts such as Teflon® and Dacron® have been frequently used for the past 50 years. While Teflon® and Dacron® work well for large arteries (e.g., the aorta) where the blood flow is high, their clinical outcome in medium/small arteries is disappointing. The technology of vascular tissue engineering holds promise in the design of responsive living conduits with properties similar to those of native tissue. In the most common vascular tissue engineering strategy, synthetic biodegradable, non-degradable, or extracellular matrix (ECM) scaffolds are infiltrated with vascular cells and cultured under physiological conditions. Tissue-engineered blood vessels are thus attractive since they are designed to be responsive both mechanically and biologically with respect to the load that changes with the hemodynamic environment. Despite considerable advances made in vascular tissue engineering over the past 30 years, the engineering of functional vascular tissues proved to be a formidable engineering task.
Scaffolds, once thought to be a passive cell support system, are now considered to be cell-instructive and must mimic the ECM. Existing vascular biomaterials make it difficult to meet the requirements of biological properties. More specifically, when the degradation products of the biomaterial are acidic, the acidic microenvironment will greatly affect cell behavior. Scaffolds must also provide biochemical cues to significantly increase the pace of tissue maturation and physical cues can affect the differentiation of stem cells. Therefore, degradable biomaterials that have near-natural degradation products, tunable degradation rates, mechanical properties, and potential for biochemical cues are needed.

The objective of this work is to design advanced and new classes of biodegradable scaffolds that can provide several of the above-mentioned features. Although the literature review focuses on vascular tissue engineering, the secondary goal is to expand the applications of these novel biodegradable scaffolds to other tissues.

1.2 Thesis outline

This thesis is structured into 6 chapters. In Chapter 2, a board review covering the strategies for tissue engineering, biomaterials, polyphosphazenes, electrospinning techniques, mechanical testing and ROS in tissue engineering is highlighted. At the end of Chapter 2, the hypothesis and objectives of the work are provided. The research findings of the listed objectives are presented in Chapters 3-5.

Chapter 3 is dedicated to the improvement of the PaAPz synthesis method, establishing electrospinning parameters, and assessment of their effectiveness in supporting the vascular smooth muscle cells differentiation of iPSC-derived mesenchymal stem cells.
In Chapter 4, an in-depth study was conducted on methionine based PαAPz, focusing on its microscale mechanical properties and morphological changes during degradation, and evaluated the material biocompatibility, along with the maturation of cells over the material scaffold.

Chapter 5 delves into the expansion of the application of methionine based PαAPz as ROS-scavenger, guide cell alignment and induce osteoblast differentiation of the iPSC-derived mesenchymal stem cells.

Finally, a summary of the work, the strengths, limitations, and future directions are outlined in Chapter 6.

### 1.3 References


Chapter 2

2 Literature review

Overview: This chapter provides background information on the strategies used in vascular tissue engineering, biomaterials used in tissue engineering and the novelty and limitations of polyphosphazenes. Furthermore, the requirements for the current advanced tissue engineering, like nanoscale topography, microscale mechanical properties and ROS scavenging, were discussed. At the end of the chapter, an outline of the scope of this work and the objectives are provided.

2.1 Strategies of tissue engineering

As stated in the introduction, vascular tissue engineering has emerged as a viable technology to fabricate arterial and peripheral blood vessel substitutes for clinical applications and improve clinical outcomes. A number of strategies have been used to engineer vascular tissues. The dominant strategy is a scaffold-guided approach. A less common strategy is cell sheet technology where cells are first grown at high density in the presence of ascorbic acid and rolled around a cylindrical mandrel to form tubes that are subsequently matured under physiological conditions. Yet, another strategy utilized is inserting a plastic rod into the peritoneal cavity of animals to recruit resident cells and form a capsule tissue, which upon removal of the rod will give a vascular tube. These approaches are briefly reviewed below.

2.1.1 Scaffold-guided vascular tissue engineering

The tissues and organs of the human body are not made up solely of cells. Outside the cell, there is an intricate network of macromolecules called the extracellular matrix (ECM). The basic function of ECM is to provide mechanical support and sites for anchoring cells.
Therefore, the most direct strategy is to engineer a tissue is to seed cells on a template scaffold that has a similar three-dimensional (3D) structure to the ECM. The most basic requirements for scaffold include (i) appropriate level and size of porosity allowing for cell migration; (ii) appropriate degradation rate to ensure that scaffold provides adequate support before cells generate their own ECM; (iii) suitable surface properties that encourage cell adhesion, growth, migration, and differentiation. Many methods have been developed to make porous scaffolds similar to ECM, such as freeze-drying, gas foaming, electrospinning, and 3D printing. The biomaterial selection and fabrication are important because topographical and physical cues provided by the scaffold are increasingly becoming important for stem cell differentiation tissue maturation. Various biomaterials, including natural and synthetic polymers, can also be utilized for vascular tissue engineering (this will be discussed further in Section 2.2).

2.1.2 Engineered tissues based on self-assembly

An alternative approach used to fabricate vascular tissue is the self-assembly process. In this approach, cells are first grown in large dishes at high density to produce their own ECM products and form sheets. The cells, along with their newly produced ECM, are then “peeled off” and rolled around a sold mandrel of the desired diameter to form tubes and then matured in a bioreactor for several weeks (> 12 weeks), making the strategy a slow process.

2.1.3 Engineered and decellularized tissue

While the scaffold-guided and the self-assembly strategies primarily aim for a live engineered tissue, one modification to either of the approaches is to decellularize after engineering the tissue. The rationale for this is based on the fact that the decellularized
tissue can serve as a universal graft since the immunologic cells are removed from it. This approach, reported by Nicklason and co-workers, uses a synthetic tubular biodegradable scaffold and seed animal or allogeneic human cells. The cell-seeded scaffold is then matured in a bioreactor. Once matured, the engineered tissue is decellularized, thus removing the immunogenic cells and making the graft “universal”. Because these decellularized tissues can be made in advance (as they do not require patient-specific cells), they can be available off the shelf. The acellular tissues can be seeded with patient-specific cells before implantation, thus shortening the timeline needed to fabricate it. It remains to be seen if this approach is better than the decellularization of pig arteries for human use instead of engineering it for decellularization.

2.2 Biomaterials for tissue engineering

Both the scaffold-guided live tissue fabrication and the decellularized tissue fabrication processes need a suitable biodegradable biomaterial from which the scaffolds can be fabricated. Natural and synthetic materials have been used to fabricate scaffolds. Natural materials commonly used in tissue engineering include collagen, fibrinogen, glycosaminoglycans (GAGs), gelatin, and cellulose. Collagen, fibrinogen, and GAGs are usually obtained from xenogenic, digested ECMs. Usually, these natural materials contain biological cues and signal molecules for cell adhesion and migration but have low mechanical properties. As an example, the most commonly used natural, collagen, has a variety of receptors that modulate cell behavior. However, many naturally occurring materials have weak mechanical properties. In addition to poor mechanical properties, natural polymers also have the disadvantage of a large batch to batch variation consisting.
Some suppliers can achieve lower batch to batch differences, the cost is usually higher, which will hinder the translation to the clinic.

Contrary to natural materials, synthetic materials, such as polyglycolic acid (PGA), polycaprolactone (PCL), and poly-L-lactide (PLLA) have the advantages of better mechanical properties, and tunable degradation rate and mechanical properties. However, they suffer from a common lack of biological activity and acidic degradation by-products. Adding biological cues and signaling molecules to the scaffold such as short-chain peptides (RGD, GRGDSP, PSHRN, and IKVAV) and peptide sequences improves their surface properties though the chemistry to do this is often complex. Despite several other synthetic materials reported for vascular tissue engineering, the quest for a better biomaterial continues. It is believed that the application of polyphosphazene to vascular tissue engineering may address some of the issues of current synthetic materials (e.g., biodegradation rate, initial mechanical properties, biocompatibility, pliability, etc.)

2.3 Polyphosphazenes in tissue engineering

Polyphosphazenes are a class of polymers whose main chain is composed of alternating nitrogen and phosphorus single bonds and double bonds. Hundreds of polyphosphazenes with different side groups have been reported. Among these, PaAPz (Scheme 2.1) are one of the most widely studied polyphosphazenes in tissue engineering due to their easy biodegradation and higher biological properties. The different PaAPz studied are those based on ethyl esters of alanine (PaAPz-A), glycine (PaAPz-G), and phenylalanine (PaAPz-F), and they have been investigated exclusively in skeletal tissue engineering.
Buffering effects of the degradation products of PαAPz are attractive because the degradation products are alcohol, amino acid, phosphates, and ammonia which are collectively non-toxic chemicals that can be metabolized by the body. Among the degradation products, phosphates and ammonia are common chemicals in buffering systems, while the amino acid by-products can serve as nutrients for cell growth. Because its degradation products are buffers the system, PαAPz have been mixed with polylactic acid to reduce the acidic environment which is produced by the degradation of polylactic acid.

In terms of biocompatibility, overall, PαAPz have demonstrated good cytocompatibility. Poly [(ethyl alanato)(p-methyl phenoxy) phosphazene] has been used to coat the surface of PCL fibers to improve the hydrophilicity of PCL, and ultimately enhanced the adhesion of mesenchymal stem cells. Endothelial cells (EC) were also cultured on a scaffold made by poly [(ethyl phenylalanato) (ethyl glycinate) phosphazene] and poly [(ethyl alanato)(p-methyl phenoxy) phosphazene].
phenylalanato) (ethyl alanato) (ethyl glycinate) phosphazene]. These PaAPz promoted EC adhesion, growth, and tissue formation into capillary structures. At present, the seeding of EC onto the graft is considered to be one of the main means to improve the patency of the transplanted blood vessel. Besides, mesenchymal stem cells (MSCs) also showed better cell adhesion than pure PCL scaffolds on PCL and PaAPz hybrid scaffolds.

The degradation rates and mechanical properties of PaAPz can be controlled by the side-chain substitution. Studies on the effects of different types of side-chain chemicals on the mechanical properties and degradation rate of polyphosphazene have been studied. For example, Allcock et al. reported that long carbon alkoxy side groups can be introduced into PaAPz to enhance elasticity. Recently, exploitation of the hydrogen bonding capacity of glycylglycine dipeptide has also been considered as an effective means that has the potential to change tensile modulus and strength. The degradation rate of PaAPz also differs depending on the type of amino acid connected to the main chain. Also, the use of other organic substances in the side chain can greatly improve the degradation rate. For example, when p-phenylphenoxy is introduced into the PNEG side chain, the time for 50% molecular weight loss is extended from about 1 week to 8 weeks.

2.3.1 Synthesis of polyphosphazenes

To date, the majority of research surrounding PaAPz focuses on orthopedic tissue engineering and drug delivery applications with limited use in vascular tissue engineering. One of the main reasons limiting the development of polyphosphazene materials is the synthesis of the precursor material poly[(dichloro)phosphazene](PDCP) requires expensive and specialized equipment before the actual PaAPz materials can be realized. Therefore,
previous work has tried to synthesize polyphosphazene under a non-strict anhydrous environment and simplify the synthesis process.\textsuperscript{46}

The overall synthesis step can be divided into two steps: synthesis of linear PDCP and macromolecular substitution of chlorine atoms. Currently, there are three main synthesis methods of linear PDCP: (i) thermal ring-opening polymerization (TROP),\textsuperscript{47} (ii) "living" cationic polymerization,\textsuperscript{48} and (iii) one-pot de jaeger polymerization.\textsuperscript{46,49} As a material for tissue engineering, polymers with relatively high molecular weights are required to ensure the material's mechanical properties. Due to the high degree of polymerization and relatively simple procedures, the TROP is currently the most popular synthetic method for polyphosphazene research in tissue engineering. In previous experiments conducted, grease-sealed synthesis of PDCP has proved successful without a glove box. However, this method is not reproducible, and the polymer formed has a relatively low molecular weight that could not be electrospun.\textsuperscript{46} So, the review of the reaction mechanism of TROP is needed. At the same time, due to the different needs and research directions of polyphosphazene in different laboratories, there is a considerable difference in the reaction time and the solvent used.

2.3.2 Thermal Ring-Opening Polymerization (TROP)

Although Stokes et al. are credited for the first synthesis of polyphosphazene by the thermal ring-opening reaction of cyclic hexachlorocyclotriphosphazene (HCCP), the material was highly cross-linked, and the chlorine atoms were difficult to functionalize and the material itself was difficult to dissolve.\textsuperscript{50} In the 1960s, Allcock et al. reported a linear polyphosphazene for the first time by conducting the reaction in a vacuum and by controlling the reaction time and temperature.\textsuperscript{51} The reaction mechanism of the thermal
ring-opening is poorly understood, but the most widely accepted mechanism is shown in Scheme 2.2.52
According to the reaction mechanism, in the initial stage of the reaction, when the components in the reactor are mainly trimers, the main reaction is a chain growth reaction (Scheme 2.2 (2)). When the number of linear polyphosphazene increases and after the chlorine on the polyphosphazene chain leaves the linear polyphosphazene may react with the trimer to produce branched polyphosphazene (Scheme 2.2 (4)) or cross-link with another linear polyphosphazene (Scheme 2.2 (5)). Therefore, the main strategy for manufacturing linear polyphosphazene is to terminate the reaction before the cross-linking reaction takes place. Even in an inert environment, it was found that the cross-linking reaction is rapid and unpredictable; thus, the maximum conversion is between 70% and 75%, and the cross-linking reaction will become uncontrollable beyond this point. Since the early experimental approach in the 1960s, continued improvements made it possible to optimize the TROP reaction such that the maximum value of $n$ in $\text{[NPCl}_2\text{]}_n$ was also increased from 15,000 to 40,000.

Although the standard preparation process of polyphosphazene requires a very strict anhydrous environment, previous work synthesized co-substituted $\text{PαAPz}$ without using a strict anhydrous environment. In this study, high-temperature grease was used to seal a custom-made glass reactor without removing the air from the glass reactor. During the PDCP formation at 230°C and time less than 25 h, 54.3% conversion was achieved before the onset of cross-linking. However, the $\text{PαAPz}$ obtained by this method was not electrospinnable, attributed to the lower molecular weight of the material. A modified synthesis approach for PDCP is needed such that the macromolecular substituted $\text{PαAPz}$ can be utilized for vascular tissue engineering.
2.3.3 Macromolecular Substitution

After synthesizing the linear PDCP, the chlorine atoms must be substituted using several side groups. In a previous study, three different macromolecular substitution methods were compared, namely, two-step method at room temperature, a two-step method at low temperature, and the newly created one-pot synthesis method at room temperature. Since the amino groups can be easily oxidized and are usually as a hydrochloride salt, the first step of macromolecular substitution reaction usually generates the free base of amino acid with a nonnucleophilic base, typically triethylamine (TEA, NEt3). The main difference between the three different methods mentioned above is on this first step. Subsequently, the amino acid ester solution will be mixed with the PDCP solution and complete the macromolecule.

2.3.4 PaAPz scaffolds

Despite advances made in the synthesis of PaAPz, there are limited reports of scaffold fabrication. Due to the wide application of polyphosphazene in bone tissue engineering, the microsphere scaffold is currently the most widely reported. However, this scaffold is also mainly used for drug delivery and bone tissue engineering and is not widely used in vascular tissue engineering. Furthermore, few attempts have been made to electrospin PaAPz. PaAPz that were electrospun are poly[bis(ethyl alanato) phosphazene], and poly[(ethyl phenylalanato) phosphazene]. The electrospinning parameters of these three electrospun PaAPz fibers are different from each other. The solvents used include THF, trifluoroethanol (TFE), and a 3:1 (v/v) THF/DMF solvent mixture.
2.4 Electrospinning

Various methods for forming tissue engineering scaffolds are available each having its advantages and disadvantages.\textsuperscript{59} Electrospinning is of particular interest in this proposed research because it can provide aligned nanofibers, which can affect the arrangement of cells, which is very important for vascular tissues.\textsuperscript{60, 61}

The traditional electrospinning device usually consists of three parts (Fig 2.1A): a high-voltage power supply, a grounded collector, and a spinneret (usually composed of a syringe and syringe pump). The electrospinning solution is kept in a syringe and pushed out at a stable and controllable speed through a syringe pump. The collector can be a flat plate or a rotating drum, but the surface of the collector needs to be conductive. Then, the syringe needle is usually connected to the positive pole of the high-voltage power supply, and the collector is connected to the negative pole or ground.\textsuperscript{62}

\textbf{Figure 2.1B} shows the specific electrospinning equipment used in this thesis. In addition to the fundamental components of electrospinning equipment, several safety features have been incorporated to mitigate potential hazards associated with electrospinning operations. The entire equipment is fitted with an enclosure box, and a switch on the box ensures that the equipment can only operate when the box is securely closed. Furthermore, the controllers regulating the speed of the collector and the voltage are positioned outside the box, allowing for adjustments to the electrospinning parameters during equipment operation. Simultaneously, the entire enclosure is equipped with a vent located above to facilitate the safe discharge of volatile organic solvents.
After connecting the spinneret to the high-voltage power source, the polymer solution in the syringes is charged. Due to the potential difference between the spinneret and the collector, the charges will migrate toward the surface of the droplet. As the voltage rises, the droplet on the spinneret will gradually be deformed by the potential difference into a shape called "Taylor cone". Afterward, as the voltage continues to rise, the droplet will eventually overcome the surface tension, leaving the spinneret and form a tiny charged jet. As the jet is accelerated by the electric field, the jet will become thinner and thinner. The solution will continuously be evaporated during this process. With the higher surface tension and viscoelastic force, the acceleration will continuously drop. When the acceleration becomes small or drops to zero, any small perturbation can destroy the straight movement.
After combining the jet path determined by the mathematical model and high-speed camera, the main cause of the instability of the jet is considered to be caused by the mutual repulsion of the charges carried by the jet itself.\textsuperscript{64, 65} This repulsion formed a lateral disturbance, which subsequently developed into an electrically driven bending instability. After several times of unstable bending, the arrangement of electrospun fibers became random.\textsuperscript{62, 66}

Although electrospinning is a very simple method to obtain nanofibers, many parameters can affect fiber morphology and diameter. These parameters can be divided into three categories: the parameters of the electrospinning settings, the parameters of the polymer solution, and the parameters from the surrounding environment.\textsuperscript{67} The parameters of the electrospinning setting include the feed flow rate, the diameter of the spinneret, the distance, and the voltage difference between the collector and the spinneret. Solution parameters include concentration, viscosity, conductivity, surface tension, the boiling point of the solution, dielectric constant, and the use of additives. Environmental conditions include temperature, humidity, and airflow around the spinneret. Therefore, there is no universal electrospinning parameter suitable for all polymers. In general, high-boiling solvents require lower feed rates and longer distances to ensure complete solution evaporation. High-viscosity, low-conductivity solutions require higher potential differences to allow Taylor cones to form.\textsuperscript{68} But at the same time, the parameters set by electrospinning will have limits, such as the maximum voltage that the power supply can provide. When the limit set by electrospinning is reached, it is necessary to adjust the solution. So, in theory, each solution can find the appropriate electrospinning setting parameters to form electrospinning fibers, but it may not be carried out within the range of
other parameters of the electrospinning settings. Therefore, a large number of attempts are required to determine the appropriate parameter range.

Compared to conventional random fiber electrospinning technology, the aligned electrospun fibers can affect the arrangement and differentiation of cells.\textsuperscript{69, 70} This structure is important when simulating the natural ECM of vascular. Another widely used structure in tissue engineering is the core-shell structure. The core-shell structure is often used for drug delivery and can load growth factors to further promote the growth of cells on the scaffold.\textsuperscript{71} At the same time, native tissues often have layers with different structures and compositions while these layers are mechanically integrated with the adjacent layer to maintain function. Therefore, gradient electrospinning of different fibers are needed.

2.4.1 Aligned electrospun fibers

Electrospinning, by default, gives randomly arranged fibers on the collector. However, there are many applications in electronics, photonics, actuators, and tissue engineering where the alignment of fibers in a preferred orientation. Therefore, many different aligned electrospinning technologies have been developed. The main strategies for forming aligned electrospun fibers are: (i) use a high-speed rotating collector to straighten the fiber and (ii) reduce the disorder of the bending zone by changing the arrangement of the electric field\textsuperscript{72}

First reported by Matthews et al. in 2002, the most common method of forming aligned electrospinning is to increase the rotational speed of the spin collector.\textsuperscript{73} In this cited study, collagen fibers were found to have a preferred alignment when the collector mandrel speed was increased to 4500 rpm (mandrel velocity was 1.4m/s). Similar studies demonstrated similar effects for polyethylene terephthalate,\textsuperscript{74} polycaprolactones,\textsuperscript{75} and polystyrene.\textsuperscript{76}
However, when the collector linear velocity was too high, the mechanical properties of the electrospun mats were compromised.\textsuperscript{77} The principle of a rotating collector to fabricate aligned electrospun fibers is believed to be the result of the drag force. As the fibers are continuous, a high-speed collector can form a drag force, thereby increasing the amount of force in the axial direction of the electrospun fiber.\textsuperscript{62} Therefore, the disturbance in the transverse direction is reduced, and the aligned electrospinning is formed. The degree of fiber alignment caused by a high-speed rotating collector decreases with increasing the spinning time (as the mat thickness increases) and is lower than other methods of inducing fiber alignment. The main cause of this phenomenon is that the charge on the electrospinning cannot be removed quickly, which affects the subsequent electrospinning.\textsuperscript{78} Also, since the fibers are parallel to the axis of the rotation axis before landing on the collector, high-speed rotation cannot effectively align these parallel fibers.

To address some of the limitations of rotating collector-induced fiber alignment, electrostatic alignment has been proposed by adjusting the electric field near the collector to minimize lateral disturbances or turn random disturbances into unidirectional disturbances (Fig 2.2A).\textsuperscript{79} One of the most effective methods is to use parallel electrodes. When the fibers are close to the parallel electrode, the electric field force will receive the force perpendicular to the parallel electrode. Due to the force in a single direction, fibers tend to line up in the direction of the force (Fig 2.2B, C).\textsuperscript{79} Because electrospinning itself is charged and moving, the electrospun jet could be seen as an electrifying solenoid during the ES process. So, the parallel magnetic collector is also used to align the electrospun fibers (Fig 2.2D).\textsuperscript{80} However, the distance between parallel electrodes is usually between 5-10 cm, and it is not easy to form a large area electrospun mat. Therefore, wire drum-type
rotating collectors have been developed (Fig 2.2E). The rotation speed of such a collector is usually below 100 rpm.

Figure 2.2. A) Setups of the parallel electrode electrospinning B) Electric field distribution of parallel electrodes C) Force analysis of electrospun fibers in the electric field generated by parallel electrodes D) Setups of the parallel magnetic poles E) Setups of the rotating wire drum collector.
Combining rotating collectors with a spindle-shaped electric field further enhances fiber alignment. When fibers approach the collector, the electric field converges, thus shrinking the bending zone and, reducing the disorder (Fig 2.3). Disk collectors and collectors with additional knife electrodes are the most common methods using this principle. In general, due to the relatively simple structure of the disc-type collector and the high degree of fiber alignment, it is currently one of the most widely used methods for collecting and aligning electrospun fibers.

Figure 2.3. (A) The electric field distribution between the needle tip and the aluminum strips. (B) setups of the counter-electrode made of parallel aluminum strips electrospinning. (C) Electric field distribution of the disk collector (D) setups of the disk collector electrospinning.
In addition to the above-mentioned methods for forming aligned electrospun fibers, a strategy of combining electrical and centrifugal forces to form aligned fibers at lower voltages (2.8-3kV) and collector speed (ca. 400 rpm). To provide a context, conventional electrospinning technology requires a voltage of 10-30kV and a speed of 3000-8000rpm for fiber alignment. The method of combining conventional electrospinning principle and 3D printing technology has also begun to emerge for aligned microfiber structures.

2.5 Mechanical test with AFM at microscale

There is growing recognition of how the mechanical properties of local ECM influence cell behaviors, including tissue development, homeostasis, repair, and disease progression. Researchers are paying more and more attention to the mechanical properties of materials used in tissue engineering. Traditional methodologies for quantifying the mechanical properties of biomaterials are measuring tensile or compressive force in a solid shaft or membrane states. However, due to the material’s internal structure and defects, such as crystal defects, and phase boundaries, the mechanical properties at the macroscale can be significantly different from that at the microscale. In the meanwhile, studies further identified the precise receptors that enable cells to detect pressure and elucidated the underlying mechanism of cellular pressure sensation. The size of the widest studied mechanoreceptor PIEZO1 proteoliposomes is approximately 20 nm in diameter. Obviously, the mechanical properties at the macroscale are insufficient to reflect the mechanical performance between cells and materials. Consequently, the development of techniques for microscale measurement of scaffolds or materials' mechanical properties is imperative.
While microscale measurement of synthetic tissue engineering scaffolds is infrequently reported, there is a body of research on the mechanical properties of biological samples such as cells and extracellular matrix. Among the various techniques employed to evaluate the mechanical properties of biological samples, atomic force microscopy (AFM) stands out for its unique ability to cover the elasticity of cells, ECM and tissue samples with submicrometer resolution while also enabling the probing of localized cell-ECM mechanical interactions at nanoscale. Therefore, AFM was posited as an effective way utilized to evaluate the mechanical properties of synthetic ECM-like scaffolds.

The process for assessing the mechanical properties of the material using AFM is shown in Figure 2.2. According to the resolution for the target assessment, probes of varying dimensions are employed to test the samples. As illustrated in Figure 2.2, when approaching the target surface, the material's viscosity and surface tension will attract the probe through intermolecular forces and draw it downwards to reach point B. Following this, the probe moved with a pre-calibrated distance, arriving at point C, thereby delineating the approaching force curve. The probe then initiates its retraction phase. Typical force curves of segments BC and CD tend to parallel to each other. Nonetheless, deviations in these curves are observed in samples with porous structures. In recent reports, the segment of the retraction force curve proximal to the material's surface was accepted to be the proper part that can be used to calculate the Young's modulus of the sample (blue dashed line in Figure 2.2).
Depending on the shape of the indenter and the sample’s surface, several models of applied mechanics can be used to calculate Young’s modulus. Among these models, the Hertz model is a classic and widely used method to determine the mechanical properties of biological samples and biomaterials.\textsuperscript{95-97} This model was first used to describe the contact between two elastic spheres.\textsuperscript{98} However, when testing the biological samples and biomaterials, the target sample is often approximated as a sphere with an infinitely large radius. Thus, the Hertz model can be simplified as shown in Equation 2-1, where $F$ is the applied load, $E$ is Young's modulus, $v$ is Poisson's ratio of the sample, $R$ is the radius of the contact area, and $h$ is the indentation depth.
2.6 ROS and ROS-scavengers in tissue engineering

Reactive oxygen species (ROS) have been considered the pivotal signaling molecules in the physiologic and pathologic processes and play an important role in cellular signal transductions and cell homeostasis interference. In tissue engineering, despite the stimuli caused by pathological microenvironments, scaffold-cell interactions and surgical implantation can all increase the ROS levels. This can lead to an elevated level of oxidative stress, inflammatory response, and cellular damage, as well as disruption in the tissue repair process.

In response to this challenge, researchers have turned their focus towards ROS-scavenging biomaterials and their potential impact on tissue engineering. Duvall et al. utilized poly (propylene sulfide) (PPS) conjugated with curcumin and observed the decreased ROS level in activated macrophages, reduced oxidative stress-induced cell death in vitro, and increased angiogenesis in a hind-limb ischemia model. Shan et al. developed ROS-scavenging hydrogels containing rapamycin which can promote the survival of mesenchymal stem cells (MSCs) under oxidative environment and induced myogenesis and macrophages polarization toward M2 phenotypes. Zhou et al. demonstrated the ROS scavenging capabilities of polypyrrole-polydopamine (PPy-PDA) by coated on hydroxyapatite scaffolds. Such porous scaffold provided excellent bone regeneration through the synergistic effects of electroactivity, cellular affinity, and antioxidant activity.
of PPy-PDA nanoparticles and osteogenic induction of hydroxyapatite NPs. Furthermore, Wang et al. prepared a series of novel chemically modified N-polyphenol substituted chitosan derivatives, which displayed antioxidative activity and promoted the osteo-differentiation of MSCs.\textsuperscript{104}

A wide range of antioxidants and free radical scavengers, including vitamins,\textsuperscript{105, 106} thiols,\textsuperscript{107} chondroitin sulfate\textsuperscript{107} and hyaluronic acid\textsuperscript{108} have been proven to reduce oxidative stress. However, the short half-life of these small molecules in circulation limits their in vivo biological functions.\textsuperscript{99} In the meanwhile, traditional macromolecular ROS-scavengers, primarily conductive polymers, are also found unsuitable for tissue engineering due to their non-degradable nature under physiological conditions.\textsuperscript{109, 110} Therefore, developing biodegradable macromolecular ROS-scavengers are needed.

The strategies for synthesizing biodegradable macromolecular ROS-scavengers can be categorized into four main types: polyphenol-based, polysaccharide-based, peptide and amino acid-based, and mimetic enzyme-based scavengers.\textsuperscript{111} Given the ease of utilizing amino acids in the synthesis or conjugation of polymers, coupled with the well-studied mechanisms of ROS scavenging, there is a growing research focus on amino acid-based ROS scavengers.\textsuperscript{112, 113}

Among the amino acid-based ROS scavengers, poly (ester amide) (PEA) polymers are currently the primary focus of research. C. Chu's et al. used L-methionine as the monomer in the synthesis of poly (ester amide) and have noticed its response to the intracellular high ROS concentration in PC3 cells.\textsuperscript{114} Additionally, Jun Wu synthesized PEA with l-arginine
and l-phenylalanine and observed the promotion of wound healing by shortening the inflammation stage in wound healing experiments with rats.\textsuperscript{115}

Similarly, PaAPz has also demonstrated strong potential as ROS scavenger effects in tissue engineering due to its biodegradability and easily modifiable structure. Cai and his team have co-substituted aniline tetramer and glycine on the polyphosphazene side chain, observing its capacity to eliminate exogenous ROS and ultimately achieving efficient bone regeneration.\textsuperscript{116} Notably, in this study, ROS scavenging function comes from the aniline tetramer, and amino acids mainly contribute to phosphazene's biodegradability. To date, there are no reports on the efficacy of amino acid-based polyphosphazene in ROS scavenging. Given that sulfur-containing amino acid (such as cysteine, cystine, and methionine), aromatic amino acids (such as tyrosine, tryptophan, phenylalanine), proline, and histidine exhibit relatively strong ROS scavenging abilities, polyphosphazenes based on these amino acids hold significant potential as ROS scavengers for tissue engineering applications.\textsuperscript{117}

\section*{2.7 Hypothesis and objectives}

\textit{Hypothesis:} Fibrous PaAPz mats provide the material and topographical cues required for stem cell vascular differentiation and ECM expression.

\textit{Objectives:}

(i) Syntheses and characterization of electrospinnable PaAPz from different $\alpha$-amino acids using a simplified approach and study vascular differentiation of stem cells.

(ii) Study electrospinning process parameters for random and aligned fibers and their morphological properties.
(iii) Investigate the microscopic mechanical properties of pure PαAPz random fibers and co-electrospun with poly(ester amide)s (PEA).

(iv) Investigate the feasibility of PαAPz prepared from methionine for its reactive oxygen scavenging potential and the effect of PαAPz temporal degradation products on osteogenic differentiation of stem cells.

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Chapter 3

Electrospun biodegradable α-amino acid-substituted poly(organophosphazene) fiber mats for stem cell differentiation towards vascular smooth muscle cells*

Overview: This Chapter focuses on refining the synthesis process of electrospinnable polyphosphazenes and explores its use in vascular tissue engineering. In this study, polyphosphazene was synthesized under easily achievable non-stringent anhydrous conditions and produced an electrospinnable polymer. Subsequently, the vascular differentiation of mesenchymal stem cells on this scaffold was also validated.

3.1 Abstract

Mesenchymal stem cells derived from human induced pluripotent stem cells (iPSC) are valuable for generating smooth muscle cells (SMCs) for vascular tissue engineering applications. In this study, biodegradable α-amino acid-substituted poly(organophosphazene) polymers were synthesized and electrospun into nano-fibrous scaffolds to evaluate their suitability as a matrix for differentiation of iPSC-derived mesenchymal stem cells (iMSC) into mature contractile SMCs. Both the polymer synthesis approach and the electrospinning parameters were studied. Three types of cells, namely iMSC, bone marrow-derived mesenchymal stem cells (BM-MSC) and primary human coronary artery SMC attached and well-spread on the materials. Although L-ascorbic acid (AA) and transforming growth factor-beta 1 (TGF-β1) were able to differentiate iMSC along the smooth muscle lineage, the electrospun fibrous mats also provided material cues

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for enhanced differentiation of iMSCs. Differentiation of iMSC to SMC was characterized by increased transcriptional levels of early to late-stage smooth muscle marker proteins on electrospun fibrous mats. These findings provide a feasible strategy for engineering functional vascular tissues.

3.2 Introduction

Biodegradable poly(organophosphazenes) substituted with α-amino acids, hereinafter abbreviated as PαAPz, are important biomaterials for drug delivery and tissue engineering applications\textsuperscript{1-3}. Unlike biodegradable polyesters (e.g., poly(lactide), poly(glycolide), and their copolymers), PαAPz releases non-acidic and buffering degradation products comprised mainly of phosphate, ammonia, and the corresponding side groups\textsuperscript{4, 5}. Several previous studies investigated PαAPz primarily for the regeneration of musculoskeletal tissues and demonstrated their ability to support osteoblast adhesion and proliferation with minimal inflammatory responses and enhanced bone growth\textsuperscript{1-3, 6-8}. To introduce porosity to bone tissue engineering PαAPz scaffolds, either dynamic solvent sintering of pre-formed microspheres\textsuperscript{9, 10} or electrospinning of PαAPz blended with nanohydroxyapatite\textsuperscript{11} have been studied. In one study\textsuperscript{12} where a PαAPz was electrospun for bone tissue engineering, the fiber diameter was large (~55µm), making it challenging for osteoblasts to see the true 3D topography of the fibers. In addition to bone cell culture, some studies investigated electrospun PαAPz for their potential use of endothelial monolayer cell culture\textsuperscript{13, 14}.

While the above-mentioned studies demonstrated the versatility of PαAPz, primarily in bone regeneration, their utility in vascular tissue engineering has not been studied. Vascular smooth muscle cells (VSMCs) are the major cellular components to engineer vascular
tissues. Since VSMCs are particularly sensitive to acidic degradation products, it is believed that PαAPz that degrade into non-acidic by-products could be excellent biomaterials for smooth muscle tissue engineering. Because harvesting primary VSMCs from patients is not feasible due to their anatomical location (e.g., coronary artery), mesenchymal stem cells (MSCs) are ideal cell sources for vascular tissue engineering. MSCs are multipotent cells commonly characterized by their ability to differentiate into cell types of mesodermal origin. MSCs can be isolated from many adult tissue sources in a non-invasive manner; however, their self-renewal and differentiation potential is dependent on the cell source of isolation. It may also be affected by donor aging and environmental stresses from in vitro cultivation. To circumvent these challenges, the use of iPSCs for generating MSCs is beneficial to preserve their high differentiation potential.

MSCs can be differentiated into VSMCs to engineer vascular tissues for morphogenesis and functional studies. VSMCs express smooth muscle phenotypic marker genes and proteins such as α-SMA and calponin that are early and mid-stage differentiation markers, and myosin heavy chain (MHC), smoothelin (SMTN) and smoothelin-B, which represent late-stage differentiation stages. Expression of early- and mid-stage markers only indicates progression towards a smooth muscle lineage and are also expressed in other cells; however, the late-stage marker proteins MHC and SMTN are exclusively expressed in mature contractile VSMCs. In view of this, biomaterials designed for vascular tissue engineering must promote the differentiation of MSCs to VSMCs by providing mechanical and topological cues. Although MSC differentiation to vascular smooth muscle cells on electrospun fibers have recently been reported, the fibers were prepared from a non-
degradable polyacrylonitrile\textsuperscript{30} or else the fibers were used only to deliver a biochemical factor\textsuperscript{31}.

The objectives of this study were twofold. First, the synthesis procedure is modified electrospinnable PαAPz by modifying the synthesis procedure. This is important since most PαAPz are generally difficult for producing electrospun fibrous mats without being blended with other polymers, and only few previous studies attempted doing this. Secondly, fibrous mats were used for differentiation of iPSC-derived MSCs towards vascular smooth muscle cells.

3.3 Experimental

3.3.1 Materials

Hexachlorocyclotriphosphazene (HCCP) was obtained from Sigma Aldrich (Milwaukee, WI) and stored in the desiccator until used. Anhydrous tetrahydrofuran (THF), glass distilled hexanes-190, were acquired from Caledon Labs (Georgetown, ON). Triethylamine (NEt\textsubscript{3}), Chloroform (CF), and (DMSO) were purchased from Sigma Aldrich. L-alanine ethyl ester hydrochloride (H-Ala-OEt•HCl) and L-phenylalanine ethyl ester hydrochloride (H-Phe-OEt•HCl), were from Alfa Aesar (Ward Hill, MA) and stored in the fridge. Krytox Performance Lubricant GPL207 high-temperature grease was purchased from DuPont (Wilmington, DE). Unless specified otherwise, all chemicals and solvents were used as received.
3.3.2 Synthesis of P\textalpha APz

Polydichlorophosphazene (PDCP) was prepared using thermal ring-opening polymerization (TROP) with three different approaches, namely: grease sealing, flame sealing, and HCCP recrystallization followed by flame sealing. In the grease sealing approach, approximately 1 g HCCP was added into a dry glass reactor fitted with a stopper and argon was purged to displace any residual air on the walls of the reactor. For the flame sealing and recrystallization (FS-R) approach, HCCP was first recrystallized by vacuum sublimation at 100°C and 20 mmHg before use. Following recrystallization, approximately 2 g of HCCP was added into a glass ampoule and then connected to a vacuum line and flame-sealed using a propane torch. Alternatively, the vacuum step could be replaced by filling the ampoules with dry argon followed by flame sealing. The sealed ampoules were placed in the oven at 230°C for 72 h. The samples were recovered from the ampoules and were dissolved in THF, and purified by precipitating in hexanes three times to remove the unreacted HCCP and crosslinked PDCP. PDCP recovery and purification were similar for grease sealing and flame sealing.

3.3.3 Macromolecular substitution

The macromolecular substitution reaction was carried out by a one-step approach at room temperature as described before\textsuperscript{32}. Briefly, after flame drying a 50 ml flask either H-Ala-OEt•HCl (1.70 g, 11.1 mmol, 2.6 equiv) or H-Phe-OEt•HCl (2.16 g, 11.1 mmol, 2.6 equiv) was added into the reaction flask, and then the flask was sealed with a rubber septum. Next, 10 ml of THF and 4 ml of triethylamine were injected into the flask through the septum. Then, PDCP (0.50 g, 4.3 mmol of N=P-Cl\textsubscript{2} units) obtained from the TROP was dissolved in 10 ml of anhydrous THF and injected into the reaction flask through the
septum. The mixture was stirred for 72 h, filtered to remove the insoluble salt, and the product was dissolved in anhydrous THF and purified by repeated precipitation in hexanes. The PαAPz polymers were then dried in a vacuum and stored at 4 °C.

3.3.4 PDCP and PαAPz characterization with $^{31}$P-NMR and $^1$H-NMR.

Nuclear Magnetic Resonance (NMR) spectroscopy was performed on a Varian INOVA 400 MHz spectrometer ($^1$H 400.1 MHz, and $^{31}$P{1H 161.8 MHz, Varian Canada Inc., Mississauga, ON). Chemical shifts are reported in parts per million (ppm). All chemicals are dissolved in chloroform (CDCl3) with a concentration of ~40mg/ml. Chemical shifts were relative to chloroform at $\delta$=7.27 ppm.

3.3.5 Preparation of PαAPz thin films

PαAPz was dissolved in THF to form a 1wt.% solution. Then 67.8 μL of the solution was dipped onto a glass coverslip with a diameter of 12 mm. After drying, all the film was sterilized under the UV light for 30 min and pre-treated with Hank’s Balanced Salt Solution (HBSS) overnight.

3.3.6 Electrospinning of PαAPz

The electrospinning equipment consists of 0.5 mL glass syringes, blunt-tip 22-gauge stainless steel needles, high voltage DC power supply (7 - 20 kV, ES30P, Gamma High Voltage, USA), syringe pump (KD101, KD Scientific, USA) and rotating collector. The PαAPz were electrospun at various concentrations and spinning parameters (Table 2.) The morphology of the PαAPz fibrous mat was evaluated using SEM (S-2600N Hitachi, Japan). Fibrous samples were sputter-coated with gold/palladium (K550X, sputter coater, Emitech
Ltd., UK) and scanned at a working distance of 9 mm and a constant accelerating voltage of 5 kV. Analysis of the SEM was performed with ImageJ software (NIH, Bethesda, MD, USA).

3.3.7 Gel Permeation Chromatography (GPC)

Gel permeation chromatography (GPC) analysis was conducted with a Shimadzu system equipped with a CMB-20A controller system and styrene-divinylbenzene columns. The sample was dissolved in dimethylformamide (DMF) with 20 mM tetrabutylammonium trifluoromethanesulfonate. The GPC columns were calibrated with low-dispersity poly(methyl methacrylate) (PMMA) standards.

3.3.8 Cell culture studies on PαAPz and smooth muscle cell differentiation

Mesenchymal stem cells derived from induced pluripotent stem cells (iPSCs), hereinafter named iMSCs (kindly donated by Dr. Dale Laird, Western University, Canada) and human bone marrow-derived mesenchymal stem cells (BMMSCs, Lonza, Walkersville, MD, USA; PT-2501) were used. iMSCs were grown on gelatin (Sigma-Aldrich Canada Co., Oakville, Ontario) coated dishes in mesenchymal stem cell expansion media (MSCEM, Cedarlane Labs, Burlington, Ontario; HMSC.E.MEDIA-450) supplemented with 10% fetal bovine serum (FBS), 1% l-glutamine and 1% penicillin/streptomycin (all from Fisher Scientific, Whitby, Ontario, Canada). Media were changed every other day. Human coronary artery smooth muscle cells (HCASMCs, Lonza, USA. CC-2583) were maintained in Smooth Muscle Cell Growth Medium 2 BulletKit (SmGM-2, Lonza, USA). Cells between passage numbers 4-11 were used for experiments. Cells cultured in humidified incubators at 37°C and 5% CO₂. Differentiation of stem cells to smooth muscle cell lineage
was induced using L-ascorbic acid (L-AA, Sigma-Aldrich Canada Co., Oakville, Ontario) and transforming growth factor (TGF-β1, R&D Systems, Minneapolis, MN, USA) in high glucose DMEM modified based on reported procedures. Briefly, iMSCs were plated either on gelatin-coated dishes for 2D study (2 × 10^4 cells/cm^2 in 6-cm Corning cell culture dish) or on gelatin-coated electrospun fibrous mats for 3D study (20 × 10^4 cells per mat with a size of 2 × 2 cm) and pre-cultured in MSCEM to reach 70% of confluence followed by changing culture media to differentiation condition (DC) composed of DMEM supplemented with 1% FBS plus 82.5μg/mL L-AA or 2 ng/mL TGF-β1. The induced smooth muscle-like cells were characterized by detection of smooth muscle marker genes and proteins using qRT-PCR, Western blot analysis as well as immunofluorescence staining. All fibrous mats were coated with 0.1% gelatin at 37ºC for 1 h before cell seeding. Non-induced iMSCs were grown in regular MSCEM as the undifferentiated growth control (GC).

3.3.9 Quantitative real-time qPCR and Western blot analysis.

Total RNA was extracted from cells using TRIZol™ Reagent (Life Technologies Inc., Burlington, Ontario) following the manufacturer’s instructions. One μg of total RNA was reverse transcribed into complementary DNA (cDNA) using the Promega™ Random Hexamers protocol (Fisher Scientific, Whitby, Ontario). qRT-PCR was conducted in four repeats using a CFX96™ Real-Time System (C1000 Touch Thermal Cycler; Bio-Rad, Mississauga, Ontario, Canada), and human genes of interest were determined with SsoAdvanced Universal SYBR® Green Supermix (Bio-Rad) according to the recommended procedures. The sequences of primers were as follows: Human Acta2 forward primer 5’-CAA GTG ATC ACC ATC GGA AAT G-3’, reverse primer 5’-GAC
TCC ATC CCG ATG AAG GA-3’. Human \textit{Cnn1} forward primer 5’-TGA AGC CCC ACG ACA TTT TT-3’, reverse primer 5’-GGG TGG ACT GCA CCT GTG TA-3’. Human \textit{Myh11} forward primer 5’-GTC CAG GAG ATG AGG CAG AAA C-3’, reverse primer 5’-GTC TGC GTT CTC TTT CTC CAG C-3’. Human SMTN forward primer 5’-CAG GAC AAC GAG AAC TGG-3’, reverse primer 5’-CAG TCA ATT CCT CCA CAT CGT-3’. Human 18S forward primer 5’-GCG GTT CTA TTT TGT TGG TTT-3’, reverse primer 5’-CTC CGA CTT TCG TTC TTG ATT -3’. The results were analyzed with the comparative threshold cycle method and normalized with human 18S as an endogenous reference.

Western blotting was performed to evaluate the expression levels of specific smooth muscle marker proteins. Cell lysates were separated via SDS-PAGE, transferred to a nitrocellulose membrane, and immunoblotted using the following primary antibodies: SM-MHC (rabbit, 1:1000; Alfa Aesar, BT-562), SMTN-B (rabbit, 1:1000; Santa Cruz, sc-28562), and β-Tubulin (mouse, 1:250; ThermoFisher Scientific, MA5-11732). Primary antibody labeling was detected using HRP-conjugated goat anti-rabbit or anti-mouse secondary antibodies and the ECL detection system.

3.3.10 Immunofluorescence microscopy

Cells were fixed in 10% formalin for 10 min at ambient temperature, followed by three washes in PBS. All samples were permeabilized with 0.1% Triton X-100 for 10 min and then were blocked in 2% BSA with 22.52 mg/mL of glycine in PBS for 30 min at ambient temperature. Samples were labeled with the following antibodies overnight at 4°C: α-SMA (mouse, 1:100; Santa Cruz, sc-32251), SM-MHC (rabbit, 1:100; Alfa Aesar, BT-562),
SMTN-B (rabbit, 1:100; Santa Cruz, sc-28562), SMTN (rabbit, 1:100; Santa Cruz, sc-166292). Primary antibody binding was detected using Alexa Fluor® 555 goat anti-rabbit IgG or Alexa Fluor® 488 goat anti-mouse IgG as secondary antibodies (1:300) addition with Alexa Fluor® 568- or Alexa Fluor® 488 phalloidin (1:100 dilution, all from Life Technologies, Burlington, Ontario, Canada) in some cases. The cells were counterstained with 4’6-diamidino-2-phenylindole (DAPI, 300 nM in PBS, Life Technologies, Canada) for labeling nuclei. Coverslips were mounted on microscope slides with PermaFluor™ Mounting Medium (Fisher Scientific™, Whitby, Ontario, Canada) and sealed with clear nail enamel. Images were taken with a Zeiss LSM 800 confocal microscope (Zeiss, Toronto, Canada) equipped with 10x/25x (water) lenses and analyzed using ZEN 3.1 (blue edition) software.

3.3.11 Statistical analysis

Data are presented as mean ± SD unless otherwise indicated. At least three independent experiments were analyzed by one-way ANOVA with Student’s two-tailed independent sample t test. All analyses were done using GraphPad 5.0 statistical software (GraphPad Software) and significance was assigned for p< 0.05

3.4 Results and Discussion

3.4.1 PαAPz synthesis and characterization

The synthesis of PαAPz is a two-step procedure. In the first step, the thermal ring-opening polymerization of HCCP at high temperature (ca. 230°C) produces PDCP. In the second step, the chlorine atoms attached to the central phosphorous are replaced with side chains via a macromolecular substitution reaction at room temperature (Figure 3.1A). The thermal
ring-opening reaction requires a strict anhydrous environment and specialized equipment. In this study, the complexity involved in the ring-opening reaction was simplified while simultaneously reducing undesirable hydrolysis and crosslinking. Since the primary objective in the ring-opening reaction is to avoid moisture, six different approaches were attempted (Table 3.1). In the first approach (GS method), high-temperature fluorinated grease were used as a potential moisture barrier when sealing the reaction content using a glass stopper. The main component of this grease is perfluoroalkyl ether which was used for food packaging and proved to have the ability to provide moisture resistance. With the GS method, the product crosslinked after 25 h reaction, so the reaction was stopped at this time. In a modification of the GS procedure, argon was purged into the glass reaction vial and sealed with the same grease and glass stopper with the expectation that the argon may displace any residual moisture/air present around the walls of the glass vial due to its high density (GS-Ar method). Indeed, the conversion of HCCP to PDCP increased from 54% to 86% when argon was used to displace air, although it was crosslinked after 54 h of reaction. For the remaining four thermal ring-opening reaction methods, flame sealing of the glass ampoule was used. Thus, flame sealing (FS), flame sealing using argon (FS-Ar), flame sealing under vacuum (FS-vacuum), and recrystallization of HCCP followed by flame sealing under vacuum (R-FS-vacuum) were explored. Both FS-vacuum and R-FS-vacuum methods produced PDCP that was not crosslinked during 92 h of reaction at 230°C. In addition, the conversion of HCCP to PDCP increased to 95%; thus, FS-vacuum and R-FS-vacuum methods were taken as appropriate methods to produce linear PDCP for the subsequent macromolecular substitution with α-amino acid esters. The thermal ring-opening polymerization time of HCCP to produce PDCP is up to 120 h, and the goal is to get very high conversions without crosslinking. Since the reaction occurs in the melt, the
occurrence of crosslinking is easily detected by visually inspecting if the melt still flows when the ampoule is held upside down.

Table 3.1: Comparison of different methods to synthesize PDCP

<table>
<thead>
<tr>
<th>Method</th>
<th>Reaction time at 230°C</th>
<th>Integration of +20ppm peak</th>
<th>Integration of -17ppm peak</th>
<th>% conversion</th>
</tr>
</thead>
<tbody>
<tr>
<td>GS</td>
<td>25 h</td>
<td>1</td>
<td>1.19</td>
<td>54</td>
</tr>
<tr>
<td>GS-Ar</td>
<td>54 h</td>
<td>1</td>
<td>6.51</td>
<td>86</td>
</tr>
<tr>
<td>FS</td>
<td>58 h</td>
<td>1</td>
<td>5.70</td>
<td>85</td>
</tr>
<tr>
<td>FS-Ar</td>
<td>93 h</td>
<td>1</td>
<td>5.32</td>
<td>84</td>
</tr>
<tr>
<td>FS-Vacuum</td>
<td>92 h</td>
<td>1</td>
<td>18.91</td>
<td>95</td>
</tr>
<tr>
<td>R-FS-Vacuum</td>
<td>92 h</td>
<td>1</td>
<td>15.38</td>
<td>94</td>
</tr>
</tbody>
</table>


$^{31}$P-NMR spectra for HCCP, linear PDCP, PαAPz and $^1$H-NMR spectrum of poly [bis (ethyl alanato) phosphazene] (PαAPz-A) are shown in Figures 3.1B &C. Consistent with the literature, HCCP shows a single peak at 21.09ppm, and PDCP, which is the product from the thermal ring-opening reaction after purification by precipitation showed a singlet at approximately -17 ppm. In Figure 1B, representative $^{31}$P-NMR spectra of PDCP from grease sealing and flame sealing methods before purification are also shown (B2, B3).
Figure 3.1. (A) Synthesis scheme of PaAPz-A. (B) $^{31}$P NMR of (1) HCCP, (2) crude PDCP from GS method, (3) Crude PDCP from FS, and (4) purified PDCP, (5) PaAPz-A. C) $^1$H NMR of PaAPz-A.
In the $^{31}$P-NMR spectrum of PαAPz after the macromolecular substitution with alanine ester, the peak at -17.27 ppm representing the phosphorous atom on PDCP disappeared and there is only a small peak at +1.41 ppm (Fig. 3.1B (5)) which lies between the reported values of +1.2 to +1.9 for PαAPz. The complete disappearance of the peak at -17.27 ppm means that most, if not all, Cl atoms in the PDCP have been replaced. In the H-NMR, all the expected peaks for the ethyl ester of alanine are shown, which confirmed the successful substitution. As reported before, the hydrogen connected to the amino group was not seen in the H-NMR spectrum. In addition, the $^1$H-NMR demonstrated that the unreacted amino acid had been fully removed during the precipitation process.

3.4.2 Electrospinning of PαAPz

Although PαAPz have been used for drug delivery studies in solid and film forms, electrospinning allows for the formation of fibers that can be utilized for tissue engineering. However, standalone fiber formation from PαAPz is often challenging except for a few reports. Therefore, a wide range of electrospinning parameters and solvent combinations to produce fibrous mats were studied (Table 3.2).
Table 3.2. Studied electrospinning parameters for PaAPz.

<table>
<thead>
<tr>
<th>Sample abbreviations</th>
<th>Solvent ratio</th>
<th>Distance (cm)</th>
<th>Voltage (kV)</th>
<th>Concentration (wt%)</th>
<th>Flow rate (ml/h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PaAPz-A</td>
<td>CF:DMSO (3:1)</td>
<td>12-15(*12)</td>
<td>15-20 (*20)</td>
<td>7.5-12.5(*10)</td>
<td>0.2-0.6(*0.2)</td>
</tr>
<tr>
<td>PaAPz-P</td>
<td>THF:CF (9:1)</td>
<td>9-15(*12)</td>
<td>12-20(*12)</td>
<td>10-15(*10)</td>
<td>0.2-0.6(*0.2)</td>
</tr>
<tr>
<td>PDCP from R-FS-vacuum method</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PaAPz-A</td>
<td>CF:DMSO (3:1)</td>
<td>12-15(*12)</td>
<td>15-20 (*15)</td>
<td>7.5-12.5(*10)</td>
<td>0.2-0.6(*0.2)</td>
</tr>
<tr>
<td>PaAPz-P</td>
<td>THF:CF (9:1)</td>
<td>9-15(*12)</td>
<td>12-20(*12)</td>
<td>10-15(*10)</td>
<td>0.2-0.6(*0.2)</td>
</tr>
</tbody>
</table>

*Optimal electrospinning parameters. CF: Chloroform, THF: tetrahydrofuran, DMSO: dimethylsulfoxide. A – alanine and P – Phenylalanine

The PaAPz-A and PaAPz-F prepared in the current study were electrospinnable from a solution of THF or CF, but that depended on the method of the PDCP preparation. Although PaAPz-A PaAPz-F were successfully synthesized from PDCP that was prepared by all the methods listed in Table 3.1, only PDCP from FS-Vacuum and R-FS-Vacuum methods resulted in PaAPz that could be consistently electrospun. PDCP prepared by grease sealing resulted in a corresponding PaAPz that led to electrosprayed particles instead of fibers attributed to hydrolysis of PDCP during its synthesis and subsequent lower molecular weight of PaAPz (shown in Table 3.3). It is known that lower molecular weight polymers often produce electrosprayed particles rather than continuous fibers due to the lower solution viscosity that makes the Taylor cone unstable during electrospinning.
Table 3.3 GPC analysis of $\alpha$APz produced by polymerization following grease sealing (GS) and recrystallization followed by flame sealing (R-FS) methods.

<table>
<thead>
<tr>
<th></th>
<th>$M_w$ (Da)</th>
<th>PDI</th>
</tr>
</thead>
<tbody>
<tr>
<td>GS</td>
<td>10,238</td>
<td>1.7</td>
</tr>
<tr>
<td>R-FS</td>
<td>51,734</td>
<td>5.0</td>
</tr>
</tbody>
</table>

Figure 3.2 shows the SEM images of the electrospun $\alpha$APz fiber mats that were synthesized from PDCP, that in turn, was prepared from the FS method. Both low magnification (large scan area) and high magnification (small scan area). The fibers were free from defects and had diameters below 0.7 µm with averages between 0.1 µm and 0.3 µm.

For the $\alpha$APz-A polymers, CF:DMSO solvent combination was used to electrospun them. When the CF alone was used, the needle tip was clogged due to the rapid evaporation of the solvent while the fibers collected also had defects due to beading. Thus 25% DMSO was added to address both challenges. As an alternative, it was also possible to electrospin $\alpha$APz-A polymers from THF:CF (9:1) ratio using the indicated parameters of Table 3.2. It is noteworthy that $\alpha$APz-P polymers were only electrospun in THF:CF (9:1) ratio.
Figure 3.2. SEM images of electrospun PaAPz derived from L-phenylalanine and L-alanine and the corresponding histogram showing the fiber diameter distribution. For each SEM image, the PDCP synthesis method (FS – flame sealing; R-FS: recrystallization followed by flame sealing) and the optimum solvent ratio are shown. Other electrospinning parameters are listed in Table 3.1
3.4.3 Short-term in vitro degradation study

PαAPz are biodegradable polymers due to the presence of α-amino acid residues. While this is a desired property for tissue engineering, rapid degradation (within 3-5 days) of the electrospun fibers is a disadvantage since infiltration of seeded cells into the porous mats will be minimal. Cell infiltration into electrospun fibers is generally challenging and early degradation exacerbates it as the fibers fuse and thus limit infiltration. To gain an insight into the early degradation of the fiber mats, samples were incubated in PBS at 37°C for up to 5 days. As presented in Figure 3.3, the fiber morphologies of PαAPz-A did not change during the 5 days incubation; however, progressive fiber thickening, flattening, and eventual fusion was observed for the PαAPz-F. After 5 days of incubation, the fibrous mats lost their porosity considerably presumably due to the propensity of L-phenylalanine to hydrolysis compared with alanine. All cell culture experiments were therefore conducted on PαAPz-A.

Figure 3.3. SEM images showing the short-term in vitro degradation study of electrospun mats fabricated from PαAPz derived from the amino acids L-phenylalanine (PαAPz-F) and L-alanine (PαAPz-A). PαAPz-A fibers lost their porous morphology faster than PαAPz-A fibers. Scale bar = 1μm and is applicable to all images.
3.4.4 Cell adhesion and morphology on PaAPz-A films

To investigate *in vitro* cell adhesion and morphology, three distinct cell types were seeded on PaAPz-A films over a 3-day period. Since primary human vascular cells are not readily available, the potential of obtaining them by differentiating bone marrow-derived mesenchymal stem cells (BMMSC) and mesenchymal stem cells (iMSC) from induced pluripotent stem cells was evaluated. Human coronary artery smooth muscle cells (HCASMC) were used as control. Confocal microscopy images shown in Figure 3.4A demonstrated that all three types of cells attached and spread on the surface of PaAPz-A films after 3 days of culture and were morphologically indistinguishable from cells cultured on the glass coverslips. All three types of cells showed abundant F-actin expression; however, BMMSCs had a better attachment, as judged by the dense cell layer observed. As the same number of cells were initially seeded and the culture time was only 3 days, the observed high cell density in BMMSC is related to high retention rather than cell growth. Although cell retention is high for BMMSC, their application as cell source is not as robust as iMSC since they require invasive procedures to obtain them, and they have replicative senescence during expansion. Compared to primary HCASMC, iMSCs retention on the PaAPz-A film was high, suggesting that these cells were viable. Thus, iMSC attachment and growth was further evaluated on the electrospun fibrous mats by F-actin fluorescence staining after culturing iMSCs on PaAPz-A fibrous mats for 2 days (Figure 3.4B1) and 4 days (Figure 3.4B2). As seen from these confocal images, iMSCs were well-spread on the fibrous mats, characterized by well-defined F-actin stress fibers with a typical small cell body that is long and thin morphology, which is characteristic of stem cells, suggesting that the scaffold microenvironment was favorable for cell attachment and growth.
Figure 3.4. (A) Fluorescence images of different cell types on PαAPz-A films and on coverslips. After 3 days of culture, F-actin is labeled by Phalloidin in green and nuclei are labeled by DAPI in blue. Scale bar = 20 μm. (B1 & B2) Fluorescence images of iMSCs after 2 days (B1) and 4 days (B2) of culture on PαAPz-A electrospun fibrous mats. Red channel is F-actin and nuclei are labeled by DAPI in blue. Scale bar = 50 μm.

3.4.5 Differentiation potential of iMSCs towards smooth muscle phenotype

After establishing that PαAPz-A fibrous mats were able to support the attachment and spreading of iMSCs, the next investigation focused on whether these cells have the differentiation potential towards the vascular smooth muscle phenotype. iMSCs were chosen because BMMSCs have a relative ease in undergoing osteogenic and adipogenic differentiation compared to vascular differentiation which is more complex42-44. Although direct differentiation of partially induced pluripotent stem cell to a VSMC has been reported45, those obtained from fully induced pluripotent cells via mesenchymal precursor
cells is the most established method\textsuperscript{46-48}. To determine the differentiation capacity of iMSCs into smooth muscle phenotype, the expression of smooth muscle-specific marker genes and proteins was analyzed under 2D conventional tissue culture conditions, and results are shown in Figure 3.5. iMSCs were plated on gelatin-coated culture dishes in stem cell culture media. Once the cells reached 70\% of confluency, the culture media were changed to the following for 7 days: (i) differentiation culture (DC) media; (iii) DC+82.5 μg/mL L-Ascorbic acid (L-AA); (iii) DC+2 ng/mL TGF-β1. A regular growth condition (GC) was used as a control. As shown in Figure 3.5A, DC media was able to significantly upregulate the expression of smooth muscle marker gene Acta2 ($p<0.05$), Cnn1 ($p<0.05$), and SMTN ($p<0.001$) but not the late-stage marker Myh11 ($p>0.05$), which suggested that iMSCs possess an intrinsic differentiation capacity towards smooth muscle phenotype. Upon the addition of AA, the expression levels of Cnn1 and SMTN were further increased.
Figure 3.5. Differentiation potential of iMSCs towards vascular smooth muscle lineage in conventional 2-D cultures. (A) iMSC mRNA expression of \( h\text{Acta}2 \), \( h\text{Cnn}1 \), \( h\text{SMTN} \), and \( h\text{Myh}11 \) was analysed using Quantitative real-time polymerase chain reaction (qRT-PCR) after cell treatment with L-AA and TGF-\( \beta \)1 for 7 days. GC stands for regular growth condition of iMSCs, and DC stands for differentiation inducible condition by exchanging culture media to DMEM with 1% FBS. (B) mRNA expression of \( h\text{Myh}11 \) by iMSCs after 8 hr and 24 hr of cultivation under DC. (C) iMSCs were cultured on coverslips under differential conditions for 7 days, MHC and SMTN-B were detectable with either AA or TGF-\( \beta \)1 pre-treatment. Green channel is F-actin and Red is MHC/SMTN-B. Nuclei are labeled by DAPI in Blue. Scale bar = 50 \( \mu \)m. (D) Western blot analysis revealed that vascular smooth muscle contractile marker SMTN-B was up-regulated by pre-treatment with TGF-\( \beta \)1 whereas MHC was slightly up-regulated by either L-AA or TGF-\( \beta \)1 pre-treatment. * \( p < 0.05 \); *** \( p < 0.001 \)
TGF-β1 is one of the major cytokines that can induce VSMC differentiation of stem/progenitor cells, alone or in combination with other factors. Interestingly, the addition of TGF-β1 into DC media did not provide a synergistic effect on mRNA expression. Moreover, the late-stage marker *Myh11* was less responsive to differentiation inducers after 7 days of culture, which suggested that *Myh11* marker gene may have been expressed earlier than 7 days. To verify the expression pattern of *Myh11*, iMSCs were differentiated for 8 h and 24 h using L-AA and TGF-β1 in DC media. As shown in Figure 3.5B, *Myh11* was notably upregulated by L-AA but was less responsive to TGF-β1. Immunofluorescence staining was subsequently performed to detect proteins of MHC and SMTN, two key smooth muscle markers, and the representative confocal images are presented in Figure 3.5C. Both marker proteins were extensively expressed in DC-treated cells with the addition of L-AA and TGF-β1. Moreover, the expressions of two SMC-specific contractile proteins were detectable after 7 days of culture using Western blot analysis (Figure 3.5D). Although MHC protein remains unchanged by either L-AA or TGF-β1 treatment, protein expression of SMTN-B, a specific differentiation marker exclusively expressed in vascular smooth muscle tissues, was upregulated with the addition of TGF-β1.

3.4.6 Differentiation of iMSCs towards smooth muscle phenotype on electrospun PaAPz-A fibrous mats.

The preceding cell differentiation results were done on conventional cell culture dishes to rule out the effect of the fiber mat topography. To determine if the microenvironment provided by electrospun PaAPz-A fibrous mats is favorable for vascular differentiation of iMSC, immunofluorescence staining was performed on key vascular smooth muscle markers (α-SMA, MHC, SMTN, and SMTN-B) that represent different stages of smooth
muscle differentiation. Stem cell culture on synthetic materials is challenging as it may transform them into subpopulations with reduced multipotency and fibrotic character; therefore, the microenvironment provided by PaAPz-A fibrous mats with the right physical cues is indispensable for the highly efficient proliferation and differentiation of iMSCs. After 7 days of differentiation on PaAPz-A fibrous mats, protein markers α-SMA (green, Figure 3.6A1) and MHC (green, Figure 3.6B1) were observed in L-AA-treated iMSCs and the fluorescence intensity of early-stage marker protein α-SMA was further enhanced in the presence of TGF-β1 (Figure 3.6A2); however, a further increase of the late-stage protein marker MHC (green, Figure 3.6B2) with the addition of TGF-β1 were not observed. After 12 days of iMSC differentiation, a more contractile phenotype of smooth muscle cells was observed, as verified by immunofluorescence staining of two contractile-specific protein markers SMTN (Figure 3.6C1) and MHC (Figure 3.6C2), respectively. In addition, SMTN-B is a long isoform of smoothelin that is specifically detectable in vascular smooth muscle tissues and used to discriminate VSMCs from myofibroblasts49, 50. Consistent with the observation of Western blot analysis in Figure 3.5D, the co-localization of SMTN-B and F-actin (Figure 3.6D1-2) confirmed the iMSC differentiation into smooth muscle phenotype on PaAPz-A fibrous mats. Overall, the immunostaining of SMTN and MHC for cells cultured on the PaAPz-A strongly indicates robust expression compared to conventional cultures shown in Figure 3.5. Furthermore, differentiated cells were aligned and elongated on the fibrous mats, suggesting directional orientation, presumably due to cues provided by the fiber topography.
Figure 3.6. Characteristic analysis of smooth muscle markers by iMSCs seeded on PαAPz-A fibrous mats addition with L-AA or L-AA/TGF-β1 in DC culture media. After 7 days of pre-differentiation, α-SMA (A1/A2) and MHC (B1/B2) were determined by immunofluorescence staining. (C1-2) Representative confocal images of SMTN (green) and MHC (red) by iMSCs after 12 days of cultivation. (D1-2) Co-localization of SMTN-B (green) and F-actin (red) by iMSCs after 12 days of cultivation. Scale bar = 50 µm.

PαAPz polymers have been previously studied almost exclusively as orthopedic biomaterials, including tendon and ligament\textsuperscript{2, 3}, but their use in vascular smooth muscle differentiation of stem cell has not been reported before. Regarding other vascular cells, to the best of the author’s knowledge, only two previous studies\textsuperscript{13, 14} reported endothelial cell interaction with PαAPz fibers. Furthermore, standalone electrospinning of PαAPz is challenging, and it is often modified with other polymers such as polycaprolactone\textsuperscript{7, 32, 51, 52}. In this study, it is the first time that iMSCs were shown to be able to differentiate into vascular smooth muscle cells on standalone electrospun PαAPz-A mats. The presence of elongated and spindle-shaped cells, as well as abundant expression of specific proteins
related the vascular smooth muscle cell lineage demonstrated differentiation. These studies provide strong evidence on the utility of electrospun \( \alpha \)-APz-A as a conducive microenvironment for stem cell differentiation.

3.5 Conclusion

In this study two \( \alpha \)-APz polymers from L-alanine and L-phenyl alanine were synthesized. Both \( \alpha \)-APz were electrospinnable and produced beads-free fibers with average diameters between 0.1\( \mu \)m and 0.3 \( \mu \)m. The electrospun fibers from L-phenyl alanine -based \( \alpha \)-APz started to degrade faster than the corresponding \( \alpha \)-APz from L-alanine. The \( \alpha \)-APz fibrous mats from L-alanine was able to support the adhesion and spreading of iMSC, BM-MSC and primary human coronary artery SMC. More importantly, electrospun fibers from \( \alpha \)-APz from L-alanine were able to promote the differentiation of iMSCs towards SMC lineage. Taken together, data in this chapter strongly suggest the utility of \( \alpha \)-APz for differentiating stem cells for tissue engineering applications.

3.6 References


Chapter 4

4 Preparation and Microscopic Mechanical Characterization of L-Methionine-Based Polyphosphazene Fibrous Mats for Vascular Tissue Engineering*

Overview: The purpose of the study herein is to explore the application of a previously unreported polyphosphazene, L-Methionine-based polyphosphazene, in vascular tissue engineering. After characterizing this polyphosphazene with NMR and testing the electrospinnability, AFM was used to test the mechanical properties of the fiber scaffolds at the microscale. Lastly, how mesenchymal stem cells differentiate on these fiber mats and their production of the extracellular matrix were studied.

4.1 Abstract

This study investigates the mechanical properties, degradation behavior, and biocompatibility of poly[(a-amino acid ester) phosphazene] electrospun fibers based on the ethyl ester of L-methionine (PaAPz-M), a material with potential applications in tissue engineering. Atomic force microscopy (AFM) was utilized to evaluate the fiber mechanical characteristics and calculate its Young’s modulus, revealing it to closely mimic the stiffness of a natural extracellular matrix (ECM). The degradation behavior of PaAPz-M scaffolds over 21 days was also studied, showing that they maintain the highly porous structure required for tissue engineering. Further evaluation of mesenchymal multipotent 10T1/2 cell and mesenchymal stem cell (MSC) behavior on the scaffolds demonstrated significant cell viability, proliferation, and successful MSC differentiation into smooth muscle cells.

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Expression of collagen and elastin by MSCs on the fiber mats highlighted potential ECM formation during scaffold degradation, confirming PαAPz-M as a promising material for vascular tissue engineering.

4.2 Introduction

Poly[(α-amino acid ester) phosphazene]s, or PαAPz, show promise in the field of tissue engineering and drug delivery due to their unique degradation characteristics. Unlike many biodegradable polyesters, such as poly(lactide) and poly(glycolide), PαAPz release non-acidic and buffering degradation products, primarily comprising phosphate, ammonia, and corresponding side groups \(^1\), \(^2\). Consequently, PαAPz have been reported to mitigate inflammatory responses triggered by acidic degradants when used synergistically with other materials \(^3\), \(^4\).

Current applications of PαAPz in tissue engineering have predominantly employed those derived from alanine and phenylalanine, with a principal focus on bone tissue engineering \(^5\)-\(^7\). This is owed to their glass transition temperatures typically being higher than physiological temperature, ensuring structural integrity and mechanical support in vivo \(^6\), \(^8\).

However, vascular tissue engineering scaffolds should be flexible to mimic native blood vessel mechanical properties \(^9\), \(^10\). Therefore, in contrast to bone tissue engineering, vascular tissue engineering may benefit from more pliable materials. Previously, methionine-based polyphosphazene, or PαAPz-M, whose glass transition temperature is approximately 9 °C, was identified as a potential candidate for this purpose \(^11\). This material is especially appealing considering the sensitivity of vascular smooth muscle cells (VSMCs) to acidic degradation products.
Although synthesis methods for PαAPz-M have been reported 11, a comprehensive study of its influence on cell affinity, differentiation, and the effect of its hydrolysis on cellular adhesion is still lacking.

In this study, the potential of PαAPz-M for vascular tissue engineering applications is evaluated. PαAPz-M were synthesized and characterized, fabricated fibrous scaffolds through electrospinning methods, and assessed their performance in vitro. To benchmark these findings, a comparative study was also conducted with poly(ester amide) (PEA), a well-documented synthetic biomaterial used in vascular tissue engineering 12, 13.

Given the central role of mechanical behavior in evaluating biomaterials for tissue engineering, the mechanical properties of PαAPz-M were explored 14-16. Macrotropic mechanical properties (e.g., those obtained from tensile testing of fibrous mats) are completely sensed by cells; instead, microscopic and mechanical properties localized at the cellular scale are more important, thus prompting us to use AFM to measure local mechanical properties at the microscale 17, 18. Compared to mechanical tests at the macroscale, measuring the local mechanical properties at the microscale is believed to provide valuable insights into the complex interplay of cell-to-cell and cell-to-material communication 18, 19.

To evaluate the degradation rate of PαAPz-M, a 21-day in vitro degradation study of the fiber mat was performed, observing alterations in its surface morphology using SEM. Subsequently, the material’s cell affinity and differentiation potential were tested. For this purpose, two types of cells were selected: a mouse embryonic multipotent mesenchymal progenitor cell line (10T1/2 cells) and human mesenchymal stem cells (MSCs). Here,
10T1/2 cells, known for their easy cultivation and potential to differentiate into VSMCs, served as excellent model cells \(^{20}\), while MSCs, on the other hand, due to their human source and potential to differentiate into VSMCs, were deemed optimal for human vascular tissue engineering \(^{21,22}\).

Overall, this research aimed to provide a systematic investigation of a methionine-based polyphosphazene derivative, PαAPz-M, starting from its synthesis and characterization to its scaffold fabrication and evaluation for vascular tissue engineering. Primary focus was placed on understanding its degradation behavior, mechanical properties, and cell compatibility, and thus its potential as a favorable scaffold for vascular tissue engineering applications.

### 4.3 Materials and methods

#### 4.3.1 Materials

Hexachlorocyclotriphosphazene (HCCP) from Sigma Aldrich (Milwaukee, WI, USA) was recrystallized under vacuum at 55 °C. Poly(dichlorophosphazene) (PDCP) was synthesized as reported previously \(^{23}\). Anhydrous tetrahydrofuran (THF) and glass distilled hexanes-190 were purchased from Caledon Labs (Georgetown, ON, USA). Triethylamine (Et\(_3\)N), chloroform (CHCl\(_3\)), and dimethyl sulfoxide (DMSO) were purchased from Sigma Aldrich. L-Methionine ethyl ester hydrochloride (H-Met-OEt·HCl) was obtained from Alfa Aesar (Ward Hill, MA, USA) and stored in the fridge. Unless specified otherwise, all chemicals and solvents were used as received.
A polyester amide (PEA), specifically 8-Phe-4, was synthesized by interfacial polymerization using sebacoyl chloride, butanediol, and L-phenylalanine as the amino acid, according to the previous publication 24.

4.3.2 Synthesis of PαAPz-M

To synthesize uncrosslinked PDCP, 2.00 g of purified HCCP was added into a flame-dried ampoule, which was then evacuated under vacuum and sealed using a propane torch. Thermal ring-opening polymerization of HCCP was performed at 230 °C for approximately 72 h, and uncrosslinked PDCP was obtained by three precipitations from THF into hexane, as reported previously 23.

To functionalize the PDCP, a one-step method was employed using H-Met-OEt·HCl. Briefly, 20 mL of the 10 wt.% PDCP in THF, 4 mL of triethylamine and 2.36 g of H-Met-OEt·HCl were added into a flame-dried flask and reacted at room temperature for 72 h. The final product, functional PαAPz-M, was purified by filtering and precipitating from THF into hexane 3 times 25.

4.3.3 PDCP and PαAPz characterization with $^{31}$P-NMR and $^1$H-NMR

The polymer structures of PEA and PαAPz-M were evaluated using a Varian INOVA 400 MHz spectrometer (Varian Canada Inc., Mississauga, ON). All chemicals were dissolved in chloroform-D (CDCl₃) with a concentration of approximately 40 mg/mL for $^{31}$P-NMR and 5 mg/mL for $^1$H-NMR. Chemical shifts are reported in parts per million (ppm) and referenced to chloroform at $\delta = 7.27$ ppm.
4.3.4 Electrospinning of PαAPz

In this experiment, pure PEA, PαAPz-M, and a 50:50 (by weight) mixture of PEA and PαAPz-M were electrospun. The electrospinning solution formula for PEA was the same as that reported before \(^\text{26}\), consisting of CHCl\(_3\):DMSO = 3:1. The electrospinning solution formula for PαAPz-M was THF:CHCl\(_3\) = 9:1. The mixture of PEA and PαAPz-M at a ratio of 50:50 was a combination of the two electrospinning solutions. The electrospinning solution was packed into a 0.5 mL glass syringe and connected to a 22 G stainless steel needle. Electrospinning flow rate was controlled by a syringe pump (KD101, KD Scientific, Holliston, MA, USA). The stainless-steel needle was connected to the positive electrode of a low-current power supply (Gamma High Voltage, Ormond Beach, FL, USA) that can provide 7–20 kV, and the rotating collector was connected to the ground to ensure a high potential difference between the electrospinning solution and the collector. Electrospinning was performed at room temperature and 20–25% relative humidity.

4.3.5 Scanning electron microscopy (SEM)

The morphology of the fibers and the thickness of the fiber mat were analyzed using a scanning electron microscope (S-3400N, Hitachi, Ltd., Tokyo, Japan). Samples were cut into a 1 × 1 cm\(^2\) square and then attached to an SEM sample holder using carbon tape. Prior to imaging, samples were coated with 5 nm of osmium using a sputter coating technique (Filgen OPC80T Osmium Plasma Coater, Filgen Nanosciences & Biosciences Inc., Jonoyama, Japan). SEM images were obtained at an acceleration voltage of 5 kV and the diameter of the fibers was analyzed using ImageJ(1.50i) software. Briefly, images were processed to enhance the contrast, while the diameter of the fibers was measured by analyzing at least 100 randomly selected fibers in 3 independent SEM figures from each
sample. The reported values represent the average of the measurements obtained from at least three independent samples.

4.3.6 Measurement of mechanical properties by AFM

Mechanical testing at the microscopic scale was conducted with an atomic force microscope (AFM) (Bruker ICON AFM) using the MultiMode platform. AC 160 silicon nitride cantilevers (Bruker) with a silicon sphere tip with a diameter of 20 μm and a wedge-shaped tip with a diameter of 7 nm were used for AFM mechanical testing. Before testing began, the actual spring constants of AFM cantilevers were measured using the thermal tuning method. Fiber mats with a thickness of 30–70 μm were chosen for mechanical testing. Prior to testing, the fiber mats were cut into pieces with dimensions of approximately 5 mm × 10 mm. The fiber mats were mounted on a glass slide using double-sided adhesive tape, and the AFM cantilever was brought into contact with the sample surface. A force–distance curve was recorded for each sample using the contact mode with a maximum indentation depth of 5 μm and a rate of 1 μm/s. At least 10 force–distance curves were recorded for each sample.

In order to evaluate the mechanical properties of the samples, the Hertz model was employed. Typically, the size of the material being tested is much larger than the contact area between the AFM probe and the material. Thus, the Hertz model can be simplified as shown in Equation 4-1, where $F$ is the applied load, $E$ is Young’s modulus, $\nu$ is Poisson’s ratio of the sample, $R$ is the radius of the contact area, and $h$ is the indentation depth.
When fitting a force curve with a Hertz model, there are two different methods of analysis. One is to raise both sides of the equation to the power of 2/3 which results in Equation 4-2. Since Young’s modulus and Poisson’s ratio are constant, \( F^{2/3} \) and \( h \) are linearly related when \( R \) does not change during the indentation.

\[
F^{\frac{2}{3}} = \left[ \frac{4}{3(1-\nu^2)} ER^2 \right]^{\frac{2}{3}} h \Rightarrow F = Sh, \text{ where}
\]

\[
S = \left[ \frac{4}{3(1-\nu^2)} ER^2 \right]^{\frac{1}{3}}
\]

\[
E = \left[ \frac{3(1-\nu^2)}{4R^2} \right] S^3,
\]

The other is to use Equation 4-3 from the Derjaguin–Muller–Toporov (DMT) model and substitute it into Equation 4-1, resulting in Equation 4-4. In Equations 4-3 and 4-4, \( R_p \) represents the diameter of the probe and is constant during the measurement \(^{30, 31}\). As a result, \( F \) and \( h \) behave linearly in the force curve.

\[
R_p^2 = Rh,
\]

\[
F = \frac{4}{3(1-\nu^2)} E(Rh)^{\frac{1}{2}} h = \frac{4ER_p}{3(1-\nu^2)} h \Rightarrow F = Sh,
\]

\[
E = \left[ \frac{3(1-\nu^2)}{4R_p} \right] S,
\]
In this study, both methods were used to determine the Young’s modulus of the fiber mats.

4.3.7 Cell culture studies and smooth muscle cell differentiation on fiber mats

Mouse embryo multipotential mesenchymal progenitor cells (C3H/10T1/2 cells; ATCC; Manassas, VA, USA) and mesenchymal stem cells derived from induced pluripotent stem cells (iMSCs, kindly donated by Dr. Dale Laird, Western University, London, ON, Canada) were used in this study. The fiber mat scaffolds were cut with aluminum foil supports, and the edges were folded to secure the mat onto the foil, creating an effective area of approximately 1 cm². The fixed fiber mats were placed in individual wells of a 24-well plate and sterilized with 70% ethanol for at least 30 min, followed by three washes with HBSS before cell seeding.

For the 10T1/2 cells, they were directly seeded onto the surface of the fiber mat at a density of 20,000 cells/cm² and cultured in high glucose DMEM supplemented with 5% fetal bovine serum and 1% penicillin/streptomycin.

Prior to iMSC seeding, the sterilized fiber mats were coated with 0.1% gelatin and incubated at 37 °C for 1 h. The iMSCs were then seeded onto the gelatin-coated fiber mats at a density of 20,000 cells/cm² and cultured in mesenchymal stem cell expansion media (MSCEM, Cedarlane Labs, Burlington, ON, Canada; HMSC.E. MEDIA-450) supplemented with 10% fetal bovine serum, 1% L-glutamine, and 1% penicillin/streptomycin (all from Fisher Scientific, Whitby, ON, Canada). The cells were incubated at 37 °C in a humidified incubator containing 5% CO₂.
To induce the differentiation of the iMSCs towards smooth muscle cell lineage, L-ascorbic acid (L-AA, Sigma-Aldrich Canada Co., Oakville, ON, Canada) and transforming growth factor-beta 1 (TGF-β1, R&D Systems, Minneapolis, MN, USA) were used, following a previously reported protocol. After seeding the iMSCs on the fiber mat, the cells were pre-cultured on the fiber mats for 3 days until reaching approximately 70% confluence. The culture media were then changed to a differentiation medium comprising high glucose DMEM (Dulbecco’s Modified Eagle Medium) supplemented with 1% FBS, 1% penicillin/streptomycin, 82.5 μg/mL L-AA, and 2 ng/mL TGF-β1.

4.3.8 Cell viability on fibrous scaffolds

The cell viability of the scaffolds was assessed using both live/dead staining and the MTT assay. After seeding the 10T1/2 cells onto the scaffolds for 7 days, the live/dead assay was conducted using a Live/Dead Cell Imaging Kit (488/570, Molecular Probes, Eugene, OR, USA; Life Technologies Corp., Carlsbad, CA, USA) following the manufacturer’s instructions. The cells were incubated with the working solution for 15 min at room temperature and, subsequently, the samples were examined using a 20× objective epifluorescence on Leica DMi8 (Leica Microsystems, Wetzlar, Germany) microscope equipped Leica Application Suite X (LAS X) software.

To evaluate the metabolic activity of viable cells, colorimetric assays were conducted using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Invitrogen, Burlington, ON, Canada). After seeding the cells onto the scaffold for 2 days, 4 days, and 7 days, the scaffolds were transferred to a new 24-well plate to eliminate the influence of cells growing on the plate. Then, 100 μL of fresh medium and 10 μL of a 5 mg/mL MTT
solution were added to each well. The samples were incubated at 37 °C for 4 h, during which time the yellow MTT salt was converted to a purple insoluble formazan salt by the dehydrogenase activity of intact mitochondria in metabolically active cells. Subsequently, 100 μL of sodium dodecyl sulfate (Invitrogen, Burlington, ON, Canada) was added and incubated for an additional 4 h to dissolve the formazan salt. Finally, the formazan salt was quantified at 570 nm (maximum absorbance) using a BioTek EL307C multi-plate reader (BioTek Instruments, Winooski, VT, USA).

4.3.9 Immunofluorescence microscopy

After 7 days of culturing or 7 days of differentiation, the MSCs were fixed at 4 °C with 4% paraformaldehyde for 15–20 min, permeabilized with 0.5% Triton X-100 in PBS for 10 min, and then blocked in 2% BSA with 22.52 mg/mL of glycine in PBS for 30 min. Then, the samples were immunostained for 1 h at room temperature for α-SMA (mouse, 1:100; Santa Cruz, CA, USA, sc-32251), SMTN (rabbit, 1:100; Santa Cruz, sc-166292), and elastin (mouse, 1:100; Santa Cruz, sc-166543). Alexa Fluor® 555 goat anti-rabbit IgG or Alexa Fluor® 488 goat anti-mouse IgG was used as the secondary antibody for fluorescent detection (1 h incubation, 1:100 dilution; all from Life Technologies, Burlington, ON, Canada) and F-actin was detected with Alexa Fluor® 568 or Alexa Fluor® 488 phalloidin (1 h incubation, 1:100 dilution; Invitrogen Canada, Burlington, ON, Canada). Lastly, cell nuclei were visualized with 4′,6-diamidino-2-phenylindole (DAPI, 300 nM in PBS, Life Technologies, Burlington, ON, Canada). Coverslips were mounted on microscope slides with PermaFluor™ Mounting Medium (Thermo Scientific™, Mississauga, ON, Canada) and sealed with clear nail enamel. Images were taken with a Leica DMi8 (Leica...
Microsystems, Wetzlar, Germany) equipped with a 20× objective and analyzed using Leica Application Suite X (LAS X) software.

4.3.10 Quantitative real-time qPCR

Total RNA was isolated using a TRIzol™ reagent (Life Technologies) according to the manufacturer’s instructions. A total of 1 μg of RNA was used to synthesize cDNA using the Promega™ Random Hexamer protocol (Fisher Scientific, Whitby, ON, Canada). Real-time quantitative PCR (RT-qPCR) was conducted in 10 μL reaction volumes, using a CFX96™ Real-Time System (C1000 Touch Thermal Cycler; Bio-Rad, Mississauga, ON, Canada). The qPCR system contained 1 μL of cDNA, 0.2 μL of primers, 5 μL of SsoAdvanced Universal SYBR® Green Supermix (Bio-Rad), and 3.8 μL of RNase-free water. The primer sequences are listed in Table 4.1.

Table 4.1 The primer sequences used in qPCR assay.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Sequence (5′-3′)</th>
<th>Reverse Sequence (5′-3′)</th>
</tr>
</thead>
<tbody>
<tr>
<td>h-Acta2</td>
<td>-CAA GTG ATC ACC ATC GGA AAT G-GAC TCC ATC CCG ATG AAG GA</td>
<td></td>
</tr>
<tr>
<td>h-Cnn1</td>
<td>-TGA AGC CCC ACG ACA TTT TT</td>
<td>-GGG TGG ACT GCA CCT GTG TA</td>
</tr>
<tr>
<td>h-SMTN</td>
<td>-CAG GAC AAC AAG GAG AAC TGG</td>
<td>-CAG TCA ATT CCT CCA CAT CGT</td>
</tr>
<tr>
<td>h-18s</td>
<td>-GCG GTT CTA TTT TGT TGG TTT</td>
<td>-CTC CGA CTT TCG TTC TTG ATT</td>
</tr>
</tbody>
</table>

4.3.11 Statistical analysis

The data are expressed as a mean and a standard deviation. A minimum of three independent experiments were performed, and statistical analysis was conducted using a one-way ANOVA combined with Student’s two-sided independent sample t-test. Statistical significance was considered when the p-value was less than 0.05.
4.4 Results and Discussion

4.4.1 Characterization

The $^{31}$P-NMR spectra of HCCP, linear PDCP, and PαAPz, and the $^1$H-NMR spectrum of PαAPz-M and PEA are shown in Figure 4.1A–C. Purified HCCP exhibited a singular peak at 21.09 ppm, whereas linear PDCP, following thermal ring-opening polymerization and purification, displayed a peak at −18.09 ppm. These characteristic peak positions concur with prior literature 32, thus confirming the successful synthesis of linear PDCP via the flame-sealing method. Upon the functionalization of PDCP with H-Met-OEt·HCl, the characteristic peak at −18.09 ppm vanished, and a new peak with diminished intensity emerged, which aligns with previous findings, thereby corroborating the success of the functionalization process 11.

In the $^1$H-NMR spectrum of PαAPz-M (Figure 4.1B), characteristic peaks associated with methionine ethyl ester were discerned between 0 and 5.0 ppm. The protons in -CH$_2$-S-CH$_3$ and ethyl carboxylate manifested peaks at approximately 4.3 and 2.1 ppm, respectively, indicating that the functionalization was successful and that the methionine ethyl ester remained unaltered during the functionalization process.

The $^1$H-NMR spectrum of PEA is presented in Figure 4.1C, and the characteristic peaks of PEA were found to agree with previous studies 33.
Figure 4.1. (A) $^{31}$P NMR of (1) HCCP, (2) crude PDCP from the flame-sealing method, and (3) purified PDCP, (4) PaAPz-M. (B) $^1$H NMR of PaAPz-M. (C) $^1$H NMR of PEA.

4.4.2 Scaffold fabrication by electrospinning

Electrospinning is one of the most frequently used methods in tissue engineering applications. Compared to other methods for scaffold formation (e.g., gas forming, solvent casting, and freeze-drying), electrospinning holds greater potential for large-scale industrial manufacturing of extracellular matrix (ECM)-like structures $^{34}$. Furthermore, the technique may be combined with 3D printing to increase printing resolution $^{35-37}$. However, compared to the other techniques, the electrospinning process has an increased complexity and imposes specific solubility and conductivity properties on each polymer–solvent
combination that can be successfully used. Therefore, the potential electrospinnability of a new material can greatly increase its attractiveness in tissue engineering.

To determine the conditions for producing bead-free electrospun fibers, screening experiments were conducted using various solvent combinations based on CHCl₃ and THF at polymer concentrations ranging from 5% to 20% and a fixed flow rate and distance to the collector of 0.4 mL/h and 12 cm, respectively. The most optimal conditions for producing bead-free electrospun fibers of PαAPz-M were found to involve a mixed solvent of CHCl₃: THF = 1:9 with a concentration between 10 and 15% and a fixed voltage of 15 kV. When the solution concentration was below 10%, electrospray occurred due to decreased viscosity; conversely, when the concentration exceeded 15%, the solution dried on the needle tip, resulting in blockage. To comparatively assess the degradation performance and cell adhesion of PαAPz-M with a previously studied material, PEA, a well-documented synthetic biomaterial utilizing amino acid as one of the constituent monomers, was also electrospun. The electrospinning parameters for PEA were consistent with those previously reported. In line with prior research showcasing the electrospinnability of PαAPz and PEA with chloroform, chloroform (CHCl₃) was employed as the predominant solvent for electrospinning the blend. By trial and error, a CHCl₃: DMSO solvent ratio of 3:1 with a distance of 12 cm, concentration of 15 wt%, and voltage of 15 kV were determined as the optimum parameters that can produce bead-free fibers. As illustrated in Figure 4.2, bead-free fibers with an approximate diameter of 400 nm were produced by pure PαAPz-M, PEA, and a blend of PαAPz-M and PEA.
Figure 4.2. SEM images of electrospun PaAPz-M, PEA, and a mixture of PaAPz-M and PEA and the corresponding histogram showing the fiber diameter distribution. Scale bar = 10 μm.

4.4.3 Mechanical properties of fibers from AFM measurements

Understanding fiber mat mechanical properties at the submicron scale, as probed by AFM, could be crucial for their application in tissue engineering. To this end, the utilization of AFM to measure the mechanical properties of decellularized ECM has been reported \(^{40,41}\). However, based on the investigations, its application in assessing the mechanical properties of porous synthetic fibrous scaffolds resembling ECMs has not received much attention.

It is pertinent to mention that some studies have employed AFM to measure the mechanical properties of natural ECMs and porous membranes with structural similarities to these fiber mats \(^{19,42}\). Additionally, within the realm of polymer systems, both AFM and traditional mechanical tensile testing methods have yielded comparatively similar results \(^{43,44}\). These
earlier investigations have laid a foundational understanding of the methods employed to determine Young’s modulus of the fiber mats. In this study, Hertz model was employed to calculate the Young’s modulus of the fiber mats using two different approaches \(^{28}\). To ascertain the appropriate application of the Hertz model, the thickness of the fiber mats was measured with SEM, as demonstrated in Figure 4.3A, and determined it to be between 30 and 70 μm (Figure 4.3B). When compared to the probe (diameter 7 nm), it was estimated that the material’s thickness was considerably greater, rendering the Hertz model applicable to this study.

Equations 4-2 and 4-5, derived from the Hertz model, indicate that when the contact area remains constant, \(F^{2/3}\) and \(h\) exhibit a linear relationship, while the \(F-h\) curve is non-linear. When the contact area varies with force, \(F\) and \(h\) display a linear relationship in the force curve \(^{28}\) Figure 4.3C illustrates the force–distance curve obtained after using AFM to examine the fiber mat. The approach force curve (black) is a segmented broken line, while the retraction force curve (red) is smooth. This suggests that when the fiber mat is compressed, the probe continuously penetrates the fiber, exhibiting a straight line. Simultaneously, the slope abruptly changes as the force increases due to its interaction and contact with other fibers, resulting in a broken line. In contrast, the retraction force curve maintains a constant contact area during retraction due to the material’s adhesion to the probe, resulting in a smooth curve. Figure 4.3D is derived from plotting \(F^{2/3}\) and \(h\), demonstrating that the red retraction force curve is linear in a larger interval of deformation, as mentioned above.

In a retraction force curve, a slope near zero force is considered a reliable representation of the material’s Young’s modulus \(^{31}\). The bold lines in Figure 4.3C,D illustrate that \(F^{2/3}-h\)
represents the average mechanical properties observed in the retraction curve, while the F-h fitting captures the local mechanical properties experienced when the surface of the fiber mat undergoes compression or stretching on a submicron scale. The high slope observed at the end of the retraction curve is commonly attributed to the viscous resistance exhibited by the material and the tip. Thus, the Young’s modulus calculated using the $F^{2/3}$-h model could be significantly higher than that of the F-h model. Despite the excellent fit achieved by the $F^{2/3}$-h model, the local mechanical properties shown by F-h fitting offer a more direct reflection of the cellular microenvironment.

Figure 4.3. (A) SEM images of thickness of PαAPz-M, PEA, and mixture of PαAPz-M and PEA mats; (B) fiber mat thickness; (C) force curves during approach (black) and retraction (red) of AFM probe; (D) force curves of $F^{2/3}$-h. Area in bold is used for fitting and calculating Young’s modulus.
Consequently, both the $F^{2/3}$-$h$ and $F$-$h$ slopes were analyzed and compared the Young’s modulus calculated using the two slopes (as shown in Table 4.2). $\text{P}\alpha\text{APz-M}$ displayed a significantly lower elastic modulus (0.4–0.8 GPa) than PEA (1.7 GPa), previously reported PCL scaffolds (1–4 GPa) \(^{45}\), and polystyrene scaffolds (1.7 GPa) \(^{46}\). However, it still surpassed the elastic modulus of decellularized ECM (pig heart 28–40 kPa and mouse lung parenchyma 25–30 kPa \(^{19}\)). The reduced stiffness of $\text{P}\alpha\text{APz-M}$ brings it closer to the mechanical properties of natural ECMs.

**Table 4.2. Young’s modulus calculated from $F$-$h$ fit and $F^{2/3}$-$h$ fit.**

<table>
<thead>
<tr>
<th>Young’s Modulus (GPa)</th>
<th>From $F$-$h$</th>
<th>From $F^{2/3}$-$h$</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 PEA 100 $\text{P}\alpha\text{APz-M}$</td>
<td>0.440 ± 0.086</td>
<td>0.884 ± 0.076</td>
</tr>
<tr>
<td>50 PEA 50 $\text{P}\alpha\text{APz-M}$</td>
<td>1.340 ± 0.141</td>
<td>4.907 ± 0.338</td>
</tr>
<tr>
<td>100 PEA 0 $\text{P}\alpha\text{APz-M}$</td>
<td>1.749 ± 0.034</td>
<td>11.436 ± 0.816</td>
</tr>
</tbody>
</table>

4.4.4 Fiber mat degradation and morphology

The biodegradability of tissue engineering materials is crucial for the success of regenerative therapies. An ideal degradation rate must balance between supporting tissue regeneration, keeping the scaffold porosity, and avoiding adverse reactions \(^{47},^{48}\). In the early stages of cell culture in tissue engineering, the surface refresh rates and porosity changes of materials during degradation are of utmost importance. Therefore, SEM was utilized to evaluate the degradation behavior of the fiber mats during a 21-day incubation experiment in PBS at 37 °C.

As illustrated in Figure 4.4, $\text{P}\alpha\text{APz-M}$’s fiber diameter significantly increased by day 3, suggesting water penetration and swelling. For blended fiber mats with 50% PEA added,
the fiber diameter remained relatively unchanged during degradation, although some slight fusion was observed. In contrast, the fiber mat composed solely of PEA fused the most during the 21-day culture period. Overall, the PαAPz-M and 50% blend maintained higher-porosity structures during the process.

The degradation performance of solid polymer surfaces is influenced by factors such as the hydrolysis rate of polymer bonds, water diffusivity, solubility, and material size\textsuperscript{49,51}. Previous reports have raised concerns regarding the effect of the polyphosphazene side group on hydrolysis rate and cellular adhesion\textsuperscript{11}. However, the degradation behavior of PαAPz-M is primarily characterized by swelling and bulk erosion, which may be attributed to the methionine hydrophilicity. This degradation pattern allows for a high degradation rate while maintaining a low surface refresh rate and preserving scaffold porosity. In contrast, the PEA scaffold exhibits more fusion, likely due to its higher solubility, which noticeably affects surface porosity during degradation. The 50% blend appears to strike a balance between solubility and water diffusivity, thereby maintaining favorable scaffold porosity throughout the 21-day degradation experiment.
4.4.5 Cell viability and adhesion on fiber mats

The ideal material for vascular tissue engineering should support vascular cells and promote good cell adhesion, cell viability, and differentiation. The viability, growth, and spreading of mesenchymal multipotent 10T1/2 cells on composite fibrous scaffolds were evaluated by MTT and live/dead assays.

Figure 4.5A displays the cell viability of 10T1/2 cells on fibrous scaffolds on day 2, day 4, and day 7. The results showed that cell viability significantly increased over time, indicating an improvement in cell metabolism. Notably, no significant difference in cell viability among the three scaffolds was observed during the seven-day culture, suggesting that the degradation rate of PaAPz-M did not influence cell adhesion and proliferation. Although the viability of the cells cultured on the fiber mat appeared lower than that on the...
culture disk surface on day 2, the viability of the cells on the two surfaces was comparable after seven days of culture. These findings indicate that the fibrous scaffolds effectively support the growth and viability of 10T1/2 cells over time. In addition, the live/dead assay results (Figure 4.5B,C) demonstrated that after seven days of culture, 10T1/2 cells populated the fiber mat surface with only a small number of dead cells appearing on any of the three materials. This result confirms that both PaAPz-M and PEA materials exhibit good cell adhesion and viability and, therefore, have potential as biomaterials for vascular tissue engineering applications. Taken together, the results of this study suggest that PaAPz-M and PEA materials exhibit similar performance in terms of cell adhesion and cell viability and that both show promise as biomaterials for further research in artificial vascular tissue engineering.

Figure 4.5. Cell viability of 10T1/2 cells on the fibrous scaffolds. (A) MTT assay on days 2, 4, and 7 of culturing; (B) percentage of living cells on the fibrous scaffolds; (C) live/dead assay of 10T1/2 cells on fiber mats. * p < 0.05; *** p <0.001.
4.4.6 Evaluation of MSC adhesion and smooth muscle cell differentiation

Building on the observations of 10T1/2 cells, the effects of the increased degradation rate and associated surface changes on the adhesion, proliferation, and differentiation of MSCs on the fiber mats were investigated. Upon staining the MSCs after three and five days of cultivation, their morphology was examined. The data pointed to a robust adhesion of MSCs on all three material surfaces. A comparison of day 3 and day 5 images (Figure 4.6) revealed healthy cell spread on the material surfaces and a significant increase in cell nuclei, suggesting an active proliferation. These results imply that the surface changes occurring during the degradation of the PαAPz-M fiber mat do not significantly impede cell adhesion and proliferation. However, the low porosity caused by the degradation of the PEA fiber mats can potentially impact the infiltration of cells.

To investigate the differentiation capacity of stem cells, L-ascorbic acid (L-AA) and TGF-β1 were employed to instigate their differentiation into smooth muscle cells on the mats. Following seven days of differentiation culture, the expression of smooth muscle marker genes, such as h-Acta2, h-cnn1, and SMTN, was examined by q-PCR. A regular growth condition (ND) on the fiber mats served as a control. The findings indicated a notable upregulation of Acta2 and cnn1 \((p < 0.05)\) after seven days of differentiation culture, although no substantial increase in SMTN expression was noted on any of the three materials \((p > 0.05)\). Importantly, no significant differences were observed among the three materials. These findings further suggest that the degradation products of PαAPz-M and the presence of phosphate as a degradation product on the material surfaces did not adversely affect the differentiation of MSCs into smooth muscle cells \((p > 0.05)\).
Figure 4.6. (A) Fluorescence images of MSC on PEA, PEA/PαAPz-M blend, and PαAPz-M fibrous mats after 3 days and 5 days of culture; F-actin is labeled by phalloidin in green and cell nuclei are labeled by DAPI in blue. (B) MSC mRNA expression of hActa2, hCnn1, and hSMTN was analyzed using RT-qPCR after cell treatment with L-AA and TGF-β1 for 7 days. ND stands for regular growth condition of iMSCs, and D stands for inducible condition of differentiation by exchanging culture medium to DMEM with 1% FBS. *** p < 0.001.

Despite the optimal performance observed in degradation experiments for the 50% mixed fiber mat, no significant differences were observed among the three materials in terms of gene expression. Therefore, for protein expression analysis, the PαAPz-M fiber mat was specifically selected, as it was exclusively reported in this study. After seven days of differentiation with L-AA and TGF-β1, the protein markers vinculin (red, Figure 4.7A2),
α-SMA (red, Figure 4.7B2), and MHC (red, Figure 4.7C2) were detected. Vinculin, an essential player in cell adhesion to the ECM, was localized to adhesion plaques. Comparing F-actin and vinculin staining in Figure 4.7A, focal contacts were clearly visible. Figure 4.7B,C clearly demonstrates the robust expression of α-SMA and MHC within the cells. These findings further bolster the strong cell adhesion and SMC differentiation of MSCs on PuAPz-M at the protein expression level.

Figure 4.7. Immunofluorescence analysis of iMSC differentiation on PuAPz-M fibrous mats with the addition of L-AA and TGF-β1 in DC culture medium after 7 days of pre-differentiation; vinculin (A1–A3), α-SMA (B1–B3), and MHC (C1–C3) are labeled in red. F-actin is labeled by phalloidin in green and nuclei are labeled by DAPI in blue.
4.4.7 Analysis of ECM expression

A key strategy in tissue engineering involves the use of biodegradable materials, with the anticipation that cells will generate their own extracellular matrix (ECM) during the material degradation process. Because of this, the expression of collagen and elastin in MSCs on PaAPz-M fiber mats was further examined. For this series of experiments, the same culture medium used in the differentiation culture was employed. L-AA, present in this differentiation culture medium, is widely recognized to promote ECM expression. In native blood vessels, collagen imparts tensile strength, while elastin provides elasticity, both crucial for the functional integrity of the vessel walls. Despite various methods yielding tissue-engineered grafts with tubular structures, many reports have noted a deficiency in elastin expression. Thus, the challenge of ensuring adequate elastin expression persists in vascular tissue engineering.

In a prior study, 10T1/2 cells expressed elastin were observed on polyurethane and PEA. Nonetheless, in the current study, as depicted in Figure 4.8, the evident expression of elastin in h-MSCs, a viable candidate for vascular substitutes, compared to 10T1/2 model cells, was a particularly exciting observation. The expression of collagen and elastin provides strong evidence of ECM formation during the degradation of PaAPz-M. This outcome further bolsters the potential of PaAPz-M in vascular tissue engineering applications.
Figure 4.8. Immunofluorescence analysis of ECM expression by iMSCs on PαAPz-M fibrous mats. A1, B1 show F-actin (red) and nuclei (blue); A2 displays collagen (green); B2 highlights elastin (green); A3, B3 are merged images.

4.5 Conclusions

The study in this chapter showed PαAPz-M to be promising potential as a biomaterial in vascular tissue engineering. It exhibits mechanical properties closer to those of the natural extracellular matrix (ECM) compared to other tested materials. While PαAPz-M fiber mats can maintain high porosity during degradation, blending them with a hydrophobic material can help reduce swelling. Moreover, these scaffolds support the viability, proliferation, and smooth muscle cell differentiation of mesenchymal stem cells (MSCs), key elements for successful tissue regeneration. Furthermore, MSCs on PαAPz-M fiber mats show collagen and elastin expression, suggesting possible ECM formation during material degradation. These results make PαAPz-M a promising candidate for future research and application in vascular tissue engineering. However, there are some limitations that warrant further investigations. First, for ECM deposition, the culture time of 14 days is relatively short, as
matrix deposition and assembly often require extended maturation times. Second, mechanical forces (e.g., fluid shear stress) that are known to influence cell behavior and matrix deposition are missing from this study since a circular mat instead of a tubular scaffold was used. These research avenues hold significant promise for PaAPz-M in vascular tissue engineering.

4.6 References


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Chapter 5

Poly [bis (ethyl methionato) phosphazene] Fiber Mats: A Novel Approach to Oxidative Stress Protection and Material-Guided Cell Behavior in Tissue Engineering*

Overview: This chapter aims to further expand the application of Poly [bis(ethyl methionato)phosphazene] Fiber Mats in tissue engineering. Based on the chemical properties of methionine, the composition of polyphosphazene degradation products and the structure of the fiber mat, its application in reactive oxygen species (ROS) scavenging, and material-directed cell behaviors were explored.

5.1 Abstract

The study in this Chapter investigates the application of poly[bis (ethylmethionato) phosphazene] (PaAPz-M) electrospun fibers in tissue engineering, focusing on its reactive oxygen species (ROS) scavenging capabilities and material-directed cell behaviors, including the influence of its degradation products on cell viability and differentiation and scaffold topography’s influence on cell alignment. The ROS scavenging ability of PaAPz-M was assessed through the DPPH assay, and then PaAPz-M’s protection against exogenous ROS was studied. Results showed enhanced cell viability on PaAPz-M fiber mats under oxidative stress conditions. The research also studied the effects of PaAPz-M degradation products on cell viability and osteogenic differentiation. It was observed that the late-stage degradation product, phosphoric acid, can significantly influence the osteogenic differentiation of MSCs. In contrast, methionine, which is the early-staged

*This chapter is to be submitted for publication.
degradation product, showed a minimal influence. Additionally, the study fabricated fiber mats that can lead to enhanced cell alignment while maintaining high porosity. Collectively, this study explored the functions of the PtαAPz-M fiber mat and expanded its application in protection against oxidative stress and guiding osteogenic differentiation and cell alignment.

5.2 Introduction

As tissue engineering evolves, the demands placed on materials extend beyond their biocompatibility and biodegradation properties. Researchers are now seeking materials that can meet the unique functional requirements of various tissues. Previous research on PtαAPz-M mainly focused on the viability of cells and differentiation with the supplementation of different biochemical induction factors. However, the possible contribution of its thioether structure in tissue engineering and the biochemical and biophysical cues provided by the scaffold are ignored.

One of the most important functions of methionine in the body is to counteract oxidative stress. Reactive oxygen species (ROS), including peroxides and free radicals, are crucial molecules in physiological processes. Nevertheless, it can detrimentally modify cellular components, impairing DNA and proteins and, consequently, obstructing new tissue formation when overexpression. However, even when using biocompatible materials, the implantation surgery could lead to trauma, foreign body reactions, and localized inflammation, resulting in the significant production of ROS.

While small molecules have been met with limited success in vivo due to their transient presence, research on ROS scavengers focuses more on macromolecular polymers.
Among them, poly(organophosphazenes), due to their biodegradability and easily tailored properties, are believed to be involved in ROS scavenging. As an example, the inclusion of aniline tetramer onto the glycine-based phosphazene and produced a biodegradable polymer with ROS-scavenging capability.\textsuperscript{5} Considering that the pure amino acid methionine is known to scavenge ROS,\textsuperscript{9, 10} and PαAPz-M showed good biocompatibility (Chapter 4),\textsuperscript{2} may serve to modulate ROS level without impairing cellular physiological processes.

In addition to traditional approaches, which mainly rely on in vitro culture supplementation of different biochemical induction factors, materials-directed differentiation, such as substrate stiffness, surface topography, and material composition, have recently been used as alternative regulators for MSC differentiation.\textsuperscript{11} In the previous Chapter, an increasing expression of vascular smooth muscle cell markers in MSCs on the PαAPz-M fiber mat was observed in the presence of growth factors.\textsuperscript{2} However, phosphoric acid, a primary degradation product of PαAPz-M, has demonstrated potential in guiding the osteogenic differentiation of MSCs.\textsuperscript{12, 13} Additionally, the influence of ROS on cell differentiation has been widely discussed.\textsuperscript{14, 15}

Meanwhile, cell alignment is present in numerous tissue, including the heart valve, vascular, tendon, and neural.\textsuperscript{16-19} Electrospun-aligned fiber is considered an ideal candidate for producing topographical features that guide cell arrangements and growth.\textsuperscript{20, 21} Although several strategies have been developed to produce aligned electrospun fibers, each method has limitations and material requirements, and the alignment highly depends on the system parameters, such as voltage and solution viscosity.\textsuperscript{22}
The objective of this study was to explore the application of PαAPz-M for tissue engineering in terms of ROS scavenging ability and the cell behavior under the influence of an exogenous ROS-generating compound. Furthermore, material-directed differentiation based on PαAPz-M, focusing on how PαAPz-M and its degradation products, and the topography of fiber mats can guide MSC differentiation without growth factors, were studied.

5.3 Material and methods

5.3.1 Materials

PEA and PαAPz-M were synthesized following previous reports. Polyester amide (PEA), specifically 8-Phe-4, was synthesized by interfacial polymerization using sebacoyl chloride, butanediol, and L-phenylalanine as the amino acids, according to previous publications. PαAPz-M was synthesized by thermal ring-opening polymerization with hexachlorocyclotriphosphazene and macromolecular substitution with L-methionine ethyl ester hydrochloride (H-Met-OEt·HCl).

Anhydrous tetrahydrofuran (THF) was purchased from Caledon Labs (Georgetown, ON, USA), and chloroform (CHCl3) was purchased from Sigma Aldrich. Hydrogen peroxide (H2O2) used as exogenous ROS was purchased from Fisher Chemical (Fair Lawn, NJ, USA).
5.3.2 Preparing electrospun fibrous scaffolds

For the preparation of electrospun fibrous scaffolds, the parameters, including voltage, concentration, flow rate and rotating speed are presented in Table 5.1. The electrospinning parameters of a 20 cm distance to the collector and a nozzle size of 22G was maintained.

5.3.3 Characterizations with SEM

The morphologies of all fiber mats were characterized using a Scanning Electron Microscope (SEM) (Model S-3400N, Hitachi, Ltd., Tokyo, Japan). Samples were affixed to a stainless steel holder using carbon adhesive tabs and subsequently coated with a 5 nm layer of osmium using a Filgen OPC80T Osmium Plasma Coater (Filgen Nanosciences & Biosciences Inc., Jonoyama, Japan). SEM images were captured at an acceleration voltage of 5 kV, utilizing the microscope's internal lens.

5.3.4 Cell culture

This study used mesenchymal stem cells derived from induced pluripotent stem cells (iMSC), generously provided by Dr. Dale Laird of Western University, London, ON, Canada. The fiber mat scaffolds, supported by aluminum foil, were carefully cut and their edges folded to secure the mats, creating an effective area of approximately 1 cm². These prepared fiber mats were individually placed in the wells of a 24-well plate and sterilized using 70% ethanol for a minimum of 30 minutes, followed by triple rinsing with HBSS to prepare for cell seeding.

Before seeding the iMSCs, the sterilized fiber mats were coated with a 0.1% gelatin solution and incubated at 37°C for 1 hour. The iMSCs were then seeded onto these gelatin-
coated fiber mats at 20,000 cells/cm² density. The cells were cultured in mesenchymal stem cell expansion media (MSCEM, Cedarlane Labs, Burlington, ON, Canada; HMSE-Media-450), supplemented with 10% fetal bovine serum, 1% L-glutamine, and 1% penicillin/streptomycin (all sourced from Fisher Scientific, Whitby, ON, Canada). The culture was maintained at 37°C in a humidified incubator with 5% CO2.

5.3.5 ROS-scavenging assays

A 2,2-Diphenyl-1-picrylhydrazyl (DPPH) scavenging assay was used to evaluate the ROS scavenging ability. DPPH were dissolved in ethanol at 5 mg/100 mL, then 0.1g of the solid PaAPz-M or PEA or 1 cm×1cm PaAPz-M or PEA fiber mats were immersed into 1.5 mL of the DPPH solution and kept in a dark for 2 hours. The DPPH solution added to a 1×1cm aluminum foil was used as the control. Then, the absorbance of the solution was measured by a UV-Vis spectrophotometer (F-7000, Hitachi, Japan) between 400-800nm. DPPH scavenging was calculated as follows:

\[
\text{DPPH scavenging} = \frac{A_c - A_s}{A_c} 
\]

Where \( A_c \) is the absorbance of the control DPPH solution and \( A_s \) is the absorbance of the DPPH solution with biomaterials.

To evaluate if the cell protection effect of the materials from exogenously generated ROS, iMSCs were cultured on aluminum foil, PEA fiber mat, and PaAPz-M fiber mat. Once the cells achieved approximately 95% confluence on the material surfaces after around 5 days after seeding, \( \text{H}_2\text{O}_2 \) at concentrations of 1, 2, 5, and 10 mM was added into the culture medium. After a 2-hour exposure to the \( \text{H}_2\text{O}_2 \)-conditioned medium, the LIVE/DEAD™
Cell Imaging Kit (488/570) was used to stain the cells on the scaffolds. Then, the fluorescent images were captured and analyzed quantitatively using ImageJ software. The live/dead stained cells were further counted to calculate cell viability.

5.3.6 Osteogenic differentiation with degradation products

In performing osteoinductive culture, cell culturing with and without scaffold was applied. iMSCs were seeded on the 24-well plates at the density of 20,000 cells/cm² in all the cases. When the cells reached 80% confluency, stock solutions with different degradation components were added into the medium. All stock solutions were adjusted to a pH of 7.4. The osteoinductive culture was continued for 7 days.

5.3.7 RNA extraction and quantitative real-time qPCR

1 ml of TRIzol™ Reagent (Life Technologies Inc., Burlington, Ontario) was utilized to extract the total RNA from each sample for the qPCR assay. The cells were lysed for 10 minutes at room temperature, and then chloroform was added at a ratio of 1:5 (chloroform: Trizol). The samples were vortexed for 15 seconds and incubated at room temperature for 15 minutes. They were then centrifuged at 4 °C and 12000×g for 15 minutes. The organic phase was removed, and the aqueous phase was transferred to another Eppendorf tube. Isopropanol was added at a ratio of 1:2 (isopropanol: Trizol) and incubated at room temperature for 10 minutes, followed by centrifugation at 12000×g for another 10 minutes at 4 °C. The isopropanol was then aspirated, and the pellet was resuspended in 75% EtOH at a ratio of 1:2 (EtOH: Trizol) and centrifuged at 7500×g for 5 minutes at 4° C. This final step was repeated twice to wash excess salts. After removing the EtOH, the pellet was air-
dried, dissolved in 25 µL of DEPC water, and quantified with nanodrop (Thermo Scientific).

The Promega™ Random Hexamers protocol (Fisher Scientific, Whitby, Ontario) was used to reverse transcribe 1µg of total RNA into complementary DNA (cDNA). qRT-PCR was performed four times using a CFX96™ Real-Time System (C1000 Touch Thermal Cycler; Bio-Rad, Mississauga, Ontario, Canada), and human genes of interest were determined with SsoAdvanced Universal SYBR® Green Supermix (Bio-Rad) as per the recommended procedures.

The primer sequences were as follows: Human Alpl forward primer 5’- CCT TCA CGG CCA TCC TAT ATG -3’, reverse primer 5’-CTG GTA GTT GTT GTG AGC GTA-3’. Human Runx2 forward primer 5’- CAC TGG GTC ACA CGT ATG ATT -3’, reverse primer 5’- AGG GAA GGG TTG GTT AG TACA -3’. Human Sp7 forward primer 5’-GCC AGT AAT CTT CAA GCC AGA -3’, reverse primer 5’-CCA TAG TGA GCT TCT TCC TGG C-3’. Human OCN forward primer 5’- CTG CAT TCT GCC TCT CTG AC-3’, reverse primer 5’-CTA TTC ACC ACC TTA CTG CCC-3’. Human 18S forward primer 5’-GCG GTT CTA TTT TGT TGG TTC TTG ATT-3’. The results were analyzed using the comparative threshold cycle method and normalized with human 18S as an endogenous reference.

5.3.8 Immunofluorescence Microscopy

The cells were treated with 10% formalin for 10 minutes at room temperature and then washed three times with PBS. To enable intracellular staining, 0.1% Triton X-100 permeated all the samples for 10 minutes. Subsequently, for 30 minutes at room
temperature, the cells were blocked in 2% BSA containing 22.52 mg/mL of glycine in PBS. For nuclear labeling, 4′6-diamidino-2-phenylindole (DAPI), obtained from Life Technologies, Canada, was used at a concentration of 300 nM in PBS. The coverslips were mounted onto microscope slides using PermaFluor™ Mounting Medium by Fisher Scientific™, Whitby, Ontario, Canada, and sealed with clear nail enamel. Images were captured using a 10x/25x water lens-equipped Zeiss LSM 800 confocal microscope (Zeiss, Toronto, Canada) and analyzed with ZEN 3.1 (blue edition) software.

5.3.9 Statistical Analysis

Mean ± standard deviation (SD) represented all quantitative data for n = 3. Tukey's test was used for statistical analysis with one-way variance analysis. The groups were considered statistically significant if the probability values of *p < 0.05.

5.4 Result and discussion

5.4.1 Evaluation of ROS-Scavenging Capacity

**Figure 5.1A** illustrates how methionine, the functional group of PaAPz-M, scavenges ROS. The thioether group in methionine can capture ROS and be oxidized into the methionine sulphoxide. Meanwhile, methionine sulphoxide can also be reverted, enabling continuous ROS regulation. The PaAPz-M synthesis only involves the amino groups of methionine. Therefore, the thioether structure is well-protected (**Figure 5.1A**). With phosphazenes' substantial methionine loading capacity, PaAPz-M can be a promising candidate for effective ROS scavenging. To evaluate this, DPPH assay, which is a reliable method for assessing antioxidant activity, was employed.
The DPPH assay measures antioxidant activity by observing the reaction of antioxidants with the DPPH radical, which leads to a color change from violet (DPPH•) to yellow (DPPH-H) (Figure 5.1B). At the concentration of 5mM, the Lambert-Beer law is obeyed over the valuable absorption range. So, the absorbance before and after the reaction with the sample is quantifiable, allowing for a direct assessment of ROS scavenging ability.

Figure 5.1. (A) Structure of PaApz-M (B) Reaction mechanism of 2,2-diphenyl-1-picrylhydrazyl (DPPH) with antioxidant. A-H = antioxidant radical scavenger; A• = antioxidant radical.
The ROS scavenging capacity of PEA, PαAPz-M solids, and fiber mats was tested in this experiment. After immersion in the DPPH solution for 2 hours, the color changes caused by characteristic reactions of DPPH and antioxidants can be observed. This color change was further substantiated quantitatively using an absorption photometer, as shown in Figure 5.2 C, D. Notably, both PEA and PEA fiber mats exhibited negligible deviation from the control, which means PEA did not have the capacity to scavenge ROS. Meanwhile, PαAPz-M and PαAPz-M fiber mats effectively neutralized nearly 50% and 100% of DPPH, respectively (Figure 5.2D). The lower scavenging fiber can be attributed to the limited PαAPz-M content in the fiber mats. Nevertheless, this data demonstrates the ROS scavenging capacity of PαAPz-M and PαAPz-M fiber mats.

Figure 5.2. (A) Mechanism scheme of Methionine scavenging ROS. (B) DPPH solution cultured with PEA and PαAPz-M solids and fiber mats. (C) Absorbance of DPPH solution cultured with each sample. (D) DPPH radical scavenging activity of each sample.

After verifying PαAPz-M 's ROS scavenging capability, its potential to protect cells from exogenous ROS was further tested. Gradient concentrations of H2O2 were added into the
culture media to simulate the exposure of exogenous ROS. Live/dead assays were used to qualify and quantify the viability of iMSC after the exposure. As shown in Figure 5.3, with the increase in H$_2$O$_2$ concentration, the red fluorescence signals representing dead cells are observed, and the cell density decreases. In the control group, a notable reduction in cell density was observed upon exposure to 1 mM H$_2$O$_2$. At 2 mM, the detachment of cells from the foil was noted, and at concentrations over 5 mM, the control group exhibited almost complete cell detachment. Cell detachment on the PEA surface was less severe. Significant detachment only occurred at concentrations over 10mM. Despite DPPH assay indicating PEA did not scavenge ROS, the enhanced cell adhesion on PEA fiber mats can be attributed to the large surface area presented by the nanofiber mats' structure. The viability of iMSCs on the PαAPz-M fiber mat showed a different scenario. At concentrations of 1mM and 2mM, some iMSCs adopted a spherical shape without significantly reducing cell density. The cell density only resembled that of PEA and control at 1mM when the concentration reached 5mM. It was only increased to 10mM that cells were grown on the PαAPz-M fiber mat without extensive cell detachment. With the exposure to the exogenous ROS, PαAPz-M fiber mats have shown a great protection to the iMSCs over it.
Figure 5.3. Evaluation of iMSC viabilities of fiber mats exposed to exogenous ROS. (A) Live/Dead assay after culture with gradient concentrations of H₂O₂. Scale bar in size of 200µm. (B) Relative cell number of the iMSC with exogenous ROS. (C) Percentage of live cells with exogenous ROS. Within the indicated groups, statistical significance exists except for those labeled with NS. NS stands for not significant.

5.4.2 Degradation products on cell viability

The degradation of PaAPZ is through the hydrolysis of amino acid ester, the cleavage of side groups, and, at last, the breakdown of the backbone. So, the soluble degradation products of PaAPz-M should be composed of the corresponding side groups and ions such
as phosphate and ammonium. Although methionine, phosphate, and ammonium are commonly found in the human body, previous studies indicate that the degradation products may accumulate in porous structures and produce a microenvironment that may influence cell behaviors. Therefore, it is essential to study the cell viability at high concentrations of these degradation products.

To study the effects of PtAPz-M degradation products on cell behavior, aqueous solutions of phosphoric acid, methionine ethyl ester, and their 1:2 combination (SD) on cell viability were compared. All stock solutions were adjusted to a pH of 7.4 and then, the stock solutions were added to the culture medium at 1, 5, and 10 mM concentrations. As shown in Figure 5.4, after 3-7 days, the metabolic activity with different additives was tested using the MTT assay. No significant change in cell metabolic activity were observed in the group by adding methionine. Notably, solutions containing 20 mM of methionine led to a slight increase in metabolic activity. In contrast, the groups supplemented with phosphoric acid (phosphate-only and phosphate-amino acid mixture) show a significant decrease in metabolic activity when concentrations of phosphoric ions are over 5mM.
Figure 5.4. Cell viability of iMSCs being cultured with media containing various concentrations of (A) Methionine, (B) Pi ion, and (C) simulating degradation product (SD). Molecular Mechanism Study of PaAPz-M on osteogenic differentiation. Within the indicated groups, statistical significance exists except for those labeled with NS. NS stands for not significant.

Recent research indicates that high levels of phosphoric acid can substantially increase the expression of osteogenic markers. Meanwhile, the ROS levels that methionine influences can also control osteogenic differentiation and calcium deposition. Therefore, osteogenic differentiation studies over using the PaAPz-M’s degradation products on osteogenic differentiation are needed and will be insightful. To investigate their impact on iMSCs’ osteogenic differentiation, the expression of osteo-specific marker genes was analyzed under 2D conventional tissue culture conditions, and results are shown in Figure 5.5. With the presence of high phosphate levels, there is a notable increase in the osteogenic markers RunX2, ALPL, SP7, and OCN (Figure 5.5A). Notably, the highest expression of Alpl is found at the concentration of 5mM of Pi ion, which is similar to the report on phosphate’s effects on bone marrow mesenchymal stem cells. This could be because ALP plays a crucial role in early osteogenesis, hydrolyzing phosphates to promote cell maturation, and its expression will diminish following the completion of the maturation. However, the contribution of methionine to osteogenesis seems minimal...
(Figure 5.5B), and only a relatively slight increase in Sp7 and RunX2 was observed. Meanwhile, the overall trend of the mixed group is similar to the Pi group, indicating that phosphoric acid led the osteogenic differentiation of iMSC, while methionine's influence is minor.

Figure 5.5. iMSC mRNA expression of SP7, RunX2, Alpl, and OCN was analyzed using RT-qPCR after cell treatment with (A) Pi ion, (B) Methionine, and (C) simulating degradation product (SD) for 7 days under 2D conventional tissue culture conditions. Within the indicated groups, statistical significance exists except for those labeled with NS. NS stands for not significant.

5.4.3 Staged degradation of PaAPz-M on osteogenic differentiation

As there is a staged degradation of PaAPz-M,\(^{35,36}\) after confirming the mechanism of how PaAPz-M’s degradation products influence iMSC’s differentiation, the osteogenic differentiation of iMSC over the fiber mats with staged degradation products of PaAPz-M
was tested. As shown in Figure 5.6A, at 37°C, solid PαAPz-M lost around 40% mass of
PαAPz-M after 21 days of degradation. To get the full degradation of PαAPz-M, materials
were degraded at 90°C and over 95% degraded into soluble content on day 5. The
degradation solution with around 40% and 95% mass loss of PαAPz-M were used to
simulate the mid-stage degradation products (MD), and fully degraded products (FD),
respectively. The degradation behavior of PαAPz-M aligns closely with previously
reported trends, albeit with a slightly accelerated rate.36, 37 This difference could be
attributed to the phosphazene polymerization method employed in this research. The
degradation products were added to the culture medium at 10 mM based on the estimated
phosphate ion concentration derived from the mass loss of the material. iMSCs on the
PαAPz-M fiber mats treated with simulated degradation solution were used as the control.

Figure 5.6B-E shows the expression of osteogenic markers SP7, RunX2, Alpl, and OCN
in iMSCs after a 7-day culture period with degradation products on the fiber mats. Although
the calculated concentration of phosphorous ions in the group with fully degraded products
is 10 mM, the expression levels of these four genes in this group were observed to be higher
than those in the group with 5 mM of SD group, yet lower than in the group with 10 mM
of SD. This could be attributed to the presence of soluble fragments in the degradation
solution, potentially leading to an overestimation of the phosphate ion concentration.
Meanwhile, the MD group did not significantly influence iMSCs differentiation. This
implies that PαAPz-M and its degradation products did not affect MSC differentiation in
the initial culture stages. In contrast, phosphate release from the material's backbone seems
to trigger osteogenic gene expression in MSCs as degradation progresses.
The different effects of the mid and late-stage degradation products on MSCs differentiation can contribute to the staged degradation behavior of polyphosphazene, wherein PαAPz initially releases amino acid groups, followed by the subsequent breakdown of its backbone, which will release phosphoric acid. Therefore, in the early stages of degradation, PαAPz-M and its degradation products will not guide stem cells to differentiate into bone cells. In the past, polyphosphazene was studied in bone tissue engineering due to its phosphate-containing degradation products. This could be one reason why polyphosphazene is mainly studied in bone tissue engineering.

Meanwhile, soft tissue calcification is often associated with pathological conditions, and it's important to recognize that such calcification can also be a natural healing response to injury.38 Indeed, in certain scenarios within the circulatory system, such phosphorous-guided calcification may play a beneficial role as well.29, 39 Therefore, the ability of PαAPz-M to guide osteogenic differentiation demonstrated in these experiments is an important advantage of its application in bone tissue engineering, but it will not hinder its application in other tissues.
Figure 5.6. Analysis of iMSC mRNA expression levels for (A) SP7, (B) RunX2, (C) Alpl, and (D) OCN using RT-qPCR. Cells were treated for 7 days on fiber mats with simulated degradation product (SD), mid-staged degradation products (MD), and fully degraded products (FD).
5.4.4 Cell orientation on aligned electrospun fibers

After studying the material-directed differentiation based on PαAPz-M and its degradation products, the topological cue that could potentially be provided by the fiber mat was further tested. In this study, aligned electrospun fibers were prepared with higher flow rates and lower voltages. This is because the increased fiber diameter and reduced electric field intensity can mitigate the impact of the bending zone on fiber arrangement. To further reduce the impact of the bending zone, the PαAPz-M and PEA were blended to increase the strength of the fiber. After identifying the electrospinning parameters, aligned fibers were produced following the parameters in Table 5.1.

Table 5.1. Spinning parameters to produce random and aligned fibers.

<table>
<thead>
<tr>
<th>Solvent ratio</th>
<th>Rotating speed (RPM)</th>
<th>Voltage (kV)</th>
<th>Concentration (wt%)</th>
<th>Flow rate (ml/h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Random CF: DMSO (3:1)</td>
<td>300</td>
<td>20</td>
<td>10</td>
<td>0.4</td>
</tr>
<tr>
<td>Aligned CF: DMSO (3:1)</td>
<td>1500</td>
<td>15</td>
<td>15</td>
<td>0.8</td>
</tr>
</tbody>
</table>

The SEM image and the corresponding cell morphology are shown in Figure 5.7. As shown in Figure 5.7B, a rise in the flow rate corresponded with an increase in fiber diameter (compare scale bars in A and B), producing fiber mats composed of aligned micron-scale fibers interspersed with randomly distributed nanofibers. After producing the aligned fiber mats, cells on both random and aligned fiber mats were stained with immunofluorescence to study the effect of scaffold topography on cell-scaffold interactions. As shown in Figures 5.7C and D, it is evident that cells on aligned fiber mats displayed an aligned arrangement, while on the random fiber mats, cells exhibited a random distribution. This
indicates that within the structure of electrospun fiber mats, part of aligned micro-sized fibers is sufficient to guide cellular arrangement.

This outcome is noteworthy as it addresses the standard limitation noted in the existing aligned fiber mat fabrication method. Most reports fabricated fibers with uniform diameters, resulting in decreased fiber mat porosity, thus hindering cellular penetration. In contrast, this scaffold displayed aligned microscale fibers interspersed with randomly distributed nanofibers, exhibiting enhanced porosity compared to traditionally aligned fibers with uniform diameters. Such structures can provide aligned topological cues with porosity, benefiting various tissues, including muscle, vascular, nerves and even bone.
Figure 5.7. Electrospun (A) random and (B) aligned fiber from the blend of PEA and PαAPz-M and cell morphologies of iMSC on random and aligned fiber after 7day of culturing.

5.5 Conclusion

This Chapter provided several lines of evidence for the versatile applications of PαAPz-M in tissue engineering. As evidenced by the DPPH assay, PαAPz-M has shown a significant ROS-scavenging capacity, positioning it as a promising material for scavenging ROS. Additionally, PαAPz-M fiber mats have also demonstrated the potential to protect cells from exogenous ROS, which could affect cell viability during maturation and implantation. In the subsequent material-directed differentiation study, it was shown that phosphoric acid plays a significant role in directing iMSCs toward osteogenic differentiation and aligned
fiber topology guides cell arrangement. Notably, phosphoric acid is the late-stage degradation product of PαAPz-M, and early-staged degradation products such as methionine did not show a significant influence on cell differentiation, which means PαAPz-M holds potential for applications in tissue engineering beyond bone regeneration. This research explored the functionality of PαAPz-M in both protecting cells from oxidative damage and material-directed cell differentiation and expanded the application of PαAPz-M in tissue engineering.

5.6 References


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Chapter 6

6 General discussion and conclusions

_Overview:_ This chapter summarizes the research, discusses the strengths and limitations of the current work, and recommends future directions.

6.1 Summary

Polyphosphazenes, in general, have a backbone composed of alternating phosphorus and nitrogen atoms. Poly [(α-amino acid ester) phosphazene] (PαAPz) is a class of biodegradable polymers incorporating amino acids as part of the phosphorus and nitrogen backbone structure. When amino acids are introduced as side groups, the resulting polymers combine the unique properties of polyphosphazenes with the biocompatibility and functionality of amino acids. The focus of this thesis was to design PαAPz and expand their application to vascular tissue engineering. Those PαAPz based on L-alanine and L-phenylalanine have been previously studied for hard tissue engineering due to their biodegradation and biological properties, but studies for soft tissues such as vascular are lacking. Contrasting with biodegradable polyesters such as poly(lactide), poly(glycolide), and their copolymers, PαAPz only releases non-toxic buffering byproducts mainly consisting of phosphates, ammonia, and corresponding side groups, in its degradation.\(^1\) Since vascular smooth muscle cells (VSMCs) are particularly sensitive to acidic degradation products, PαAPz could be excellent biomaterials for vascular tissue engineering. In addition, the side chain of poly phosphazene can be easily modified through...
the macromolecular substitution, allowing for the tailoring of its properties, such as mechanical degradation rates, mechanical properties, or the introduction of new functionalities, including conductivity and ROS-scavenging capabilities, to PαAPz.

Despite the apparent advantages of PαAPz, its application in vascular tissue engineering also presents several challenges. Firstly, the polymerization of HCCP trimer to produce the PDCP intermediate requires a strict anhydrous environment, which has constrained the development of polyphosphazene materials. Although several methods can synthesize polyphosphazenes under conventional conditions, these methods yield polyphosphazenes with low molecular weight, which poses challenges for producing electrospun scaffolds for tissue engineering. Secondly, bone tissue engineering scaffolds should exhibit stiffness to offer adequate support, while vascular tissue engineering scaffolds should be more flexible to mimic the properties of the native tissue. The most widely studied PαAPz are based on L-alanine and L-phenylalanine which have the mechanical properties more suitable for bone tissue engineering. Thus, identifying new amino acids that can produce flexible PαAPz are needed. Thirdly, the differentiation of stem cells towards mature vascular smooth muscle cells can also be challenging on synthetic materials. More importantly, the expression of late-stage differentiation makes such as myosin heavy chain (MHC), smoothelin (SMTN) and smoothelin-B and extracellular matrix (ECM) components such as collagen and elastin are difficult. Finally, with the development of tissue engineering, there is an increasing demand for materials with advanced functions, such as ROS-scavenging and topography clues. In this context, the development of PαAPz to meet these contemporary requirements also essential.
In Chapter 3, the synthesis methodology for biodegradable α-amino acid-substituted poly(organophosphazene) polymers were improved and the electrospinning parameters to produce nano-fibrous scaffolds were refined. Then their effectiveness as matrices for inducing the differentiation of induced mesenchymal stem cells (iMSCs) into mature, contractile smooth muscle cells (SMCs) was assessed. Across all three cell types examined – iMSCs, bone marrow-derived mesenchymal stem cells (BM-MSCs), and primary human coronary artery SMCs – robust attachment and spreading on the scaffolds were observed. Interestingly, this study proves that while L-ascorbic acid (AA) and transforming growth factor-beta 1 (TGF-β1) are capable of directing iMSCs towards a smooth muscle lineage, the electrospun fibrous mats can provide a more conducive environment for enhanced differentiation. This process was evidenced by the upregulated transcriptional levels of early and late-stage differentiation marker proteins.

In Chapter 4, the objective was to explore the microscopic mechanical properties of PαAPz-M for vascular differentiation of stem cells and ECM expression. Atomic Force Microscopy (AFM) was employed to assess the mechanical properties of PαAPz-M fibrous mats. The results indicated a stiffness close to that of the natural extracellular matrix (ECM). Additionally, the PαAPz-M scaffolds retained their morphological and dimensional stability during the 21 day degradation study, showing their suitability for long-term maturation. In terms of cellular behaviors, both mesenchymal multipotent 10T1/2 cells and mesenchymal stem cells (MSC) demonstrated significant viability and proliferation over the fiber mats. Notably, during the MSC differentiation and maturation, not only the expression of SMC marker but also the synthesis of ECM proteins such as collagen and elastin, suggesting potential ECM production and remodeling.
In Chapter 5, the role of PαAPz-M as ROS scavenging material, the effect of PαAPz-M degradation products to induce osteogenic differentiation, and the provision of topographical cues, were explored. After the conformation ROS-scavenging capacity of PαAPz-M through DPPH assay, it was observed that MSCs cultured on the PαAPz-M mat were effectively protected from exogenous ROS. Subsequently, the effects of degradation products of PαAPz-M and aligned fibers on cell behavior were studied. It was found that the degradation products of PαAP-M can promote osteogenic differentiation.

6.2 Strengths

This is the first study exploring PαAPz for their potential use in vascular tissue engineering. Biomaterials for vascular tissue engineering are still challenging due to vascular smooth muscle cells (VSMCs) sensitivity to acidic degradation products and natural blood vessels' complex structure and functions. One of the first steps of this research was to characterize the suitability of PαAPz for the cells used in vascular tissue engineering. Moreover, the improved synthesis method of PαAPz provided a more practical approach in a non-strictly anhydrous environment that enables the fabrication of PαAPz fibrous scaffolds. This strict anhydrous environment is a common hurdle in PαAPz synthesis.

Diverging from its traditional use in bone and skeletal tissue engineering, this work also introduces a new type of PαAPz, PαAPZ-M, which is more suitable for vascular tissue engineering applications. Despite the synthesis method, the specific application of PαAPz-M to tissue engineering and the effects of its sulfur groups on cell behaviors had not been explored yet. This research not only studied its suitability for VTE in terms of scaffold fabrication, mechanical properties, and cell viability but also discovered the ROS-
scavenging capacity of PαAPz-M, deriving from its thioether structure. Notably, AFM was used to obtain the mechanical properties of PαAPz-M for the first time.

Although using stem cells is a well-known strategy in tissue engineering, reports on their differentiation on synthetic fibrous mats are limited.\(^2\) In this work, the focus has been the expressions of late-stage VSMC markers and extracellular matrix. In addition to traditional VSMC differentiation approaches, which mainly rely on in vitro culture supplementation of different biochemical induction factors, topographical cues are incorporated into the scaffold design, representing an innovative strategy to direct MSC alignment.

6.3 Limitations and future directions

Although the studies in this thesis provided several insights on the role of PαAPz, there are some limitations that warrant further investigations. First, the culture time of 14 days for ECM deposition is relatively short, as matrix deposition and assembly often require extended maturation times. Second, mechanical forces (e.g., fluid shear stress) that are known to influence cell behavior and matrix deposition are missing from this study since a circular mat instead of a tubular scaffold was used. These research avenues hold significant promise for PαAPz-M in vascular tissue engineering. Other possible limitations and recommendations are discussed below.

6.3.1 New amino acids and substitutions in PαAPz

The PαAPz used in this work was based on L-alanine, L-phenylalanine, and L-methionine. To avoid cross-linking during the macromolecular substitution, it is crucial to use molecules with only one nucleophilic group to synthesize PαAPz.\(^3\) Among the 20 standard amino acids, only glycine, valine, proline, leucine, and isoleucine can be used without
protecting group before the substitution. However, beyond these common amino acids, synthetic amino acids also present suitable options for PαAPz preparation. Moreover, with the protection and deprotection reactions, amino acids with multiple nucleophilic groups, such as cysteine, can also be used to functionalize polyphosphazene. Concurrently, the co-substitution with other organic compounds can create polyphosphazenes with new functions, such as conductivity or enhanced elasticity. Developing new phosphazene makes it possible to customize the properties and integrate new functions of a phosphazene to meet different requirements in tissue engineering.

6.3.2 Advanced scaffold fabrication strategies

Exploring advanced methods for scaffold fabrication could also be the future work of this study. In this work, electrospinning was used to fabricate the scaffold. Even though electrospinning is a versatile technique in producing 3D nanofibrous structures, cell infiltration is still the main challenge for electrospun scaffolds. Beyond traditional fabrication methods like freeze-drying and particle leaching, innovative approaches based on rapid prototyping could also be a good choice for PαAPz scaffold fabrication. Notably, a hybrid method of electrospinning and printing could be an up-and-coming technique for PαAPz scaffold fabrication. This approach could effectively overcome the limitations of the low resolution of 3D printing and the uncontrollable shapes of electrospinning, offering the potential to construct a nano- to macroscale scaffold simultaneously. Combined with existing reports on electrospun polyphosphazenes, this will be a promising research direction in the future.
6.3.3 Tubular scaffolds and tissue maturation in bioreactors

In this study, the fiber mats were mainly used to test the application of PαAPz in tissue engineering. Future work should involve producing a tubular scaffold that can mimic the structure of natural blood vessels. In addition to simulating the three-layered structure of natural blood vessels, tubular structures are fundamental to studying the influence of physical cues, such as fluid shear stress and pressures, on cell differentiation.\(^7\)\(^8\) While several promising methods can produce a tubular scaffold, the long maturation period and the delivery of oxygen and nutrition still pose a challenge that necessitates more sophisticated bioreactor technologies.\(^9\) Finally, after the maturation of the engineered blood vessels in vitro, it is necessary to test its function in vivo.

6.4 References


Appendices

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Electrospun Biodegradable α-Amino Acid-Substituted Poly(organophosphazene) Fiber Mats for Stem Cell Differentiation towards Vascular Smooth Muscle Cells

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Preparation and Microscopic Mechanical Characterization of L-Methionine-Based Polyphosphazene Fibrous Mats for Vascular Tissue Engineering

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