Synthesis of Crosslinkable Poly(ester amide)s for Cell Encapsulation and Delivery

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Abstract

Tissue engineering using adipose-derived stromal cells (ASCs) shows promise for soft tissue regeneration. Biodegradable polymers are potential biomaterials as they support the growth and delivery of cells. Specifically, poly(ester amide)s (PEAs) are a class of biodegradable polymers with tunable structures that have been shown to exhibit low cytotoxicity and support the growth of various cell types. This thesis involved the development of new water soluble amino acid-based PEAs with crosslinkable moieties to enable formation of hydrogel scaffolds for ASC encapsulation. These hydrophilic phenylalanine-based and alanine-based PEAs were synthesized by solution polycondensation and photo-crosslinked into a series of hydrogels with and without poly(ethylene glycol) dimethacrylate, methacrylated chondroitin sulphate or decellularized adipose tissue, and hydrogel properties including gel content, equilibrium water content, and swelling were examined. ASC viability and adipogenesis were studied in selected PEA hydrogels, and the overall results demonstrated the potential of these new water soluble PEAs as biomaterials for adipose tissue engineering, as they provided a supportive environment for ASCs survival and adipogenic differentiation in culture.

Keywords

Adipose-derived stromal cells, biodegradable polymers, poly(ester amide)s, hydrogels, decellularized adipose tissue, adipogenesis, adipose tissue engineering.
Summary for Lay Audience

Using cells to regenerate lost or damaged tissues is a promising alternative to current transplant approaches. In particular, adipose-derived stromal cells (ASCs), which can be isolated from waste fat tissue, are a special type of cells that can be converted into fat cells, and hold promise for fat regeneration for soft tissue reconstruction and cosmetic plastic surgery purposes. To aid in the delivery of these cells into the body and to support their growth, materials that are able to break down into nontoxic elements can be used to hold the cells. In this project, we have picked a class of materials called poly(ester amide)s (PEAs) that are capable of producing nontoxic components as they break down. The PEAs were made to be able to dissolve in water and to contain specific chemical features in order to form a hydrogel, which is a gel material that can take in a large amount of water and resembles the structure of soft tissues such as fat. ASCs were incorporated in these PEA hydrogels and observed for their behavior inside the gels in terms of their survival and conversion to fat cells. However, using natural biological materials can often help with directing cell behaviors. Therefore, we added a material that has features similar to the environment of fat cells called decellularized adipose tissue (DAT) to the PEA hydrogels, which was also extracted from fat tissue. DAT was proposed to provide beneficial qualities to the overall hydrogels and better support the survival and fat cell conversion of the ASCs inside the gels. Based on the results, we found that the ASCs inside the hydrogels made from PEAs and PEAs with DAT survived well in culture and were able to convert into fat cells. As a result, the materials that we developed in this work showed potential to be used for fat regeneration.
Co-Authorship Statement

The work described in this thesis is the result of a collaborative effort from the author, coworkers at Western University as well as supervisors Dr. Elizabeth Gillies and Dr. Lauren Flynn. The following details the individual contribution for each chapter.

Chapter 1, the introduction was written by the author and revised by Dr. Elizabeth Gillies and Dr. Lauren Flynn.

Chapter 2 describes a project proposed by Dr. Elizabeth Gillies and Dr. Lauren Flynn. All of the chemical synthesis and characterization experiments, hydrogel fabrication and characterization, cell isolation and culture setup, adipose tissue decellularization, cell encapsulation studies, cell viability and glycerol-3-phosphate dehydrogenase activity analyses as well as BODIPY staining were conducted by the author. Thermal data for the polymers was collected by Dr. Amir Rabiee Kenaree, a postdoctoral fellow in the Gillies lab, while the chromatographic runs were performed by Aneta Borecki, a technician in the Gillies lab. The chapter was written by the author and revised by Dr. Elizabeth Gillies and Dr. Lauren Flynn.

Chapter 3, the conclusion was written by the author and revised by Dr. Elizabeth Gillies and Dr. Lauren Flynn.
Acknowledgments

From the start, I would like to express my gratitude to my supervisors, Dr. Elizabeth Gillies and Dr. Lauren Flynn for their guidance and support. I have experienced many difficulties with my research project throughout the past two years, and your mentorship and encouragement have helped me get through every one of them.

I would also like to thank all of the past and present members from both Gillies and Flynn labs, for providing a caring and cooperative environment. You have all made my graduate school journey very memorable. I especially want to thank Dr. Kai Cao and Dr. Arthi Shridhar for teaching me the technical skills and offering wonderful advices for my research. Additionally, a huge thank you to Charmainne Cruje and Nadia Sharma, from colleagues turning into lifetime friends. I will deeply treasure all of our memories inside and outside of the lab. I also want to show my gratitude to Megan, Thao and Jamie who are the amazing friends I have made in London.

Thank you to my thesis examiners, Dr. James Wisner, Dr. Joe Gilroy and Dr. David O’Gorman for their time on reading my thesis.

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<tbody>
<tr>
<td>ABAM</td>
<td>Antibiotic-antimycotic</td>
</tr>
<tr>
<td>Ala-PEA</td>
<td>L-alanine based poly(ester amide)</td>
</tr>
<tr>
<td>APS</td>
<td>Ammonium persulphate</td>
</tr>
<tr>
<td>Arg-PEA</td>
<td>L-arginine based poly(ester amide)</td>
</tr>
<tr>
<td>ASCs</td>
<td>Adipose-derived stromal cells</td>
</tr>
<tr>
<td>ATR</td>
<td>Attenuated total reflectance</td>
</tr>
<tr>
<td>BAAD</td>
<td>Bis-α-(L-amino acid)-α, ω-alkylene diester</td>
</tr>
<tr>
<td>BAECs</td>
<td>Bovine aortic endothelial cells</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>C/EBPα/β/δ</td>
<td>CCAAT/enhancer binding protein-alpha/beta/delta</td>
</tr>
<tr>
<td>CH₂Cl₂</td>
<td>Dichloromethane</td>
</tr>
<tr>
<td>D</td>
<td>Dispersity</td>
</tr>
<tr>
<td>DAT</td>
<td>Decellularized adipose tissue</td>
</tr>
<tr>
<td>DMA</td>
<td>N,N-dimethylacetamide</td>
</tr>
<tr>
<td>DMAP</td>
<td>4-dimethylaminopyridine</td>
</tr>
<tr>
<td>DMF</td>
<td>N,N-dimethylformamide</td>
</tr>
<tr>
<td>DSC</td>
<td>Differential scanning calorimetry</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>Et₃N</td>
<td>Triethylamine</td>
</tr>
<tr>
<td>EWC</td>
<td>Equilibrium water content</td>
</tr>
<tr>
<td>FABP4</td>
<td>Fatty acid binding protein-4</td>
</tr>
<tr>
<td>FAS</td>
<td>Fatty acid synthase</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>FGF</td>
<td>Fibroblast growth factor</td>
</tr>
<tr>
<td>FT-IR</td>
<td>Fourier-transform infrared</td>
</tr>
<tr>
<td>GLUT4</td>
<td>Glucose transporter type-4</td>
</tr>
<tr>
<td>GPDH</td>
<td>Glycerol-3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>HCASMCs</td>
<td>Human coronary artery smooth muscle cells</td>
</tr>
<tr>
<td>IGF</td>
<td>Insulin like growth factor</td>
</tr>
<tr>
<td>LPL</td>
<td>Lipoprotein lipase</td>
</tr>
<tr>
<td>MCS</td>
<td>Methacrylated chondroitin sulphate</td>
</tr>
<tr>
<td>MMP</td>
<td>Matrix metalloproteinase</td>
</tr>
<tr>
<td>Mn</td>
<td>Number average molar mass</td>
</tr>
<tr>
<td>MSCs</td>
<td>Mesenchymal stromal cells</td>
</tr>
<tr>
<td>MWCO</td>
<td>Molecular weight cutoff</td>
</tr>
<tr>
<td>NMR</td>
<td>Nuclear magnetic resonance</td>
</tr>
<tr>
<td>p-TSA</td>
<td>p-toluenesulfonic acid</td>
</tr>
<tr>
<td>P4</td>
<td>Passage 4</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffer saline</td>
</tr>
<tr>
<td>PDLA</td>
<td>Poly(D-lactic acid)</td>
</tr>
<tr>
<td>PDLLA</td>
<td>Poly(D,L-lactic acid)</td>
</tr>
<tr>
<td>PEA</td>
<td>Poly(ester amide)</td>
</tr>
<tr>
<td>PEG</td>
<td>Poly(ethylene glycol)</td>
</tr>
<tr>
<td>PEGDMA</td>
<td>PEG-dimethacrylate</td>
</tr>
<tr>
<td>PGA</td>
<td>Poly(glycolic acid)</td>
</tr>
<tr>
<td>Phe-PEA</td>
<td>L-phenylalanine based poly(ester amide)</td>
</tr>
<tr>
<td>Phe8-PEA</td>
<td>L-phenylalanine based poly(ester amide) with 1,8-octanediol</td>
</tr>
<tr>
<td>Acronym</td>
<td>Full Form</td>
</tr>
<tr>
<td>---------</td>
<td>-----------</td>
</tr>
<tr>
<td>PLA</td>
<td>Poly(lactic acid)</td>
</tr>
<tr>
<td>PLGA</td>
<td>Poly(lactic-co-glycolic)</td>
</tr>
<tr>
<td>PLLA</td>
<td>Poly(L-lactic acid)</td>
</tr>
<tr>
<td>PMMA</td>
<td>Poly(methyl methacrylate)</td>
</tr>
<tr>
<td>PMSF</td>
<td>Phenylmethylsulfonyl fluoride</td>
</tr>
<tr>
<td>PPARγ</td>
<td>Peroxisome proliferator-activated receptor-gamma</td>
</tr>
<tr>
<td>ppm</td>
<td>Parts per million</td>
</tr>
<tr>
<td>PTFE</td>
<td>Polytetrafluoroethylene</td>
</tr>
<tr>
<td>RGD</td>
<td>Arginine-glycine-aspartic acid</td>
</tr>
<tr>
<td>RT-qPCR</td>
<td>Real-time reverse transcription polymerase chain reaction</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulphate</td>
</tr>
<tr>
<td>SEC</td>
<td>Size exclusion chromatography</td>
</tr>
<tr>
<td>SPB</td>
<td>Sorenson’s phosphate buffer</td>
</tr>
<tr>
<td>TCPS</td>
<td>Tissue culture polystyrene</td>
</tr>
<tr>
<td>TEMED</td>
<td>Tetramethylethylenediamine</td>
</tr>
<tr>
<td>T₉</td>
<td>Glass transition temperature</td>
</tr>
<tr>
<td>TGA</td>
<td>Thermogravimetric analysis</td>
</tr>
<tr>
<td>Tₘ</td>
<td>Melting temperature</td>
</tr>
<tr>
<td>T₀</td>
<td>Onset degradation temperatures</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
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</tbody>
</table>
Chapter 1

1 Introduction

1.1 Biodegradable Polymers

In the past few decades, significant advancements have been made in the development of biomaterials to try to achieve better outcomes in their biomedical applications. Materials ranging from metals and ceramics to glasses and polymers have been explored for their potential as biomaterials.\textsuperscript{1,2} Polymers are one of the most promising classes of biomaterials due to their relative ease of chemical modification that provides a high diversity of physical and mechanical properties.\textsuperscript{1} Specifically, biodegradable polymers show great potential in the biomedical field, as these materials can be broken down and metabolized or excreted, eliminating the requirement for removal by surgical procedures if a temporary implant is required.\textsuperscript{1,2} This class of polymers can be degraded enzymatically or through non-enzymatic hydrolysis. Hydrolytically-degradable polymers contain specific chemical groups, such as anhydrides, esters, amides or ureas.\textsuperscript{3} Enzymatically-degradable polymers often consist of naturally occurring monomers, such as amino acids, monosaccharides, glycolic acid or lactic acid.\textsuperscript{3} However, several factors need to be taken into consideration regarding the degradation of polymers, including their molar mass, hydrophilicity, crystallinity, and the pH of the surrounding medium.\textsuperscript{4} Biodegradable polymers can be developed from natural or synthetic polymers.

1.1.1 Biopolymers

Natural biodegradable polymers are referred to as biopolymers, such as collagen and chitosan, which are polymers formed by living organisms (Figure 1.1).\textsuperscript{5} Collagen is the most abundant protein found in the human body.\textsuperscript{6} It has a triple helical structure made from three polypeptide $\alpha$ chains, with each $\alpha$ chain composed of the repeating units of glycine-X-Y, where X and Y are commonly proline and 4-hydroxyproline, respectively.\textsuperscript{7} Collagen can be degraded enzymatically by matrix metalloproteinases (MMPs).\textsuperscript{8} Moreover, it has been reported to be used in a variety of biomedical applications, including as a scaffold in tissue engineering.\textsuperscript{1,6} For instance, collagen-based scaffolds have been developed for skin engineering due to their ability to support cellular
adhesion and proliferation. In contrast, chitosan is the deacetylated derivative of chitin, found in the shells of arthropods, which has a structure of a linear polysaccharide composed of \(N\)-glucosamine and \(N\)-acetyl-glucosamine. Chitosan can be degraded \textit{in vitro} by a number of enzymes including chitosanase, lysozyme and papain, with lysozyme as the primary degrading enzyme \textit{in vivo}. Furthermore, chitosan is capable of water absorption, oxygen permeability and facilitating haemostasis, the process of stopping bleeding; therefore it has been heavily investigated as a wound dressing material. However, biopolymers present limitations in terms of tuning their mechanical and chemical properties, whereas, synthetic polymers offer the advantage of property control as their structures can be more easily modified.

**Figure 1.1.** Chemical structures of a) collagen and b) chitosan.

### 1.1.2 Synthetic Polymers

To design a biodegradable biomaterial suitable for biomedical applications, it should present the following properties: 1) not cause an inflammatory or toxic response, 2) have a degradation time matching with the requirements for the target application, 3) produce non-toxic degradation products that can be metabolized or excreted from the body, 4) possess suitable mechanical
properties for the intended applications, and 5) have suitable permeability and processability for the indicated application.\textsuperscript{16} A main challenge for developing such materials is the safety of the degradation products coming from the biodegradable polymers. This barrier has motivated interest in creating synthetic polymeric biomaterials such as poly(\(\alpha\)-hydroxy acid)s and poly(amino acid)s, which are derived from natural metabolites in the human body.\textsuperscript{17}

In the context of biomaterials, poly(\(\alpha\)-hydroxy acid)s have been extensively investigated. The commonly used poly(\(\alpha\)-hydroxy acid)s in biomedical applications are poly(glycolic acid) (PGA) and poly(lactic acid) (PLA) (Figure 1.2).\textsuperscript{17} PGA has a high crystallinity of 45-55\%, and thus exhibits a high Young’s modulus with low solubility in most organic solvents.\textsuperscript{18} It has a glass transition temperature (T\(_g\)) of 35-40 °C, which is defined as the temperature that the polymer changes from a hard, glassy material to a soft, rubbery material.\textsuperscript{19} It also has a high melting point (T\(_m\)) greater than 200 °C.\textsuperscript{19} Due to its favourable properties, PGA was utilized to develop the first biodegradable suture in 1969 under the trade name of DEXON\textsuperscript{®}.\textsuperscript{20} On the other hand, the monomer of PLA is a chiral molecule and it polymerizes into four different morphological forms: poly(L-lactic acid) (PLLA), poly(D-lactic acid) (PDLA), the racemic poly(D,L-lactic acid) (PDLLA) and meso-PLA.\textsuperscript{21} However, among these four forms, only PLLA and PDLLA demonstrated potential in the biomedical field due to their properties.\textsuperscript{1} PLLA possesses a T\(_g\) of 60-65 °C and a T\(_m\) of about 175 °C.\textsuperscript{18} It is a hard, crystalline polymer with a high Young’s modulus at approximately 4.8 GPa, which motivated its investigation in load bearing applications including orthopaedic fixation devices.\textsuperscript{18} Meanwhile, PDLLA, is an amorphous polymer that has a T\(_g\) of 55-60 °C with a lower Young’s modulus of about 1.9 GPa and a faster degradation rate than PLLA.\textsuperscript{19} These features have made it a better choice for drug delivery applications.\textsuperscript{22}

![Chemical structures of poly(glycolic acid) and poly(lactic acid).](image)

**Figure 1.2.** Chemical structures of a) poly(glycolic acid) and b) poly(lactic acid).

Poly(amino acid)s are attractive candidates for biomedical applications due to their structural similarities with proteins. The use of amino acids offers properties including supporting cell-
material interactions, the possibility to functionalize the pendant groups to allow for drug attachment, and enzymatic biodegradation leading to metabolically degradable building blocks.\textsuperscript{23} Nevertheless, despite the biological advantages, many poly(amino acid)s have not been able to progress towards clinical applications due to their high crystallinities, low degradation rates, unfavourable mechanical properties, and immunogenicities.\textsuperscript{24} To translate the advantages of amino acids and to overcome the limitations of their polymer forms, amino acids have been integrated into the backbone of several types of polymers including poly(ester amide)s (PEAs) which will be discussed in detail below.\textsuperscript{23,25}

1.2 Poly(ester amide)s

PEAs are a class of biodegradable polymers that can be prepared from a number of different monomer components, and they have been shown to exhibit suitable properties for a diverse range of biomedical applications.\textsuperscript{26-28} By definition, they are polymers containing ester and amide linkages that both contribute to biodegradability, while the amide linkages can provide enhanced mechanical and thermal properties relative to polyesters.\textsuperscript{26,27} Depending on the selection of monomers, different types of PEAs can be produced, such as polydesipeptides, copolymers consisting of $\alpha$-hydroxy acids and $\alpha$-amino acids; PEAs formed from diamide-diol, diester-diamine, diamide-diester and ester-diamine monomers with dicarboxylic acids derivatives or diols; and $\alpha$-amino acid-based PEAs comprising $\alpha$-amino acids, diols and dicarboxylic acids (Figure 1.3).\textsuperscript{26,27} In particular, $\alpha$-amino acid-based PEAs have attracted much attention in the field of biomedical research. The incorporation of amino acids into the PEA backbone introduces favourable biological properties that are often lacking in synthetic polymers.\textsuperscript{23} In addition, the individual components of these PEAs offer flexibility in tuning their chemical, biological and mechanical properties as they can be easily modified to achieve properties required for different applications.\textsuperscript{26,28}
Figure 1.3. Chemical structures of different types of PEAs: a) polydesipeptides and PEAs derived from monomers of b) diamide-diol, c) diester-diamine, d) diamide-diester and e) ester-diamine, and f) amino acid-based PEAs.

1.2.1 PEA Synthesis Methods

PEAs derived from α-amino acids can be synthesized by melt polycondensation, solution polycondensation and interfacial polymerization. Melt polycondensation is a two-step reaction that first involves the formation of a diamide-diester under mild conditions, followed by the reaction between the synthesized diamide-diester with a diol at a temperature over 200 °C with vacuum to favour the condensation process and to achieve a high molar mass polymer (Scheme 1.1). This procedure is considered to be advantageous for industrial production as polymer purification is typically not required. However, the disadvantages of this method include the required purification of diamide-diester, as well as the use of high reaction temperatures that can cause undesirable side reactions if the monomers are not pure or have functional side groups.
Scheme 1.1. Synthesis of amino acid-based PEAs by melt polycondensation: a) formation of diamide-diester monomer and b) melt polycondensation between diamide-diester monomer and diol.

Solution polycondensation, on the other hand, was developed to resolve the undesirable side reactions of melt polycondensation reactions and utilizes mild reaction conditions. This method is also known for generating high polymerization rates and high molar masses with minimal side reactions. There are three steps for this synthetic route (Scheme 1.2), including: 1) preparation of an activated ester from either dicarboxylic acids or diacyl chloride; 2) preparation of bis-α-(L-amino acid)-α, ω-alkylene diesters (BAAD) from α-amino acids and diols through Fischer esterification, in the presence of p-toluenesulfonic acid monohydrate (p-TSA), which catalyzes the reaction and protects the amino group as its salt; and 3) polymerization of the two prepared monomers at a temperature less than 80 °C. The drawbacks of this method include the requirements of pure monomers and careful control of stoichiometric balance in order to produce high molar mass PEAs, as well as the necessity to purify the polymer.
Scheme 1.2. Synthesis of amino acid-based PEAs by solution polycondensation: a) formation of an activated ester monomer, b) formation of BAAD monomer and c) solution polycondensation between the activated ester monomer and BAAD monomer.

While melt and solution polycondensations occur in a single phase, interfacial polymerization occurs at the interface of two immiscible phases that are typically water with an organic solvent. This method is a two-step reaction that involves the preparation of BAAD in the presence of \( p \)-TSA, and then the reaction of the synthesized monomer in the aqueous phase with a diacyl chloride in an organic solvent, often at room temperature to yield the desired polymer (Scheme 1.3). Typically, an inorganic base is used to neutralize the generated hydrogen chloride in order to prevent unwanted side reactions. Diacyl chlorides with long aliphatic or aromatic chains must be used to ensure their highly-preferential partitioning into the organic phase to avoid hydrolysis of the respective compound to its unreactive diacid form. Finally, the organic solvent must be carefully selected to allow for precipitation of the polymer at the targeting molar mass. Out of the three methods, solution polycondensation is the most commonly used synthetic route to prepare \( \alpha \)-amino acids-based PEAs, as it favours high polymerization rates under mild conditions.
Scheme 1.3. Synthesis of amino acid-based PEAs by interfacial polymerization: a) formation of BAAD monomer and b) interfacial polymerization between BAAD monomer and diacyl chloride.

1.2.2 Application of PEAs in Tissue Engineering

PEAs based on α-amino acids have been demonstrated by various research groups to exhibit low cytotoxicity.34–39 Given their biodegradability, they have become popular choices to be applied in tissue engineering. For instance, the Gillies and Mequanint groups have prepared a series of PEAs containing L-phenylalanine and L-alanine, as well as analogues of these PEAs with pendant functional groups through the incorporation of L-lysine by both solution and interfacial polymerizations.34 It was demonstrated that the PEAs were able to support human coronary artery smooth muscle cells (HCASMCs) attachment, spreading and proliferation, which makes them promising materials for vascular tissue engineering scaffolds. In a subsequent study, these amino acid-based PEAs were processed into ultrathin films that were shown to promote the formation of focal adhesions in HCASMCs seeded on the film surfaces.35 These findings indicated that these PEAs can promote integrin signalling, which is important for cell survival, migration and proliferation, and further expands their potential as scaffolds for vascular tissue engineering.

Horwitz et al. synthesized PEAs deriving from L-lysine to provide pendant amines or L-aspartic acid to provide pendant carboxylic acids, and a neutral PEA (L-phenylalanine) and studied their ability to support bovine aortic endothelial cells (BAECs) viability, proliferation and adhesion, as well as their inflammatory response in vitro.36 It was found that all three forms of PEAs were
noncytotoxic and did not induce the activation of J774 mouse peritoneal macrophages. The results also demonstrated that the amino-functionalized PEA better supported BAECs adhesion, growth and monolayer formation as compared to the other two forms. These results suggested the potential of the PEAs to be used in tissue engineering as vascular grafts.

Furthermore, Cui and coworkers have developed a copolymer of PEAs based on L-leucine and L-glutamic acid with electroactive tetraaniline that had good electroactivity, mechanical properties and biodegradability. The study revealed that the tetraaniline-grafted PEAs were noncytotoxic and supported the adhesion and proliferation of mouse preosteoblastic MC3T3-E1 cells. These copolymers were also found to promote the differentiation of MC3T3-E1 cells when stimulated by pulsed electrical signal, which suggested their potential for application in vivo as a bone repair scaffold material in tissue engineering.

1.3 Hydrogels in Biomedical Applications

Hydrogels are defined as three-dimensional networks generated from hydrophilic polymers with the capability of absorbing large amounts of aqueous fluid. They have become attractive materials since the 1960 discovery made by Wichterle and Lim that poly(2-hydroxyethyl methacrylate)-based hydrogels could be used in soft contact lens. Since then, there has been an enormous growth in the use of hydrogels in biomedical applications including drug/gene delivery, wound healing and tissue engineering. For example, a hydrogel bead composed of N-succinyl chitosan/alginate has been reported for the controlled release of nifedipine. Hydrogel dressings derived from both natural (e.g. gelatin or agar) and synthetic (e.g. polyacrylamide or polyvinylpyrrolidone) polymers have also been established to be used for wound care. Moreover, a number of biodegradable hydrogels have been found to support cell and tissue growth. Overall, there is great versatility in the design of hydrogels for a variety of applications, hence they play a vital role in the field of biomedical research.

1.3.1 Crosslinking Methods

Hydrogel networks can be generated through physical or chemical crosslinking mechanisms. Physically crosslinked hydrogels are formed by hydrogen bonding, hydrophobic interactions and ionic interactions. However, the network of these hydrogels can be disrupted due to their
relatively weak, non-covalent bonds.\textsuperscript{54} Covalently crosslinked hydrogels, on the other hand, typically exhibit the properties of stronger, more permanent networks.\textsuperscript{54} There are several approaches that can be used to form covalently crosslinked hydrogels, including photopolymerization, azide-alkyne cycloaddition and Diels-Alder cycloaddition.\textsuperscript{55-57} Photopolymerization is one of the most widely used methods in biomedical applications due to the potential to have rapid formation of a crosslinked network at room temperature with spatial and temporal controls during the reaction process.\textsuperscript{58} Photopolymerization is initiated by light and requires the presence of unsaturated groups to undergo a free radical chain-growth polymerization when exposed to photo irradiation (Figure 1.4).\textsuperscript{59} Moreover, the formation of photo-crosslinked hydrogels often employs the use of photoinitiators (e.g. Irgacure 2959) to start polymerization with ultraviolet (UV) absorption ranging between 300-400 nm.\textsuperscript{59,60}

\textbf{Figure 1.4.} Photo-crosslinking mechanism initiated by Irgacure 2959: a) formation of radical from Irgacure 2959 upon UV absorption and initiating b) polymerization to form a crosslinked network.

\subsection*{1.3.2 Hydrogel Design in Tissue Engineering}

Hydrogels are commonly used scaffolds for tissue engineering due to their high water content resembling that of soft tissues, highly tunable structures, high permeability to oxygen, nutrients and other water soluble metabolites, as well as their ability to allow for minimally invasive cell delivery at the target site.\textsuperscript{61,62} Hydrogel networks can derive from either natural or synthetic polymers. Natural polymers (e.g. collagen) possess desirable properties for tissue engineering,
including inherent biodegradability and bioactivity.\textsuperscript{61} Nevertheless, hydrogels derived from natural polymers can exhibit poor mechanical properties and can potentially cause immunogenic reactions.\textsuperscript{63,64} On the other hand, synthetic polymers offer the advantage of tailored properties due to their ease of structural modifications. For instance, the Chu group has developed water soluble cationic PEAs based on L-arginine (Arg) with varying methylene groups in the diol or diacid segment, and found that the difference in structures can influence the overall properties of the polymer (Figure 1.5).\textsuperscript{38} In a subsequent study, a hybrid hydrogel was generated through photo-crosslinking of the Arg-PEA with poloxamer (a triblock copolymer consisting of one poly(propylene oxide) and two poly(ethylene glycol)) diacrylate to investigate if the addition of Arg-PEA improved cellular interactions as compared to the pure poloxymer hydrogel.\textsuperscript{39} The study showed that the hybrid hydrogel enhanced the attachment and proliferation of Detroit 539 human fibroblast cells, as well as the viability of encapsulated BAECs, indicating that the introduction of Arg-PEA positively influenced the cell behavior within the system. Unlike natural polymers, synthetic polymers can also provide more reproducible physical and chemical properties, which is important for scaffold fabrication in tissue engineering.\textsuperscript{61}

![Figure 1.5. Chemical structure of Arg-PEA.](image)

Although property tunability from synthetic hydrogels is a desirable characteristic, they present a major limitation as tissue-engineering scaffolds, which is the lack of biological cues. To improve their biological properties, bioactive molecules have been used to modify synthetic hydrogels to mediate cell functions.\textsuperscript{65–67} The incorporation of bioactive molecules into the hydrogel network can enhance the biological function of the scaffold, as it mimics the extracellular matrix environment for supporting cell growth.\textsuperscript{61,63} Stile and Healy have developed a peptide-modified hydrogel system from poly(N-isopropylacrylamide) containing the Arg-Gly-Asp (RGD) peptide sequence.\textsuperscript{68} Their study demonstrated that the peptide-modified hydrogel better supported the
proliferation of encapsulated rat calvarial osteoblasts than the unmodified hydrogel. Ultimately, the development of a hybrid hydrogel scaffold containing both biological and synthetic materials is a promising approach for tissue engineering.

1.4 Adipose Tissue Engineering

The currently available methods to restore soft tissue loss caused by disease or injury are autologous fat transplantation and implantable fillers, but they often result in complications including donor site morbidity and volume loss of the injected fat tissue.\textsuperscript{69} To address these issues, the development of adipose tissue engineering serves as a potential strategy for reconstructive and cosmetic plastic surgery in replacing lost or damaged soft tissue within the subcutaneous layer.\textsuperscript{70,71} This method employs a three-dimensional biomaterial scaffold embedded with regenerative cell populations, such as adipose-derived stromal cells (ASCs), that provides a microenvironment composed of biological signals to support cell growth leading to tissue formation.\textsuperscript{72}

1.4.1 Adipose-derived Stromal Cells

ASCs, identified in 2001, are defined as mesenchymal stromal cells (MSCs) found in the adipose tissue that can be differentiated into adipogenic (fat), chondrogenic (cartilage) and osteogenic (bone) lineages in culture.\textsuperscript{73} Their abundance and accessibility are very advantageous for research in tissue engineering, as they can be collected from various sites including breast and abdomen through lipo-suction or lipo-reduction procedures in which the tissues are usually discarded as waste.\textsuperscript{73–75} Upon processing, one gram of adipose tissue can yield up to 500-fold more MSCs than bone marrow aspirate.\textsuperscript{76} In addition, ASCs display immunomodulatory properties, promote angiogenesis, the process of forming new blood vessels, and secrete growth factors that stimulate the recruitment and differentiation of tissue resident cells that can contribute to tissue regeneration.\textsuperscript{77,78} The differentiation of ASCs is directly related to the culturing conditions containing selective lineage-specific induction factors.\textsuperscript{73} Here, we will focus the adipogenic differentiation of ASCs, which is described in the next section.
1.4.1.1 Adipogenesis

Adipogenesis is promoted when ASCs are seeded at high cell density that eventually leads to growth arrest.\(^\text{79}\) This initial growth arrest can be stimulated by the addition of pro-differentiative hormones and cytokines \emph{in vitro}, along with triggering the signaling cascade of adipogenic differentiation (Figure 1.6).\(^\text{80}\) The main transcriptional regulators involved in this differentiation process are members of the CCAAT/enhancer binding protein (\(C/EBP\)) and peroxisome proliferator-activated receptor (\(PPAR\)) families.\(^\text{79,81,82}\) This process starts with the activation of early transcription factors, \(C/EBP\beta\) and \(C/EBP\delta\) in pre-adipocytes, followed by the stimulation of the principal transcription factors of adipogenesis which are \(C/EBP\alpha\) and \(PPAR\gamma\).\(^\text{79,81,82}\) These two transcription factors work together to maintain a positive feedback loop, while \(C/EBP\alpha\) also works to maintain growth arrest as well as to inhibit ASCs proliferation. As the process leads to the terminal differentiation stage, the formation of adipocytes is observed and can be characterized by the expression of adipogenic genes including lipoprotein lipase (\(LPL\)), glycerol-3-phosphate dehydrogenase (\(GPDH\)), glucose transporter type-4 (\(GLUT4\)), fatty acid synthase (\(FAS\)), fatty acid binding protein-4 (\(FABP4\)), insulin receptor, malic enzyme and acetyl CoA carboxylase.

![Figure 1.6. A schematic representation of ASC adipogenic differentiation.](image)

1.4.1.2 Scaffolds for Adipose Tissue Engineering

Both natural and synthetic polymers have been used as scaffolds for adipose tissue engineering. A variety of implantable scaffolds have been developed from different polymers and exhibited positive responses in supporting the adipogenic differentiation of seeded ASCs.\(^\text{83–86}\) For example, Itoi \emph{et al.} have demonstrated the potential of collagen as a scaffold for adipogenesis, with fat formation observed in ASC-seeded collagen sponges at 8 weeks after subcutaneous implantation in nude mice.\(^\text{83}\) Moreover, another group prepared a scaffold from poly(lactic-co-
glycolic) acid (PLGA) and showed that the scaffold promoted the differentiation of seeded ASCs into adipocytes 5 weeks after implantation in Lewis rats.\textsuperscript{84}

Hydrogels are also commonly used as scaffolds for adipose tissue engineering and can offer the advantage that they can be injected at the defect site through minimally invasive means, followed by \textit{in situ} crosslinking, thus minimizing the risks of infections and scarring from implantation.\textsuperscript{72} For instance, Patel \textit{et al.} have found that poly(ethylene glycol) (PEG)-based hydrogels modified with peptides improved ASC adhesion and proliferation as compared to a system with no peptides.\textsuperscript{87} Uriel \textit{et al.} have investigated the potential of adipose protein-derived hydrogels to induce adipogenesis, and observed higher adipose levels than for Matrigel\textsuperscript{TM}, a commercially available basement membranes-based product extracted from mouse sarcoma, both \textit{in vitro} and \textit{in vivo}.\textsuperscript{88} Based on these studies, the design of a composite hydrogel incorporating bioactive molecules that supports cell survival and promotes the differentiation of progenitor cells towards the adipogenic lineage holds potential as a strategy to enhance fat formation for tissue engineering applications.

\section*{1.5 Tissue Decellularization}

The extracellular matrix (ECM) is a complex and dynamic environment containing proteins and polysaccharides.\textsuperscript{89,90} It is crucial in mediating cellular functions including adhesion, migration, viability, proliferation and differentiation. Therefore, cell behavior is highly dependent on the ECM environment, as it not only provides structural support, but also presents the biochemical and biomechanical signals that are important mediators of cellular activities. Moreover, the composition and organization of the ECM is tissue-specific, as the environment is different from one tissue to another. As described in the earlier section, the design of tissue-engineering scaffolds with integrated bioactive moieties to mimic the properties of the natural ECM may direct cellular functions, leading to tissue formation.\textsuperscript{61} Numerous studies have been conducted with the utilization of ECM-derived molecules such as collagen, laminin or fibronectin for the design of bio-scaffolds.\textsuperscript{91–93} However, these systems do not replicate the complexity of the native ECM. As a result, tissue decellularization to isolate tissue-specific ECM using approaches that strive to preserve the structural architecture and biochemical composition of the ECM while
removing cellular contents has emerged as a promising approach for the development of cell delivery vehicles for tissue regeneration.\textsuperscript{94,95}

### 1.5.1 Adipose Tissue Decellularization

A variety of decellularized ECMs have been prepared from different tissue types, including adipose tissue, dermis and bone.\textsuperscript{96–98} Specifically, decellularized adipose tissue (DAT) has been studied extensively as a biomaterial for adipose tissue regeneration, which can be applied to restore soft tissue loss within the subcutaneous layer.\textsuperscript{99} Upon decellularization, a number of vital proteins and polysaccharides are conserved in the resulting DAT. Components that provide the integrity, structure and function of adipose tissue found in DAT include structural proteins such as collagen types I–VI, adhesive glycoproteins such as laminin and fibronectin, glycosaminoglycans and proteoglycans.\textsuperscript{100} A number of growth factors are also found in the DAT that are associated with regulating adipocyte differentiation, angiogenesis and homeostasis upon stimulation including fibroblast growth factor 2 (FGF2), insulin like growth factor 1 (IGF1) and vascular endothelial growth factor (VEGF).\textsuperscript{100}

DAT is obtained after a decellularization procedure involving a series of physical, chemical and enzymatic treatments. Particularly, the Flynn lab has developed a novel detergent-free method for adipose tissue decellularization that successfully preserves the essential elements of the adipose tissue ECM.\textsuperscript{96} Within the protocol, physical treatments employ the use of freeze-thaw cycles for lysing the cells, and the combination of hydrostatic pressure with hypotonic solutions to further break down the cells.\textsuperscript{96,101} The chemical treatments involve the use of isopropanol washes to remove lipids from the adipose tissue, while the enzymatic steps utilize trypsin, a serine protease, and ethylenediaminetetraacetic acid (EDTA), a chelating agent that disrupts cell-cell and cell-ECM binding, along with the use of nucleases (DNase and RNase) to cleave nucleic acids.\textsuperscript{96,101} There are other groups that have reported the use of detergents such as sodium dodecyl sulphate (SDS) and Triton X-100 in their adipose decellularization protocols to extract cellular contents.\textsuperscript{102,103} However, a concern associated with the use of detergent is the potential cytotoxicity resulting from residual detergents remaining within the tissues, in which is not an issue for the detergent-free protocol.\textsuperscript{104}
As the development of decellularized ECM bio-scaffolds has become an attractive approach for regenerative research, several studies have demonstrated the instructive effects of DAT on the lineage-specific differentiation of ASCs. For example, the Flynn group has shown that DAT-derived scaffolds, microcarriers and foams provide an inductive effect by promoting the adipogenic differentiation of ASCs both in vitro and in vivo. Wang and coworkers also found that the injection of DAT-based microparticles containing ASCs into an engineered fat graft implanted in a Fischer rat model promoted fat formation at 8 weeks. In addition, further processing of the DAT into micronized particles allows the ECM to be incorporated into hydrogel carriers. The Flynn lab has previously demonstrated that composite hydrogels derived from methacrylated chondroitin sulphate (MCS) with incorporated DAT particles enhanced the adipogenic differentiation of encapsulated ASCs relative to MCS controls. Overall, these findings show the promising role of decellularized tissue-specific ECM in stem cell differentiation and ultimately, in tissue regeneration.

1.6 Project Motivation, Objective and Hypothesis

1.6.1 Project Motivation

PEAs have been studied for their potential in biomedical applications including tissue engineering due to their biodegradability and the ease of tuning their structure and properties. However, only a few examples of PEA hydrogels for cell encapsulation have been reported due to the challenges of obtaining water soluble PEAs. These PEAs are cationic and difficult to reproducibly synthesize based on past experience in our lab. Moreover, although it has been demonstrated that PEAs exhibit low cytotoxicity and support various cell types, the behavior of ASCs in PEA systems has not yet been investigated. Given the potential cell-instructive properties of DAT and its ability to be incorporated into hydrogels when processed into particles, here we propose the design of hybrid hydrogels based on PEAs and DAT that will offer the structural control of synthetic polymers and the biological cues of natural materials to support and control the differentiation of ASCs.
1.6.2 Project Objective and Hypothesis

The goals of this thesis were to develop water soluble PEAs with crosslinkable moieties for the fabrication of hydrogel carriers for ASC encapsulation, and to study the potential of the PEA systems with and without DAT as a cell-instructive scaffolds for ASC delivery for applications in adipose tissue engineering.

The hypothesis for this project is that PEAs derived from L-phenylalanine and L-alanine will support the viability of encapsulated ASCs and the incorporation of DAT into the PEA hydrogels will enhance cell viability and adipogenic differentiation in culture.

Chapter 2 describes the detailed procedures for the development of hydrophilic PEAs, followed by hydrogel fabrication using these PEAs through photo-crosslinking. After the hydrogels were characterized, DAT particles were incorporated into these polymeric scaffold systems and their properties were studied. In addition, human ASCs were encapsulated into the PEA systems with and without DAT incorporation (Figure 1.7), and ASC viability and adipogenic differentiation within these scaffolds were studied.

![Figure 1.7](image)

**Figure 1.7.** Schematic representation of photo-crosslinking of PEAs with the incorporation of DAT and ASC encapsulation.

Chapter 3 summarizes the key results obtained from the previous chapter and discusses the future directions of this project.
1.7 References


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Chapter 2

2 Development of Poly(ester amide)-based Hydrogels for Adipose-derived Stromal Cell Encapsulation

2.1 Introduction

The development of biodegradable polymers as biomaterials has become an attractive approach in the biomedical field. While the chemical flexibility of polymers allows researchers to create materials with a wide range of properties, biodegradable polymers provide the additional advantage of being able to be broken down and absorbed or excreted, eliminating the need for removal by surgical procedures.\textsuperscript{1,2} However, the design of biodegradable biomaterials comes with many challenges, as the materials should possess a number of essential properties required for biomedical applications.\textsuperscript{3} Firstly, the material should be nontoxic and should not cause a negative host response. Secondly, the material should be tailored to degrade once its function is complete and the degradation products should be nontoxic. Next, the material should have mechanical properties tuned for the targeted application. Finally, the material should also have the appropriate permeability and processability for the intended use.

Poly(ester amide)s (PEAs) are a class of biodegradable polymers containing ester and amide linkages in their backbones. They often possess properties of both polyesters and polyamides, including the biodegradability of polyesters and high mechanical strength and thermal stability of polyamides.\textsuperscript{4,5} Specifically, PEAs derived from amino acids, diols and dicarboxylic acids serve as promising materials for biomedical applications. The incorporation of amino acids into the PEA backbone introduces favorable biological properties, such as supporting cell-material interactions, and enzymatic biodegradation leading to metabolizable building blocks.\textsuperscript{6} As the properties of these polymers can be easily tailored by chemical modifications, a number of amino acid-based PEAs have been developed to be applied in a variety of applications.\textsuperscript{4,5,7} For instance, the Gillies and Mequanint groups demonstrated that PEAs containing L-phenylalanine, L-alanine and L-lysine were able to support human coronary artery smooth muscle cell attachment, spreading and proliferation, making them promising materials for vascular tissue engineering scaffolds.\textsuperscript{8} del Valle \textit{et al.} studied the controlled release of ibuprofen from PEAs.
based on L-alanine, sebacic acid and 1,12-dodecanodiol. In addition, the Chu group has developed a water soluble PEA formulation from cationic L-arginine that has been explored for applications including gene delivery and wound healing. To the best of our knowledge, this is the only reported water soluble, amino acid-based PEA reported to date. Hence, further research on the development of water soluble PEAs is warranted to expand our knowledge on their material properties and explore their applications for cell delivery and tissue engineering.

Tissue engineering for tissue regeneration and treating diseases aims to regenerate damaged tissues using scaffold biomaterials in combination with cells. However, the lack of cell delivery platforms that ensure the retention, survival and maintenance of cellular function over the longer term remains a significant challenge. Hydrogels are commonly used as scaffolds for tissue engineering due to their high water content resembling that of soft tissue, structural tunability, high permeability to oxygen, nutrients and other water soluble metabolites, and in some cases their ability to allow for minimally-invasive cell delivery at the target site.

In recent years, the use of bioactive molecules in the design of hydrogels has gained attention with the focus on improving the biological function of the scaffolds. Hydrogels derived from synthetic polymers lack the biological cues to guide the behavior of encapsulated cells, and the addition of bioactive molecules can help to mimic the extracellular matrix (ECM) environment and mediate cellular functions. However, the ECM is a highly complex and dynamic environment containing a diverse array of extracellular macromolecules, and it is a significant challenge to mimic the complexity of the native ECM environment through the incorporation of bioactive molecules within the scaffold. A promising alternative approach is to incorporate decellularized tissue-specific ECM into synthetic scaffold systems as it has been reported to enhance cell viability and help to direct cell function. Specifically, the Flynn group and others have demonstrated that biomaterials derived from decellularized adipose tissue (DAT) can enhance the viability of adipose-derived stem cells (ASCs) and promote the adipogenic differentiation of ASCs both in vitro and in vivo. These findings show great promise for the role of decellularized tissue-specific ECM in stem cell differentiation and ultimately, in tissue regeneration.
Here we proposed the development of a new water soluble PEA with crosslinkable moieties to enable \textit{in situ} gelation for hydrogel formation and cell encapsulation. The water solubility was achieved by the incorporation of poly(ethylene glycol) (PEG) blocks into the L-phenylalanine-based and L-alanine-based PEAs at a specific molar ratio. The properties of the hydrogels fabricated from pure PEAs, as well as those synthesized with crosslinker groups or DAT particles were studied. The potential of these PEA systems as biomaterials for adipose tissue regeneration was investigated by evaluating ASC attachment and viability following encapsulation within the scaffolds, along with determining whether the PEAs-based scaffolds supported the differentiation of ASCs towards the adipogenic lineage both with and without DAT incorporation.

2.2 Experimental

2.2.1 General Experimental Details

Poly(ethylene glycol) (2000 g/mol) and \textit{p}-nitrophenol were purchased from Alfa Aesar (Haverhill, MA, USA). L-Alanine, 4-dimethylaminopyridine (DMAP) and \textit{p}-toluenesulfonyl chloride were purchased from AK Scientific Inc. (Union City, CA, USA). Chondroitin sulphate (50,000 g/mol) was purchased from LKT Laboratories (St. Paul, MN, USA). \textit{NH}_4\textit{OH} and \textit{MgSO}_4 were purchased from Caledon Laboratories (Georgetown, ON, Canada). Toluene was distilled over sodium and benzophenone under nitrogen at atmospheric pressure to remove water before use. Triethylamine (Et_3N), dichloromethane (CH_2Cl_2) and \textit{N,N}-dimethylacetamide (DMA) were distilled over calcium hydride under nitrogen at atmospheric pressure to remove water before use. All other chemicals were purchased from Sigma Aldrich (St. Louis, MO, USA). All solvents and chemicals were used as received unless otherwise noted.

Dialysis was performed using Spectra/Por 6 dialysis tubing from Spectrum Laboratories (Rancho Dominguez, CA, USA) with molecular weight cutoff (MWCO) of 10 kg/mol or 3.5 kg/mol. Size exclusion chromatography (SEC) was performed using an instrument equipped with a Waters 515 HPLC pump, a Waters In-Line Degasser AF, two PLgel mixed D 5\textmu m (300 x 1.5 mm) columns connected to a corresponding PLgel guard column, and a Wyatt Optilab Rex RI detector operating at 658 nm. Samples were dissolved in \textit{N,N}-dimethylformamide (DMF) containing
10 mM LiBr and 1% v/v Et$_3$N at a concentration of ~5 mg/mL. Each sample was filtered through a 0.22 µm polytetrafluoroethylene (PTFE) syringe filter prior to injection using a 50 µL loop. Samples were run at a flow rate of 1 mL/min for 30 min at 85 °C. Molar mass calibrations were carried out using poly(methyl methacrylate) (PMMA) standards. Thermogravimetric analysis (TGA) was performed on a TA Q50 instrument with a heating rate of 10 °C/min up to a maximum temperature of 1000 °C under nitrogen. Differential scanning calorimetry (DSC) was performed on a TA Q2000 instrument with a heating/cooling rate of 10 °C/min under nitrogen. $T_m$ was obtained from the second heating cycle. $^1$H and $^{13}$C nuclear magnetic resonance (NMR) spectra were recorded on either a 400 HMz Bruker AvIII HD instrument or a 600 MHz Varian INOVA instrument in CDCl$_3$, D$_2$O or DMSO-d$_6$. Chemical shifts are reported in parts per million (ppm) with the solvent signals as reference. Fourier-transform infrared (FT-IR) spectra were obtained using a PerkinElmer FT-IR Spectrum Two instrument with attenuated total reflectance (ATR) sampling.

2.2.2 Synthesis of Monomers

2.2.2.1 Synthesis of di-$p$-toluenesulfonic acid salt monomer, 1a

The compound was synthesized as previously reported$^{31}$ A suspension of L-phenylalanine (12 g, 73 mmol, 2.2 equiv.) with $p$-toluenesulfonic acid monohydrate (15 g, 79 mmol, 2.4 equiv.) in dry toluene (120 mL) was refluxed and stirred at 130 °C in a flask equipped with a Dean-Stark trap for 2 h to remove the residual water. 1,4-butanediol (2.9 mL, 33 mmol, 1.0 equiv.) was then added to the solution and refluxed for 48 h. The resulting material was filtered and recrystallized from water (300 mL) following its hot filtration. The product was then collected by vacuum filtration and dried in vacuo to yield a light yellow powder. Yield: 60%. $^1$H NMR (600 MHz, DMSO-d$_6$): $\delta$ 8.40 (br s, 5H), 7.48 (d, 4H, $J = 7.4$ Hz), 7.33-7.23 (m, 10H), 7.12 (d, 4H, $J = 7.8$), 4.30 (t, 2H, $J = 6.9$), 3.99 (s, 4H), 3.15-3.00 (m, 4H), 2.29 (s, 6H), 1.39-1.28 (m, 4H). Spectral data agreed with those previously reported.

2.2.2.2 Synthesis of di-$p$-toluenesulfonic acid salt monomer, 1b

The compound was synthesized as previously reported$^{31}$ A suspension of L-phenylalanine (5.0 g, 30 mmol, 2.2 equiv.) with $p$-toluenesulfonic acid monohydrate (6.8 g, 36 mmol, 2.4 equiv.) in
dry toluene (110 mL) was refluxed and stirred at 130 °C in a flask equipped with a Dean-Stark trap for 2 h to remove the residual water. 1,8-octanediol (2.0 g, 15 mmol, 1.0 equiv.) was then added to the solution and refluxed for 48 h. The resulting material was filtered and recrystallized from water (100 mL) following its hot filtration. The product was then collected by vacuum filtration and dried in vacuo to yield a white powder. Yield: 70%. 1H NMR (600 MHz, DMSO-d6): δ 8.43 (br, s, 4H), 7.50 (d, 4H, J = 7.9 Hz), 7.34-7.20 (m, 10H), 7.13 (d, 4H, J = 8.2 Hz), 4.25 (dd, 2H, J1 = 7.7 Hz, J2 = 6.1 Hz), 4.04-3.98 (m, 4H), 3.16-2.98 (m, 5H), 2.28 (s, 6H), 1.44-1.37 (m, 4H), 1.17-1.05 (m, 8H). Spectral data agreed with those previously reported.

2.2.2.3 Synthesis of di-\textit{p}-toluenesulfonic acid salt monomer, 1c

The compound was synthesized as previously reported.\textsuperscript{31} A suspension of L-alanine (5.0 g, 57 mmol, 2.2 equiv.) with \textit{p}-toluenesulfonic acid monohydrate (11 g, 62 mmol, 2.4 equiv.) in dry toluene (100 mL) was refluxed and stirred at 130 °C in a flask equipped with a Dean-Stark trap for 2 h to remove the residual water. 1,8-octanediol (3.7 g, 26 mmol, 1.0 equiv.) was then added to the solution and refluxed for 48 h. The resulting material was filtered and recrystallized from isopropanol (30 mL) following its hot filtration. The product was then collected by vacuum filtration and dried in vacuo to yield a white powder. Yield: 68%. 1H NMR (600 MHz, DMSO-d6): δ 8.27 (br s, 6H), 7.49 (d, 4H, J = 8.1 Hz), 7.12 (d, 4H, J = 7.8 Hz ), 4.19-4.08 (m, 6H), 2.29 (s, 6H), 1.62-1.58 (m, 4H), 1.38 (d, 6H, J = 7.2 Hz), 1.34-1.26 (m, 8H). Spectral data agreed with those previously reported.

2.2.2.4 Synthesis of di-\textit{p}-nitrophenyl fumarate monomer, 2

The compound was synthesized as previously reported.\textsuperscript{32} A solution of \textit{p}-nitrophenol (8.3 g, 60 mmol, 2.0 equiv.) and Et\textsubscript{3}N (8.4 mL, 60 mmol, 2.0 equiv.) in acetone (100 mL) was cooled to -78 °C using a dry ice/acetone bath. Fumaryl chloride (3.2 mL, 30 mmol, 1.0 equiv.) in acetone (40 mL) was added dropwise over a period of 2 h. The reaction mixture was then allowed to reach room temperature and stirred overnight. The resulting solution was precipitated in water (800 mL), and then filtered, washed and dried under high vacuum. The crude product was then purified by recrystallizing in acetonitrile (400 mL) to yield a white solid. Yield: 60%. 1H NMR
(600 MHz, DMSO-d$_6$): $\delta$ 8.37 (d, 4H, $J = 9.1$ Hz), 7.60 (d, 4H, $J = 9.1$ Hz), 7.27 (s, 2H). Spectral data agreed with those previously reported.

### 2.2.2.5 Synthesis of PEG ditosylate

PEG (2000 g/mol, 40 g, 40 mmol, 1.0 equiv.) was dried *in vacuo* with P$_2$O$_5$ at 80 °C overnight before dissolving in CH$_2$Cl$_2$ (150 mL). Et$_3$N (25 mL, 180 mmol, 4.5 equiv.) and DMAP (2.4 g, 20 mmol, 0.5 equiv.) were added to the solution, and then it was cooled to 0 °C upon addition of $p$-toluenesulfonyl chloride (34 g, 180 mmol, 4.5 equiv.). The reaction mixture was then allowed to reach room temperature and stirred overnight. The resulting solution was filtered to remove salts. It was then washed with 1 M HCl (2 x 40 mL) and the collected aqueous layer was washed with CH$_2$Cl$_2$ (2 x 100 mL). The combined organic layer was dried over magnesium sulphate (MgSO$_4$), concentrated and precipitated in cold ethyl ether (400 mL). The product was then collected by vacuum filtration and dried *in vacuo* to yield a white, tacky solid. Yield: 75%. $^1$H NMR (600 MHz, CDCl$_3$): $\delta$ 7.81 (d, 4H, $J = 8.3$ Hz), 7.35 (d, 4H, $J = 8.0$ Hz), 4.16 (t, 4H, $J = 4.2$ Hz), 3.65 (s, 172H), 2.45 (s, 6H). Spectral data agreed with those previously reported.$^{33}$

### 2.2.2.6 Synthesis of PEG diamine macromonomer, 3

PEG ditosylate (30 g, 13 mmol, 1 equiv.) was added to ammonium hydroxide (105 mL, 790 mmol, 61 equiv.), and the solution was stirred at room temperature for 5 days. The solution was then washed with CH$_2$Cl$_2$ (5 x 100 mL). The organic layer was dried over MgSO$_4$, concentrated and precipitated in cold diethyl ether (300 mL). The product was then collected by vacuum filtration and dried *in vacuo* to yield a white powder. Yield: 84%. $^1$H NMR (600 MHz, CDCl$_3$): $\delta$ 3.65 (s, 172H), 3.53 (t, 4H, $J = 5.2$ Hz), 2.88 (t, 3H, 5.2 Hz). Spectral data agreed with those previously reported.$^{33}$

### 2.2.3 Synthesis of Polymers

#### 2.2.3.1 Synthesis of Phe-PEA

Monomer 1a (0.30 g, 0.42 mmol, 0.30 equiv.), monomer 2 (0.50 g, 1.4 mmol, 1.0 equiv.) and macromonomer 3 (2.0 g, 0.98 mmol, 0.70 equiv.) were dissolved with stirring in dry DMA (8.0 mL) at 60 °C. Et$_3$N (0.43 mL, 3.1 mmol, 2.2 equiv.) was added dropwise to the solution, and
then it was stirred at 70 °C for 6 h. The resulting solution was concentrated, re-dissolved in CH₂Cl₂ and precipitated in cold diethyl ether (250 mL). The crude product was purified by dialysis against DMF (500 mL) for 48 h using a 10 kg/mol MWCO membrane. The solution was then concentrated, re-dissolved in CH₂Cl₂ and precipitated in cold diethyl ether (250 mL). The product was centrifuged, the liquid decanted, and then the solid was dried in vacuo to yield a white solid. Yield: 70%. 

1H NMR (400 MHz, DMSO-d₆): δ 8.92-8.87 (m, 0.69H), 8.50-8.46 (m, 1.64H), 7.30-7.19 (m, 4.29H), 6.86-6.83 (m, 2.00H), 4.58-4.52 (m, 0.78H), 3.98 (s, 1.44H), 3.70-3.66 (m, 1.46H), 3.51 (s, 190H). FT-IR: 3510, 3295, 2879, 1739, 1641 cm⁻¹.

13C NMR (100 MHz, DMSO-d₆): δ 171.3, 163.8, 163.6, 136.9, 132.6, 131.8, 129.0, 128.6, 126.6, 69.8, 69.6, 68.9, 53.9, 36.8, 24.4. FT-IR: 3510, 3295, 2879, 1739, 1641, 1547 cm⁻¹. SEC: Mₙ = 19.9 kg/mol, Mₙ = 49.2 kg/mol, D = 2.46, Tₘ = 36 °C.

2.2.3.2 Synthesis of Phe8-PEA

Monomer 1b (0.066 g, 0.084 mmol, 0.30 equiv.), monomer 2 (0.10 g, 0.28 mmol, 1.0 equiv.) and macromonomer 3 (0.40 g, 0.19 mmol, 0.70 equiv.) were dissolved with stirring in dry DMA (2.0 mL) at 60 °C. Et₃N (0.10 mL, 0.61 mmol, 2.2 equiv.) was added dropwise to the solution, and then it was stirred at 70 °C for 6 h. The resulting solution was concentrated, re-dissolved in CH₂Cl₂ and precipitated in cold diethyl ether (100 mL). The crude product was purified by dialysis against DMF (200 mL) for 48 h using a 10 kg/mol MWCO membrane. The solution was then concentrated, re-dissolved in CH₂Cl₂ and precipitated in cold diethyl ether (100 mL). The product was centrifuged, the liquid decanted, and then the solid was dried in vacuo to yield a white solid. Yield: 58%. 

1H NMR (400 MHz, DMSO-d₆): δ 8.89-8.85 (m, 0.69H), 8.50-8.45 (m, 1.58H), 7.29-7.18 (m, 4.23H), 6.85-6.82 (m, 2.00H), 4.56-4.51 (m, 0.66H), 4.00-3.97 (m, 1.36H), 3.69-3.67 (m, 1.25H), 3.51 (s, 187H), 3.07-2.89 (m, 2.70H), 1.46 (s, 1.49H), 1.19 (s, 3.61H). 

13C NMR (100 MHz, DMSO-d₆): δ 163.8, 163.6, 136.9, 133.3, 132.6, 129.0, 128.2, 126.6, 69.8, 69.6, 68.9, 64.6, 54.9, 53.9, 36.7, 28.5, 27.9, 25.2. FT-IR: 3522, 3295, 2879, 1739, 1641, 1547 cm⁻¹. SEC: Mₙ = 20.1 kg/mol, Mₙ = 56.6 kg/mol, D = 2.81, Tₘ = 37°C.
2.2.3.3 Synthesis of Ala-PEA

Monomer 1c (0.26 g, 0.42 mmol, 0.30 equiv.), monomer 2 (0.50 g, 1.4 mmol, 1.0 equiv.) and macromonomer 3 (2.0 g, 0.98 mmol, 0.70 equiv.) were dissolved with stirring in dry DMA (8.0 mL) at 60 °C. Et$_3$N (0.43 mL, 3.1 mmol, 2.2 equiv.) was added dropwise to the solution, and then it was stirred at 70 °C for 6 h. The resulting solution was concentrated, re-dissolved in CH$_2$Cl$_2$ and precipitated in cold diethyl ether (250 mL). The crude product was purified by dialysis against DMF (500 mL) for 48 h using a 10 kDa MWCO membrane. The solution was then concentrated, re-dissolved in CH$_2$Cl$_2$ and precipitated in cold diethyl ether (250 mL). The product was centrifuged, the liquid decanted, and then the solid was dried in vacuo to yield a white solid. Yield: 77%.

$^1$H NMR (400 MHz, DMSO-$d_6$): $\delta$ 8.82-8.78 (m, 0.59H), 8.51-8.45 (m, 1.46H), 6.89-6.83 (m, 2.00H), 4.34-4.29 (m, 0.64H), 4.08-3.97 (m, 1.41H), 3.71-3.65 (m, 1.98H), 3.51 (s, 177H), 1.54 (s, 1.41H), 1.31-1.22 (m, 5.12H). $^{13}$C NMR (100 MHz, DMSO-$d_6$): $\delta$ 172.3, 163.8, 132.6, 69.8, 69.6, 68.9, 64.4, 47.9, 28.5, 28.0, 25.2, 16.9. FT-IR: 3521, 3296, 2880, 1737, 1639, 1546 cm$^{-1}$. SEC: $M_n = 18.9$ kg/mol, $M_w = 56.1$ kg/mol, $\bar{D} = 2.96$, $T_m = 34$ °C.

2.2.4 Synthesis of Crosslinkers

2.2.4.1 Synthesis of PEG-dimethylacrylate

The synthesis of PEG-dimethylacrylate was modified from a previously reported procedure.$^{34}$ PEG (2000 g/mol, 10 g, 10 mmol, 1.0 equiv.), triethylamine (7 mL, 50 mmol, 5.0 equiv.) and DMAP (0.3 g, 2.5 mmol, 0.25 equiv.) were dissolved in CH$_2$Cl$_2$ (20 mL). The solution was then cooled to 0 °C and methacryloyl chloride (5.2 g, 50 mmol, 5.0 equiv.) in CH$_2$Cl$_2$ (10 mL) was added dropwise over a period of 30 min. The reaction mixture was then allowed to reach room temperature and stirred overnight. The resulting solution was filtered to remove salts. It was then washed with water (10 mL) and the combined aqueous layers were washed with CH$_2$Cl$_2$ (2 x 20 mL). The combined organic layers were dried over MgSO$_4$, concentrated, and precipitated in cold diethyl ether (150 mL). The product was then collected by vacuum filtration and dried in vacuo to yield a white, tacky solid. Yield: 57%. $^1$H NMR (600 MHz, CDCl$_3$): $\delta$ 6.12 (s, 2H), 5.57 (s, 2H), 4.45-4.25 (m, 4H), 3.76-3.73 (m, 4H), 3.64 (s, 193H), 1.94 (s, 6H). FT-IR: 2881, 1717 cm$^{-1}$. Spectral data agreed with those previously reported.
2.2.4.2 Synthesis of Methacrylated Chondroitin Sulphate

The compound was prepared as previously reported. Chondroitin sulphate (0.20 g) was added to 0.1 M sodium phosphate monobasic buffer (1.0 mL) and dissolved with stirring. Then, 6 M of NaOH was carefully added to adjust the solution to ∼pH 10 before addition of methacrylic anhydride (60 µL). The reaction was stirred for 1 h at room temperature with constant pH adjustment to maintain a pH of ∼10. The resulting solution was then precipitated in ethanol (10 mL). The product was filtered and purified through dialysis against water (2 L) in 3.5 kg/mol MWCO membrane for 48 h. The final solution was lyophilized for 48 h to yield a white solid. 1H NMR (600 MHz, D2O): 6.19 and 5.75 ppm are the two protons attached to the double bond, and 1.95 ppm is the methyl protons adjacent to double bond. Percentage of methacrylation determined from 1H NMR was 17%. Spectral data agreed with those previously reported.

2.2.5 Human Adipose Tissue Procurement and Processing

Adipose tissue samples were obtained from female patients undergoing elective lipo-reduction surgeries involving the breast or abdomen at the University Hospital and St. Joseph’s Health Care Hospital in London, ON, Canada (HREB 105426). Within 2 h of extraction, the samples were transported to the lab on ice in sterile phosphate buffer saline (PBS) supplemented with 20 mg/mL bovine serum albumin (BSA), and processed for ASC isolation or decellularization using previously reported methods, as described below.

2.2.5.1 Human Adipose-derived Stromal Cell Culture

Human ASCs were isolated from adipose tissue samples within 2 h of procurement using previously published methods. The tissue samples were first minced using surgical scissors, and then added to collagenase digest solution composed of 2 mg/mL collagenase type II (Worthington Biochemical, Lakewood, NJ), 25 mM HEPES (Bioshop Inc., Burlington, ON), 3 mM glucose and 20 mg/mL BSA in Kreb’s Ringer bicarbonate buffer (pH 7.4) to undergo agitation (100 rpm) for 45 min at 37 °C. Next, undigested tissue segments were removed by filtering the sample through a 250 µm stainless steel filter. The collected filtrate was allowed to gravity sediment for 5 min to form two layers. The upper layer of adipocytes was removed by aspiration and left with the fraction containing the adipose precursor cells within the stromal
vascular cell population. Then, an equal volume of proliferation medium comprised of DMEM:Ham’s F-12 medium supplemented with 10% fetal bovine serum (FBS; Thermo Scientific Hyclone, Cat. # SH30396) and 100 U/mL penicillin and 0.1 mg/mL streptomycin (1% pen-strep) was added to the samples to inactivate the collagenase. The samples were centrifuged (1200 xg, 5 min, 25 °C) and resuspended in erythrocyte lysing buffer composed of 0.154 M NH₄Cl, 0.1 mM EDTA and 10 mM KHCO₃ in sterile deionized water. The sample suspension was agitated for 10 min at room temperature. Following agitation, the cells were centrifuged again and resuspended in proliferation medium before filtering through a 100 µm nylon filter (Corning Inc., Corning, NY). Finally, the cells were plated in tissue culture flasks (Corning Inc., Corning, NY) and incubated for 24 h at 37 °C with 5% CO₂, followed by PBS rinses to remove any non-adherent cells or fragments. Fresh proliferation medium was provided every 2-3 days. When the cells reached 80% confluence, they were passaged using 0.25% trypsin/0.1% EDTA (Gibco®, Invitrogen, Burlington, ON) and re-plated in new flasks at 6,000-7,000 cells/cm². Passage 4 (P4) ASCs were used for all cell encapsulation studies, and each study was repeated with three different donors (N = 3). The summary of cell donor information used for each biological study is listed in the Appendix (Table A1).

2.2.5.2 Adipose Tissue Decellularization

Adipose tissue samples were decellularized following an established detergent-free protocol. The decellularization solution volume used in this procedure was based on the ratio of 50 g of tissue per 100 mL of solution. The collected tissue samples were first transferred into hypotonic 10 mM Tris-EDTA buffer (pH 8.0) supplemented with 1% (v/v) antibiotic-antimycotic (ABAM; Gibco®, Invitrogen, Burlington, ON) and 0.27 mM phenylmethylsulfonyl fluoride (PMSF), and then frozen at -80 °C. Next, the tissue samples were thawed at 37 °C under agitation (120 rpm). This was repeated two more times for a total of three freeze-thaw cycles. Following the last freeze-thaw cycle, the tissues were added to enzymatic digestion solution #1 comprised of 0.25% trypsin/0.1% EDTA with 1% ABAM and incubated overnight. Then the samples were subjected to polar solvent extraction for 48 h to remove the lipid content, by transferring into absolute isopropanol supplemented with 1% ABAM and 0.27 mM PMSF. Subsequently, the samples were rinsed three times (30 min each) in Sorenson’s phosphate buffer (SPB; pH 8.0) solution composed of 8 g/L NaCl, 200 mg/L KCl, 1 g/L Na₂HPO₄ and 200 mg/L KH₂PO₄ with 1%
ABAM before 6 h incubation in enzymatic digestion solution #1. Repeated three 30 min rinses in SPB solution was applied to the samples, and then they were incubated overnight in enzymatic digestion solution #2 containing 55 mM Na$_2$HPO$_4$, 17 mM KH$_2$PO$_4$, 4.9 mM MgSO$_4$•7H$_2$O, 15,000 U DNase Type II (from bovine pancreas), 12.5 mg RNase Type III A (from bovine pancreas) and 2000 U lipase Type VI-S (from porcine pancreas) with 1% ABAM. The next day, the samples were rinsed three times (30 min each) in SPB solution, subjected to a final polar solvent extraction for 8 h, and rinsed three times (30 min each) again in SPB solution. The resulting DAT was then rinsed three times (30 min each) in 70% ethanol followed by repeated rinses in sterile deionized water. Finally, the DAT was frozen at -80 °C and lyophilized prior to further processing.

2.2.5.3 Cryo-milling of Decellularized Adipose Tissue

DAT was pooled from three different donors before cryo-milling using previously established protocols. The collected DAT particles were stored at 4 °C until further use. Prior to hydrogel fabrication, the DAT particles were decontaminated in 70% ethanol overnight, and then were rinsed three times consecutively for 1 h in PBS.

2.2.6 Fabrication of Phe-PEA/Ala-PEA based Hybrid Hydrogels

Pure (Phe-PEA and Ala-PEA alone) and hybrid (Phe-PEA/Ala-PEA + PEGDMA/MCS) hydrogels were fabricated without ASCs for physical characterization studies. All hydrogels were made with a prepolymer solution concentration of 10% (m/v) in PBS. For the hybrid hydrogels, the prepolymer solutions contained 7.5% (m/v) of Phe-PEA or Ala-PEA and 2.5% (m/v) of PEGDMA or MCS. The photoinitiator, 2-hydroxy-4’-(2-hydroxyethoxy)-2-methylpropiophenone (Irgacure 2959) was added last to the solution at a concentration of 0.15% (m/v). The prepolymer solution was then transferred to a 1 mL syringe (Thermo Scientific, Waltham, MA, USA), and photo-crosslinked with long-wavelength ultraviolet light (365 nm) at an intensity of 16 mW/cm$^2$ for 4 min (2 min on each side of the syringe). Pure Phe-PEA and Ala-PEA hydrogels were also prepared with the incorporation of DAT particles. Milled DAT particles were added to the prepolymer solutions at a concentration of 2% (m/v) and the crosslinking was performed as described above.
2.2.7 Characterization of the Hydrogel Properties

2.2.7.1 Gel Content and Equilibrium Water Content

Gel content and equilibrium water content (EWC) were measured in cell-free hydrogels (n = 3) using methods adapted from a previously established source. After photo-crosslinking, the initial mass \(m_i\) of each hydrogel was recorded and the theoretical mass \(m_t\) of polymers involved in crosslinking was calculated as \(m_i \times 0.1\). The hydrogels were swollen in PBS for 24 h, and the swollen mass \(m_s\) was recorded to determine the EWC. Then, the hydrogels were rinsed three times successively in distilled water for 3 h to remove remaining non-crosslinked materials and salts from PBS. Following the rinses, the hydrogels were frozen in liquid nitrogen, lyophilized, and their dry masses \(m_d\) were measured. The gel content and EWC were calculated using equations (1) and (2):

\[
\text{Gel content} = \frac{m_d}{m_t} \times 100\% \quad (1)
\]

\[
\text{EWC} = \frac{m_s - m_d}{m_s} \times 100\% \quad (2)
\]

2.2.7.2 Mass Swelling Ratio

Mass swelling ratio was measured (n = 3) using previously reported methods. After the hydrogels were prepared, their masses \(m_i\) were recorded. The hydrogels were then swollen to equilibrium in PBS at 37 °C, and the swollen mass was recorded \(m_s\). The mass swelling ratio was calculated according to the following equation:

\[
\text{Mass swelling ratio} = \frac{m_s - m_i}{m_i} \times 100\% \quad (3)
\]

2.2.7.3 Compression Testing

Samples (n = 3) were prepared in 1 mL syringes as described above. Before compression testing, the dimensions of the swollen cylindrical hydrogels were recorded using calipers, with each sample having a height to width ratio of ~2. Unconfined stress-strain measurements were conducted using a UniVert system (CellScale, Waterloo, ON, Canada), equipped with a 0.5 N load cell. During the measurement, the samples were immersed in a 37 °C PBS bath, preloaded
at 0.01 N at every cycle, and compressed to a total strain of 20% at a rate of 0.6%/s. The nominal stress was calculated by dividing the applied force by the initial cross-sectional area of the sample. The compressive modulus was determined from the slope of the linear region of the stress-strain curve between 10 and 15% strain.

2.2.8 hASC Photo-encapsulation and Culture within Phe-PEA/Ala-PEA based Hydrogels

The prepolymer solutions of Phe-PEA, Phe-PEA + DAT, Ala-PEA and Ala-PEA + DAT systems were prepared as described in Section 2.2.6. ASCs were encapsulated within the hydrogels at P4 through suspension in proliferation medium at a concentration of 1 x 10^7 cells/mL. The final solution volume contained 80% prepolymer and 20% ASC suspension by volume was then photo-crosslinked by the methods described above. Immediately after crosslinking, the hydrogels were cut into 25 µL in size, each containing ~2.5 x 10^5 cells, transferred into 12-well inserts (Greiner Bio-one, Germany), and cultured in proliferation medium at 37 °C with 5% CO₂. After 24 h, the hydrogels were changed into serum-free adipogenic differentiation medium to induce differentiation. The adipogenic differentiation medium contained the following supplements: DMEM:Ham’s F-12 nutrient mixture, 1% pen-strep, 33 µM biotin, 17 µM pantothenate, 10 µg/mL transferrin, 100 nM hydrocortisone, 66 nM insulin, and 1 nM triiodothyronine, with 0.25 mM isobutylmethylxanthine and 1 µg/mL troglitazone incorporated into the differentiation medium for the first 72 h. Fresh medium was provided to all samples every 2-3 days.

2.2.9 Cell Viability

Viability of the encapsulated ASCs in the Phe-PEA-based and Ala-PEA-based hydrogels with 0% and 2% DAT was assessed at 24 h after encapsulation and at 7 days after the induction of adipogenic differentiation (n = 3, N = 3) using the LIVE/DEAD® Viability/Cytotoxicity Kit for mammalian cells (Life Technologies Inc., Burlington, ON, Canada). DAT particles could be detected by confocal microscopy due to autofluorescence. During the preparation, the hydrogels were rinsed twice in PBS before incubation in the staining solution comprised of 2 µM calcein AM and 4 µM ethidium homodimer-1 for 45 min at 37 °C. Then, the hydrogels were rinsed two times with PBS before imaging utilizing a Zeiss Multiphoton LSM 510 META confocal
microscope (ZEISS, Canada). Images were captured at 10X magnification, and a mosaic stitch technique was used to capture the complete cross-sectional area of the hydrogel. For each sample, a total of 5 layers were collected, and each layer was separated by 75 µm. Image J analysis software was used to quantify the number of live and dead cells in each layer in order to calculate the average cell viability for each group.

2.2.10 Glycerol-3-phosphate Dehydrogenase (GPDH) Activity

Quantitative assessment of ASC adipogenesis was conducted by measuring intracellular GPDH enzyme activity in the Phe-PEA-based and Ala-PEA-based hydrogels with 0% and 2% DAT hydrogels at 7 days after adipogenic induction (n = 3, N = 3) using a GPDH Enzyme Activity Measurement Kit (Kamiya Biomedical Corporation, Cat. # KT-010, Seattle, WA, USA). Moreover, ASCs cultured on tissue culture polystyrene (TCPS) 6-well plates in proliferation medium (negative control) and adipogenic differentiation medium (positive control) were included in the study. During the preparation, the hydrogels were rinsed three times in PBS, followed by the addition of 500 µL of enzyme extracting reagent (pH 7.4) provided with the kit. The hydrogels were then broken into smaller pieces by crushing them using a plastic pestle. The crushed samples were lysed using an ultrasonic homogenizer (Model-100, Fisher Scientific), and centrifuged (12,800 xg, 5 min, 4 °C) to isolate the cytosolic fraction containing the intracellular GPDH enzyme. To normalize the GPDH activity levels, the total cytosolic protein content for all samples was measured using the Pierce 660 Protein Assay Kit (Thermo Scientific, Waltham, MA, USA) with an albumin standard. Immediately, the samples were measured for total protein content and GPDH activity using a CLARIOstar® spectrophotometer (BMG Labtech, Ortenberg, Germany). One unit of GPDH activity was defined as the activity required to oxidize 1 µM of NADH in 1 min.

2.2.11 BODIPY Staining

Qualitative assessment of ASC adipogenesis was performed using BODIPY® 493/503 staining (Thermo Scientific, Waltham, MA, USA) to capture intracellular lipid accumulation in the ASCs encapsulated in the Phe-PEA-based and Ala-PEA-based hydrogels with 0% and 2% DAT hydrogels at 7 days after adipogenic induction (n = 3, N = 3). As mentioned earlier, DAT
particles could be visualized due to autofluorescence. The BODIPY® stock solution was prepared in DMSO at 1 mg/mL, and diluted in PBS by a factor of 1:500. The hydrogels were rinsed two times in PBS and then incubated in the staining solution at 37 °C for 1 h. Following incubation, the hydrogels were rinsed twice in PBS before imaging utilizing a Zeiss Multiphoton LSM 510 META confocal microscope, with images collected at 25X magnification using a water immersion objective.

2.2.12 Statistical Analysis

Data are reported as the mean ± standard deviation (SD), and the statistical analyses were performed using GraphPad Prism 6 (GraphPad Software, San Diego, CA, USA) by one-way or two-way ANOVA with a Tukey’s post-hoc test. Differences were considered statistically significant at p < 0.05.

2.3 Results and Discussion

2.3.1 Synthesis and Characterization of Monomers

The monomers used to prepare the PEAs were synthesized as previously reported (Scheme 2.1).31,32 The original goal was to synthesize a series of PEAs that differed by the length of diol component (4 carbons vs 8 carbons) and the type of amino acid (phenylalanine vs alanine), and to explore the effects on the properties from their structural differences. However, the synthesis of di-p-toluenesulfonic acid salt monomer deriving from L-alanine and 1,4-butanediol resulted in very low yield, hence only the polymerization from the other three monomers 1a-c were further pursued. Di-p-nitrophenyl fumarate, monomer 2 was synthesized to provide the crosslinkable component of PEAs due to its double bond. Additionally, since L-alanine and L-phenylalanine are naturally hydrophobic, hydrophilic poly(ethylene glycol) (PEG) diamine, macromonomer 3 was incorporated to introduce water solubility to the polymer. All monomers were characterized by 1H NMR spectroscopy and the spectral data matched with the literature values (Figures A1-A6).31–33
Scheme 2.1. Synthesis of a) di-p-toluenesulfonic acid salt monomers 1a-1c, b) di-p-nitrophenyl fumarate monomer 2 and c) PEG diamine macromonomer 3.

2.3.2 Synthesis and Characterization of Polymers

The polymers are named by the abbreviations of the three letter amino acid designation from monomer 1: Phe-PEA, Phe8-PEA (phenylalanine monomer with 8 carbons in the diol segment) and Ala-PEA. All polymerization reactions were carried out in optimized solution co-polycondensation conditions that were derived from a procedure reported previously (Scheme 2.2). The challenges that were involved during the process of polymerization included finding the molar ratio of monomer 1 and macromonomer 3 required to produce water soluble PEAs, and the determination of a reaction time to prevent the crosslinking between the monomers as the previously-reported 48 h reaction time for the previous PEAs resulted in the formation of insoluble gel-like materials. The crosslinking could possibly occur by Michael addition between the amine groups of monomer 1 or macromonomer 3 with the double bond of monomer 2 or through a free radical mechanism.
Synthesis of three different PEAs.

Water solubility of PEAs was achieved by varying the amounts of monomer 1 and macromonomer 3. Three different ratios of the two monomers were examined: 50:50, 40:60 and 30:70 of monomers 1:3. It was found that 0.7 molar equivalent of macromonomer 3 was the minimum amount required to achieve water solubility of the polymer for Phe-PEA and Ala-PEA. However, Phe8-PEA was not water soluble for any of the prepared ratios, and was therefore excluded from further studies. In addition, after conducting a kinetic study of this polymerization reaction, it was found that a 6 h reaction time yielded no formation of insoluble materials with reproducible molar mass achievable for the resulting polymers. Overall, the best of the evaluated conditions for the PEA synthesis were: molar ratio of the three monomers (1:2:3) equal to 0.3:1.0:0.7, 70 °C reaction temperature, 6 h reaction time with dry N,N-dimethylacetamide (DMA) as the solvent and triethylamine (Et₃N) as the base.
The polymers were characterized by $^1$H NMR spectroscopy, $^{13}$C NMR spectroscopy, FT-IR spectroscopy, SEC, TGA and DSC. The number average molar mass ($M_n$) and dispersity ($D$) data were obtained from SEC analysis using refractive index detection and were determined relative to PMMA standards (Table 2.1). Both Phe-PEA and Ala-PEA had $M_n$ values close to 20 kg/mol and $D$ of 2.46 and 2.96 respectively. The SEC data indicated that polymerization had occurred between the monomers as the elution time for PEAs were much shorter than for the starting 2000 g/mol PEG (Figure 2.1). The $D$ values were relatively high for solution polycondensation, which might be attributed to a small amount of crosslinking. However, as the PEAs were designed to be incorporated in subsequent steps into a network, this was not problematic.

**Table 2.1.** SEC and thermal analysis results for the two different PEAs.

<table>
<thead>
<tr>
<th>Type of PEAs</th>
<th>$M_n$ (kg/mol)</th>
<th>$D$</th>
<th>$T_o$ ($^\circ$C)</th>
<th>$T_m$ ($^\circ$C)</th>
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<tr>
<td>Phe-PEA</td>
<td>19.9</td>
<td>2.46</td>
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<td>36</td>
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<td>Ala-PEA</td>
<td>18.9</td>
<td>2.96</td>
<td>362</td>
<td>34</td>
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**Figure 2.1.** Overlay of SEC traces of the two different PEAs in comparison to 2000 g/mol PEG.
The chemical structures of the two synthesized PEAs were confirmed by $^1$H NMR, $^{13}$C NMR and FT-IR spectroscopic methods. The $^1$H NMR data were in agreement with the anticipated chemical structures of the PEAs (Figure 2.2). Both spectra contained the -NH- peaks from the amide bonds generated by monomer 2 with monomer 1 and/or macromonomer 3, which were observed at ~8.80 and ~8.45 ppm, respectively. The spectra also had the =CH- peak at 6.84 ppm corresponding to the fumaric acid component (monomer 2). The spectrum of Ala-PEA differed from that of Phe-PEA due to the absence of aromatic proton peaks from 7.30 to 7.19 ppm, and the peaks of -CH$_2$- adjacent to the phenyl ring from 3.07 to 2.89 ppm, as well as the upfield shift of peak 6 because of the difference in the structure of the amino acid. Moreover, each spectrum contained the characteristic peaks corresponding to the type of monomer 1 that was used, and they all had the PEG peaks, indicating the incorporation of macromonomer 3 into the overall PEA structures. Combined, the integrations of the peaks corresponded closely to the specific molar ratio of monomer 1:2:3 at 0.3:1.0:0.7 (Figures A7 and A9). The $^{13}$C NMR spectra further confirmed the structures of the PEAs as they contained all the expected peaks (Figures A10 and A12).

**Figure 2.2.** $^1$H NMR spectra of a) Phe-PEA and b) Ala-PEA in DMSO-d$_6$ (400 MHz).
The IR spectral data was also consistent with the successful formation of PEAs using the optimized polycondensation conditions (Figure 2.3). The two spectra had the same characteristic peaks corresponding to the amide N-H stretch at 3300 cm⁻¹, the ester C=O stretch at 1740 cm⁻¹, the amide C=O stretch at 1640 cm⁻¹, and the amide N-H bend at 1550 cm⁻¹. Additionally, they all had a broad peak near the region of ~3500 cm⁻¹ corresponding to an amine N-H stretch, and a strong peak at 2880 cm⁻¹ corresponding to alkane C-H stretches deriving from the PEG component (macromonomer 3) of PEAs as well as other C-H bonds on the other monomers. The presence of amine N-H stretching indicated that some polymers have amine termini. Furthermore, they all had a small peak around 3070 cm⁻¹ corresponding to the alkene =C-H stretch from monomer 2. However, the C=C stretch peak in the region of 1620-1680 cm⁻¹ was not visible as it likely overlapped with the carbonyl peaks. Although, IR spectroscopy was unable to fully confirm the presence of the double bond from monomer 2, it was strongly evident in the NMR spectra.

Figure 2.3. FT-IR spectra of a) Phe-PEA and b) Ala-PEA.

To assess the thermal properties of the PEAs, they were subjected to both TGA and DSC analyses (Table 2.1). Both PEAs had onset degradation temperatures (Tₒ) near 360 °C
(Figure 2.4). Based on the DSC results, it was clear that the PEAs were predominantly in crystalline rather than amorphous states due to the presence of melting peaks in both traces (Figure 2.5). Their glass transition temperatures ($T_g$) may be detected below -40 °C, however this was limited by the instrument. They had melting temperatures ($T_m$) that only differed by 2 °C as they were 36 °C and 34 °C for Phe-PEA and Ala-PEA respectively. The reason why the PEAs are highly crystalline could be due to the presence of the PEG component which is highly crystalline.40 Overall, Phe-PEA and Ala-PEA shared similar thermal properties despite their differences in the length of the diol component and the type of amino acid.

![Figure 2.4. TGA traces of Phe-PEA and Ala-PEA.](image)

![Figure 2.5. DSC traces of a) Phe-PEA and b) Ala-PEA.](image)
2.3.3 Fabrication and Characterization of PEA-based Hydrogels

After the successful synthesis of water soluble, unsaturated polymers Phe-PEA and Ala-PEA, they were then tested to determine if they were capable of gelation. Crosslinkers including PEG-dimethacrylate (PEGDMA) or methacrylated chondroitin sulphate (MCS), which are commonly used for hydrogel formation, were also incorporated in the formulations to aid the crosslinking of PEAs and to tune the properties of the resulting hydrogels (Figure 2.6).\textsuperscript{34,35} Both PEGDMA and MCS were synthesized based on procedures previously reported and they were characterized by \textsuperscript{1}H NMR spectroscopy (Figures A14 and A15).\textsuperscript{34,35} Therefore, a series of hybrid hydrogels from Phe-PEA/Ala-PEA and PEGDMA/MCS precursors were investigated.

![Chemical structures of a) PEGDMA and b) MCS.](image)

\textbf{Figure 2.6.} Chemical structures of a) PEGDMA and b) MCS.

The scaffold fabrication involved Irgacure 2959 as the photoinitiator at a concentration of 0.15% (m/v), PEGDMA or MCS at a concentration of 0 or 2.5% (m/v), and PEAs at a concentration of 7.5 or 10% (m/v). Upon gelation, it was found that the hybrid hydrogels containing PEGDMA or MCS were very brittle, as their network structures fractured when pressure was applied to remove them from the syringe mold (Figure 2.7). However, the two pure PEA systems produced uniform gels suitable for the measurement of mechanical properties and ASC encapsulation. For this reason, these two systems were selected for the incorporation of DAT particles, to investigate the potential effects of ECM composition on the ASC response within the PEAs systems. DAT was added to the pure Phe-PEA and Ala-PEA formulations at 2% (m/v) as larger percentages led to disruption of the crosslinking.
Figure 2.7. Representative images of a series of hydrogel systems: a) Phe-PEA, b) Phe-PEA + PEGDMA, c) Phe-PEA + MCS, d) Ala-PEA, e) Ala-PEA + PEGDMA and f) Ala-PEA + MCS. Scale: 5 mm

All of the hydrogel groups (pure PEAs, PEAs + PEGDMA/MCS/DAT) were characterized by the measurement of gel content, EWC and mass swelling ratio. Gel content is the mass percentage of material successfully incorporated into the network, while EWC expresses the swelling degree of the crosslinked network with water relative to the dry weight of gel. For gel content, there was a common trend that all Phe-PEA-based systems had a greater gel content than the Ala-PEA-based systems (Figure 2.8a). However, the difference was only statistically significant for the systems with PEGDMA. Additionally, incorporating DAT did not have a noticeable effect on the gel content. The gel content was also similar when MCS was added. Meanwhile, the Ala-PEA hydrogels had consistently higher EWC than the Phe-PEA hydrogels, although the differences were only statistically significant when DAT was incorporated (Figure 2.8b). The higher EWC may be attributed to the difference in the amino acid monomers between the two polymers. Although the Ala monomer 1c has a longer aliphatic diol chain than the Phe monomer 1a, methyl side chains on Ala are much less hydrophobic than the phenyl side chains of Phe. However, it may also relate to the lower gel content for the Ala-PEA hydrogels, which likely correlates with a lower density of crosslinking. Overall, the hydrogels with PEGDMA and MCS incorporated had similar EWC to the pure PEA and PEA + DAT systems. As expected, the mass swelling ratios across the groups shared a similar trend to their EWC. Ala-PEA-based systems swelled more than the Phe-PEA-based systems, though the difference was only statistically significant in the case of the hydrogels with DAT incorporated (Figure 2.8c). Additionally, it was found that the PEA hydrogels containing MCS swelled more than the other
groups. This increased degree of swelling can be attributed to the presence of charges on the MCS, which results in an influx of water through osmosis.

![Graphs showing gel content, equilibrium water content, and mass swelling ratio of hydrogels.](image)

**Figure 2.8.** (a) Gel content of the hydrogels (n=3). * denotes a statistically significant difference between Phe-PEA + PEGDMA and Ala-PEA + PEGDMA. (b) Equilibrium water content of the hydrogels (n=3). * denotes a statistically significant difference between Phe-PEA + DAT and Ala-PEA + DAT. c) Mass swelling ratio of the hydrogels (n=3). * denotes a statistically significant difference between Phe-PEA + DAT and Ala-PEA + DAT. Data was analyzed by one-way ANOVA with a Tukey’s post-hoc comparison of the means (p < 0.05).
Compressive moduli were only measured for the pure Phe-PEA and Ala-PEA hydrogels with and without 2% DAT, as the hydrogels with PEGDMA or MCS incorporated could not be measured due to their brittleness and poor structural integrity. Higher compressive moduli were obtained for Phe-PEA-based systems in comparison to the Ala-PEA-based systems both with and without DAT incorporated (Figure 2.9). The higher moduli for the Phe-PEA hydrogels may result from their trend towards lower EWC and swelling relative to the Ala-PEA systems, as this would result in a higher density of polymer in the network, leading to higher stiffness. Hydrophobic interactions between the phenyl group in the Phe domains, may also impart higher stiffness. Specifically, the modulus of the pure Phe-PEA system was significantly higher than that of the other three systems. Moreover, for both Phe-PEA and Ala-PEA, the incorporation of DAT reduced the compressive moduli, and a significant difference was observed between pure Ala-PEA hydrogels and Ala-PEA + DAT hydrogels. Although the systems with and without DAT had very similar gel contents, this result suggested that the incorporation of DAT may have introduced defects or inhomogeneities into the networks.

![Compressive Moduli](image)

**Figure 2.9.** Compressive moduli of pure PEAs and PEAs + DAT systems (n = 3). *denotes a statistically significant difference between Ala-PEA and Ala-PEA + DAT. **denotes that Phe-PEA was statistically different than all other groups. Data was analyzed by one-way ANOVA with a Tukey’s post-hoc comparison of the means (p < 0.05).
2.3.4 Cell Viability

The viability of ASCs encapsulated in the four different hydrogel systems (Phe-PEA and Ala-PEA with and without 2% DAT) was assessed at 24 h post-encapsulation, as well as at 7 days after adipogenic induction (Figure 2.10a). Representative images of the hydrogels containing ASCs 24 h post encapsulation, where live cells appear green and dead cells appear red, are displayed in Figure 2.11. Large red-labeled structures were observed in hydrogel systems with DAT incorporated. Based on a cell-free control (Figure A16), we believe that these red structures result from the non-specific binding of the ethidium homodimer-1 stain to the DAT, and thus a size threshold was used for counting the average number of dead cells in each sample. At 24 h after encapsulation, all four hydrogel systems had viabilities higher than 75% with no significant differences observed between the systems. These results were in agreement with those of a previous study involving the encapsulation of ASCs into an MCS hydrogel system, and indicated that incorporating DAT did not influence the early cellular response. Overall, the viability results at 24 h suggested that both Phe-PEA-based and Ala-PEA-based systems provided a supportive environment for the encapsulated ASCs.

While not statistically significant, from 24 h to 7 days, there was a trend towards decreasing viability across the groups and a slightly lower average number of viable cells per xy plane in the hydrogels (Figure 2.10b). It has been shown that cell density plays an important role in mediating adipogenic differentiation, as high cell density was found to be more favorable towards adipogenesis. Importantly, there were no significant differences in the average number of viable cells between the hydrogel groups at either time point, supporting that differences in cell density would not contribute to differences in ASC differentiation towards the adipogenic lineage in the later studies.
Figure 2.10. (a) ASC viability analysis of pure PEAs and PEAs + DAT systems at 24 h after encapsulation and at 7 days after induction of adipogenic differentiation (n = 3, N = 3), with no significant differences observed between the four hydrogel systems at either time point. (b) Average number of viable ASCs per xy plane within the pure PEAs and PEAs + DAT systems, with no significant differences observed at either time point.
Figure 2.11. Representative images of ASCs encapsulated in Phe-PEA, Phe-PEA + DAT, Ala-PEA and Ala-PEA + DAT hydrogel systems at 24 h, displaying the distribution of live (green; calcein-AM) cells and dead (red; ethidium homodimer-1) cells along with DAT particles (blue due to autofluorescence): a) macroscopic view. Scale: 500 µm and b) microscopic view. Scale: 100 µm
2.3.5 Glycerol-3-phosphate Dehydrogenase (GPDH) Activity

Quantitative analysis of adipogenesis within the pure PEA and PEA + DAT systems was assessed by evaluating the GPDH enzyme activities in the encapsulated ASCs at 7 days after adipogenic induction (Figure 2.12). Due to cell donor variability, the data acquired from each donor is presented separately for each hydrogel system. Based on the data, there were no significant differences found in the GPDH activity levels between the hydrogels fabricated with Phe-PEA-based versus Ala-PEA-based systems, indicating that all of the groups similarly supported adipogenic differentiation of the encapsulated ASCs. Additionally, when considering the mechanical properties of these four hydrogel systems, their compressive moduli (20-110 kPa) are greater than that of the native adipose tissue which is approximately 1-4 kPa.\(^{41}\) Although it would be predicted to be favorable for the scaffolds to match the modulus of the native tissues, a previous study demonstrated that MCS hydrogel systems with compressive moduli ranging from 40 to 80 kPa were able to support ASC adipogenesis.\(^{27}\) The same study also demonstrated the inductive effect of DAT in promoting adipogenic differentiation by using 5 wt% of DAT.\(^{27}\) Their observation was consistent with other reported studies that investigated the potential of adipose tissue ECM in directing tissue-specific differentiation of ASCs.\(^{26,29,42}\) Based on the literature, we also expected to observe that incorporating the DAT in the PEA systems would have a positive effect in terms of adipogenesis, as stated in the hypothesis. However, no significant differences were shown with the DAT incorporated in both the Phe-PEA and Ala-PEA systems, which could be attributed to the limitation of this polymer system that was only able to incorporate 2 wt% of DAT while maintaining the stability of the hydrogel. This amount of DAT might be too little to induce an effect in adipogenic differentiation of ASCs as compared to the amount incorporated into the MCS in the previous study.\(^{27}\)
Figure 2.12. GPDH activity of Phe-PEA, Phe-PEA + DAT, Ala-PEA and Ala-PEA + DAT hydrogel systems at 7 days after induction of adipogenic differentiation (n = 3, N = 3), with data showing responses from all three donors for each hydrogel system. The mean value for each group was represented by a bar. No significant differences were observed between the four hydrogel systems.
2.3.6 BODIPY Staining of Intracellular Lipid Accumulation

Intracellular lipid accumulation in the pure PEAs and PEAs + DAT systems was visualized by using a BODIPY stain at 7 days after adipogenic induction (Figure 2.13). Adipogenesis was observed in all four hydrogel systems, as rounded green lipid droplets were detected by confocal microscopy. Similar to the quantitative GPDH analysis, the collected qualitative images suggested that all of the groups similarly supported the encapsulated ASCs differentiating into adipocytes, though a slightly higher number of lipid droplets was observed in the pure Phe-PEA system qualitatively. Notably, the lipid droplets were formed in close proximity to the DAT in both DAT incorporated systems. This was also observed in other studies, with clusters of differentiating ASCs containing lipid droplets found around the DAT.\textsuperscript{27,28} However, the incorporation of DAT within both PEA systems did not have a positive effect on the quantitative and qualitative markers of ASC adipogenesis in this study. Again, the lack of significant effects of the DAT may be attributed to the fact that only a low amount of DAT (2 wt%) could be incorporated within the PEAs while ensuring stable hydrogel formation. The previously-reported study utilized 3 and 5 wt% of DAT in the MCS hydrogel system, but significant differences in adipogenic differentiation were only observed for the 5 wt% formulation both quantitatively and qualitatively.\textsuperscript{27} Therefore, 2 wt% of DAT incorporated in the PEA systems might not be sufficient to promote adipogenic differentiation of the encapsulated ASCs.
Figure 2.13. Representative images of BODIPY-stained (green) intracellular lipid droplets in ASCs from three different donors, encapsulated in Phe-PEA, Phe-PEA + DAT, Ala-PEA and Ala-PEA + DAT hydrogel systems at 7 days after induction of adipogenic differentiation. Qualitatively more intracellular lipid droplets were observed for the pure Phe-PEA system. In the composites incorporating the DAT, the cells containing lipid droplets were generally found around the DAT particles (blue due to autofluorescence). Scale: 50 µm
2.4 Conclusions

New water soluble PEAs with crosslinkable moieties were successfully synthesized from a phenylalanine-based monomer, alanine-based monomer, p-nitrophenyl fumarate and a PEG diamine. The PEAs were only water soluble when a 70:30 PEG:amino acid monomer ratio was used and for Phe-PEA and Ala-PEA. A series of hydrogels were fabricated from the two PEAs including sample groups incorporating PEGDMA, MCS and DAT particles. Phe-PEA-based systems had a trend towards greater gel contents, lower swelling and higher compressive moduli when compared to the Ala-PEA-based systems, potentially due to the phenyl group of Phe-PEA being more hydrophobic than the methyl group of Ala-PEA though statistically significant differences between the two polymers were only observed for selected examples. ASC viability and adipogenesis were investigated in the Phe-PEA and Ala-PEA systems both with and without DAT incorporation. Similar viabilities were observed for both PEA systems, indicating that these new PEAs both provided a supportive environment for ASCs. Adipogenic differentiation was assessed in the two different PEA systems both quantitatively and qualitatively by measuring GPDH enzyme activity and staining for intracellular lipid accumulation, respectively. All of the groups were observed to similarly support ASC adipogenesis. Overall, this study demonstrated the potential of these new water soluble PEAs as biomaterials for adipose tissue engineering. Future work will focus on measuring adipogenic gene expression to further quantify adipogenesis within the PEA systems, running a time course study for assessing adipogenic differentiation of ASCs as 7 days is not long enough to observe a mature phenotype, and optimizing the hydrogel formulation to increase the amount of DAT incorporated.
2.5 References


(13) Alapure, B. V.; Lu, Y.; He, M.; Chu, C.-C.; Peng, H.; Muhale, F.; Brewerton, Y.-L.; Bunnell, B.; Hong, S.; Severe. Accelerate Healing of Severe Burn Wounds by Mouse Bone Marrow Mesenchymal Stem Cell-Seeded Biodegradable Hydrogel Scaffold


Chapter 3

3 Conclusions and Future Work

Overall, this thesis presented the synthesis of new water soluble, amino acid-based PEAs with crosslinkable moieties for hydrogel fabrication and explored their potential as biomaterials for adipose tissue engineering. The water solubility of the polymers was achieved by carefully controlling the molar ratio of PEG and amino acid monomers. Moreover, the resulting Phe-PEA and Ala-PEA polymers could be reproducibly synthesized with an $M_n$ of approximately 20 kg/mol and $Đ$ of 2-3 through a tailored reaction condition. Using the double bond incorporated into the PEA backbone, the polymers could be crosslinked photochemically to form stable hydrogels. The properties of these PEA-based hydrogels could be tuned by the addition of other components including PEGDMA, MCS and DAT particles. However, based on the structural uniformity of the hydrogels, formulations containing only the PEAs as well as PEAs with DAT particles were most promising and were therefore selected for the measurement of mechanical properties and for ASC encapsulation. The viability and adipogenic differentiation of ASCs from three different human donors were studied in Phe-PEA and Ala-PEA hydrogels with and without DAT. Overall, the results demonstrated that these new PEA hydrogels supported the viability of ASCs and their differentiation towards the adipogenic lineage, and given their property tunability and potential to be biodegradable, these PEAs are promising candidates for adipose tissue engineering.

There are a few aspects that can be addressed in terms of the future work for this project. First, DAT has been demonstrated to exhibit tissue-specific instructive effects in promoting ASC adipogenic differentiation.\textsuperscript{1-4} However, the amount of DAT (2 wt%) that could be stably incorporated into the PEA systems studied in this project was quite low compared to the previous study that used 5 wt% DAT within MCS hydrogels to produce significant effects on adipogenic differentiation.\textsuperscript{1} Therefore, it would be advantageous to optimize the hydrogel formulation to enable the incorporation of higher amounts of DAT. Several approaches can be explored, such as using a different crosslinking method that may allow more DAT to be incorporated into PEA hydrogels. Thermally initiated crosslinking using ammonium persulphate (APS) and tetramethylethylenediamine (TEMED) may be a good alternative method as it has been used in
our lab to generate a composite hydrogel containing MCS and DAT.\textsuperscript{5} In addition, decreasing the concentration of PEA to 8 wt% may lead to a less viscous solution and allow more DAT to be added by improving dispersion in the prepolymer solution. Secondly, to be able to more fully analyze the ASC adipogenic response, negative controls of the various hydrogel groups should be included in the study with scaffolds maintained in proliferation medium for 7 days to assess possible inductive effects of the DAT and/or 3-D culture conditions. In addition, performing real-time reverse transcription polymerase chain reaction (RT-qPCR) analysis of adipogenic gene expression (e.g. \textit{PPAR}\textsubscript{y} and \textit{LPL}) would be beneficial to further quantify ASC adipogenic differentiation within these PEA systems. Furthermore, running a time course study that extends to 14 or 21 days to allow for the formation of more mature adipocytes would be valuable to evaluate these PEA systems as long-term delivery vehicles for ASCs for adipose tissue engineering applications. Finally, as the results in this thesis demonstrated that these new PEAs overall provided a supportive environment for ASCs, it would be interesting to explore their capacity to support ASC differentiation towards the chondrogenic and osteogenic lineages, including within composites incorporating decellularized ECM from cartilage and bone.
3.1 References


Figure A1. $^1$H NMR spectrum of monomer 1a in DMSO-d$_6$ (600 MHz).
Figure A2. $^1$H NMR spectrum of monomer 1b in DMSO-d$_6$ (600 MHz).

Figure A3. $^1$H NMR spectrum of monomer 1c in DMSO-d$_6$ (600 MHz).
Figure A4. $^1$H NMR spectrum of monomer 2 in DMSO-$d_6$ (600 MHz).

Figure A5. $^1$H NMR spectrum of PEG ditosylate, reaction intermediate of macromonomer 3 in CDCl$_3$ (600 MHz).
**Figure A6.** $^1$H NMR spectrum of macromonomer 3 in CDCl$_3$ (600 MHz).

**Figure A7.** $^1$H NMR spectrum of Phe-PEA in DMSO-d$_6$ (400 MHz).
Figure A8. $^1$H NMR spectrum of Phe8-PEA in DMSO-$d_6$ (400 MHz).

Figure A9. $^1$H NMR spectrum of Ala-PEA in DMSO-$d_6$ (400 MHz).
Figure A10. $^{13}$C {$^{1}$H} NMR spectrum of Phe-PEA in DMSO-d$_6$ (100 MHz).

Figure A11. $^{13}$C {$^{1}$H} NMR spectrum of Phe8-PEA in DMSO-d$_6$ (100 MHz).
Figure A12. $^{13}\text{C} \{^1\text{H}\}$ NMR spectrum of Ala-PEA in DMSO-d$_6$ (100 MHz).
Figure A13. Characterizations of Phe8-PEA: a) SEC trace, b) FT-IR spectrum, c) TGA trace and d) DSC trace.
Figure A14. $^1$H NMR spectrum of PEGDMA in CDCl$_3$ (600 MHz).

Figure A15. $^1$H NMR spectrum of MCS in D$_2$O (600 MHz).
Figure A16. Representative images of the overlapping between large aggregates of dead cell and DAT signals in a cell-free control sample, Ala-PEA + DAT. Scale: 100 µm

Table A1. Summary of cell donor information for all biological studies.

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Appendix 2: Research Ethics Board Approval

Western University Health Science Research Ethics Board
HSREB Delegated Initial Approval Notice

Principal Investigator: Dr. Lauren J. Hume
Department & Institution: Schulich School of Medicine and Dentistry, Anatomy & Cell Biology, Western University

HSREB File Number: 1105426
Study Title: Phase I/II Study Involving Liposomes Conjugated with Adipose-dervied Stem Cells
Sponsor: Canadian Institutes of Health Research

HSREB Initial Approval Date: August 13, 2014
HSREB Expiry Date: August 13, 2016

Documents Approved and/or Received for Information:

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<td>2014/07/23</td>
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<td>Western University Protocol</td>
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<td>Letter of Information &amp; Consent</td>
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The Western University Health Science Research Ethics Board (HSREB) has reviewed and approved the above named study, as of the HSREB Initial Approved Date above.

HSREB approval for this study remains valid until the HSREB Expiry Date noted above, conditional to timely submission and acceptance of HSREB Continuing Ethics Review. An Updated Approval Notice is required prior to the HSREB Expiry Date, the Principal Investigator is responsible for submitting and obtaining an HSREB Updated Approval Form in a timely fashion.


Members of the HSREB who are named as Investigators in research studies do not participate in discussions related to, nor vote on such studies when they are presented to the REB.

The HSREB is registered with the U.S. Department of Health & Human Services under the IRB registration number #IRB00000000.

Ethics Officer to Contact for Further Information:

<table>
<thead>
<tr>
<th>Ethics Officer</th>
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This is an official document. Please retain the original in your files.
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