Characterization of Hybrid Synthetic/Adipose-Derived ECM Scaffolds

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ABSTRACT

Evolving out of a need to address growing concerns regarding current methods of tissue transplantation, the field of tissue engineering seeks to facilitate the regeneration of viable tissue through the use of cellular scaffolds. The first aim of this thesis was to provide a summary of the current literature on advances in biomaterial synthesis and pertaining methods of stem cell delivery in tissue engineering. Improvements in the processing of decellularized tissue and the expansion of synthetic hydrogels as platforms for stem cell encapsulation have led to the development of extracellular matrix (ECM)-based hybrid hydrogels. These stem cell scaffolds are currently being explored as biomaterials for the purpose of tissue regeneration.

The second aim of this thesis was to fabricate a hybrid synthetic/adipose-derived ECM hydrogel. Decellularized adipose tissue was incorporated, at varying concentrations, with a thiol-acrylate fraction that was then polymerized to produce hydrogels via a Michael addition reaction. Hydrogels were characterized based on their ability to support the proliferation, maintain the viability and retain the multipotency of human adipose-derived stem cells (hASCs). Cells encapsulated in hydrogels containing high concentrations of ECM demonstrated greater expression of human potency markers compared to cells encapsulated in ECM-free synthetic hydrogels or in Matrigel®, indicating that adECM hydrogels hold promise as a cost-effective platform for mesenchymal stem cell multipotency maintenance for tissue engineering applications.

Inspired by the findings that adipose-derived ECM can be converted into a cytocompatible hydrogel after combination with a synthetic fraction, efforts were conducted in order to improve the performance of the hybrid synthetic/adipose-derived ECM hydrogel platform. ECM was thiolated prior to hydrogel synthesis in order to promote more uniform dispersion. Thiolated adipose-derived ECM hydrogels were characterized based on their ability to maintain hASC viability. It was found that hASCs seeded on hydrogels containing higher concentrations of thiolated adECM (tadECM)
demonstrated decreased viability compared to tadECM-free hydrogels. As these results may be caused by incomplete thiol-acrylate conversion, increasing the thiol concentration of the tadECM prior to hydrogel synthesis may lead to improved outcomes.
1. CURRENT BIOMATERIALS AND PERTAINING STEM CELL DELIVERY METHODOLOGIES IN TISSUE ENGINEERING LITERATURE REVIEW

The field of tissue engineering seeks to regenerate viable tissue through the use of cell-scaffold systems. When employing stem cells for the generation of functional tissue, these scaffolds have the potential to promote cellular expansion and to affect cellular lineage commitment. This brief review aims to provide insight into the various types of biomaterials used for cell scaffold synthesis. In addition, this work offers a summary of the considerations for stem cell scaffold design, pertinent methods employed for stem cell delivery, and examples of current stem cell scaffold systems.

1.1 Tissue Engineering, Mesenchymal Stem Cells, and Cellular Scaffold Design

Every day, thousands of surgical procedures are performed to replace or repair damaged or injured tissue.1 Evolving out of a need to address concerns pertaining to tissue transplantation, including the limited availability of healthy tissue, the field of tissue engineering seeks to facilitate the generation of viable tissue though the combination of cells and a cellular scaffold.1,2 Mesenchymal stem cells (MSCs) are multipotent stromal cells most commonly derived from adult bone marrow or adipose.3 Due to their ability to be easily grown and selectively differentiated in vitro, MSCs are widely used in the field of tissue engineering.4 It has been demonstrated that MSCs have the ability to differentiate into a variety of cell types, including adipocytes,5 myocytes,4 osteoblasts,6 and chondrocytes.7 To date, two dimensional (2D) cell culture has been the most common platform for in vitro culture of MSCs. However, cellular differentiation and tissue development is a three-dimensional (3D) process—native cells interact with each other, the ECM, and their surrounding microenvironment in a 3D fashion.8 The additional dimensionality influences the spatial organization of cell surface proteins, which ultimately effects gene expression and cellular behavior.9 In addition, 3D conditions modulate the transport of nutrients, gases, and effector proteins differently than a 2-D environment.10 As a result, cells cultured in a 3D
environment differ genetically, physiologically, and morphologically from cells cultured in 2D. The inability of 2D culture to accurately model the 3D environment of native tissue has motivated the development of 3D cellular scaffolds for cell-based studies in the fields of stem cell research and tissue regeneration.

Tissue engineering scaffolds can be divided into three distinct groups of biomaterials—ceramics, synthetic polymers, and natural polymers. The ideal stem cell scaffold fulfills several design requirements. First, the scaffold is biodegradable, meaning that it is able to be broken down in vivo, preferably at the same rate as host's cells' colonization of the scaffold. Secondly, the biomaterial should be cytocompatible, meaning cells function normally once they adhere to or are encapsulated in the scaffold. The material should also be broadly biocompatible and its by-products should also evoke a minimal host response, once implanted, as to not cause rejection of the implant by the host or inhibit the healing process. Additionally, the biomaterial's mechanical properties should closely match that of the tissue surrounding the defect site. Furthermore, the morphology and structure should incorporate interconnected pores to facilitate cell proliferation, nutrient diffusion, and waste removal. Pores should be small enough to maintain a high specific surface area, allowing for efficient cellular adhesion, but large enough to facilitate cell migration through the scaffold. Moreover, in order to become commercially and clinically sustainable, scaffold synthesis methods should have the ability to be translated into reproducible and robust manufacturing processes. Finally, scaffolds designed for stem cell colonization should be able to effectively direct desired cellular fate. To date, numerous varieties of scaffolds have been used in combination with MSCs in an effort to regenerate various tissue types.
1.2 Naturally-Derived Biomaterials for Tissue Engineering

1.2.1 Commonly Used Naturally-Derived Polymers

The need for biodegradable and biocompatible materials to act as stem cell scaffolds has led to the widespread use of naturally-derived biomaterials for tissue regeneration. These biomimetic scaffolds can be categorized as either protein-based or polysaccharide-based polymers. Due to their capacity to mimic native extracellular matrix (ECM), protein-derived polymers, such as gelatin,22 collagen,23 fibrin,24,25 and silk,26 have the potential to support cellular organization, migration, and proliferation during the process of tissue generation. For example, El-Jawhari et al. demonstrated that the incorporation of collagen into bone marrow-derived MSC scaffolds significantly improved cellular attachment and proliferation in vitro.27

Alternatively, polysaccharide-derived polymers can be obtained from microbial, vegetal, or animal sources.28 It has been demonstrated that these biomaterials, such as hyaluronan,29 chitosan,30 starch,31 and alginate,32 have the capacity to maintain cellular viability and support stem cell differentiation. Yoon et. al found that hyaluronic acid-based scaffolds supported chondrogenic differentiation of adipose-derived MSCs cultured with bone morphogenetic protein-2 (BMP-2).33 In addition, polysaccharide-derived polymers have low toxicity and can be produced relatively inexpensively.34,35 However, although naturally-derived biomaterials have been widely used as stem cell scaffolds, their poor mechanical tunability and sometimes high batch-to-batch variation has led to further research aimed at addressing these shortfalls.36

1.2.2 Decellularized Tissue Scaffolds

The ECM of the human body is a gel-like, fibrous network that provides mechanical support and biochemical cues to cells that makeup the body’s tissues.37,38 ECM—which includes structural proteins, growth factors, proteoglycans, glycosaminoglycans, and proteolytic enzymes—has been implicated in cellular proliferation, differentiation, morphogenesis, adhesion, and migration.39 The recognition of ECM as being integral
to directing stem cell behavior has led to the production and use of scaffolds derived from decellularized tissue. These protein-based biomaterials offer the capability to simulate the role that native ECM plays in the process of tissue generation. Once tissue is harvested from allogeneic or xenogeneic donors, it is decellularized, often using detergents, chemical agents, enzymes or a combination of these. Nuclease and detergents may also be utilized to remove residual DNA. Removing the donor’s cells and cell remnants from the scaffold allows for a drastically lessened host immune response, post-implantation, compared to current organ transplant procedures. For some applications, such as whole-organ scaffolds, in which preserving ECM structure and bioactivity are paramount, tissue processing ends after decellularization. However, in instances when ECM derivatives, such as structural proteins, are required for scaffold synthesis, tissues are further digested, typically by employing chemical or enzymatic methods. It has been shown that tissue can be decellularized and processed in such a way that the risk of an adverse host immune response is minimized while scaffolds largely maintain their bioactivity profile. Such scaffolds have been used to facilitate the regeneration of multiple types of functional tissues. Table 1.1 demonstrates current clinical products that are composed of decellularized tissues. Although ECM-derived scaffolds offer the capability to serve as highly bioactive grafts, like many other naturally-derived scaffolds, their relatively limited mechanical adjustability and batch-to-batch variation hinders their versatility as platforms for tissue regeneration.
Table 1.1 - Clinical products composed of decellularized tissue.55

<table>
<thead>
<tr>
<th>Product</th>
<th>Tissue Source</th>
<th>Application Focus</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alloderm®</td>
<td>Human dermis</td>
<td>Soft tissue</td>
</tr>
<tr>
<td>Strattice™</td>
<td>Porcine dermis</td>
<td>Soft tissue</td>
</tr>
<tr>
<td>Zimmer Collagen Repair Patch™</td>
<td>Porcine dermis</td>
<td>Soft tissue</td>
</tr>
<tr>
<td>TissueMend®</td>
<td>Bovine dermis</td>
<td>Soft tissue</td>
</tr>
<tr>
<td>Oasis®, Surgisis®</td>
<td>Porcine small intestine</td>
<td>Soft tissue</td>
</tr>
<tr>
<td>Restore™</td>
<td>Porcine small intestine</td>
<td>Soft tissue</td>
</tr>
<tr>
<td>FortaFlex®</td>
<td>Porcine small intestine</td>
<td>Soft tissue</td>
</tr>
<tr>
<td>Meso BioMatrix™</td>
<td>Porcine mesothelium</td>
<td>Soft tissue</td>
</tr>
<tr>
<td>MatriStem®</td>
<td>Porcine urinary bladder</td>
<td>Soft tissue</td>
</tr>
<tr>
<td>GraftJacket®</td>
<td>Human dermis</td>
<td>Soft tissue, chronic wounds</td>
</tr>
<tr>
<td>OrthAdapt®, Unite®</td>
<td>Equine pericardium</td>
<td>Soft tissue, chronic wounds</td>
</tr>
<tr>
<td>NeoForm™</td>
<td>Human dermis</td>
<td>Breast</td>
</tr>
<tr>
<td>AlloPatch HD™, FlexHD®</td>
<td>Human dermis</td>
<td>Tendon, breast</td>
</tr>
<tr>
<td>CopiOs®</td>
<td>Bovine pericardium</td>
<td>Dentistry</td>
</tr>
<tr>
<td>Lyoplant®</td>
<td>Bovine pericardium</td>
<td>Dura mater</td>
</tr>
<tr>
<td>CorMatrix ECM™</td>
<td>Porcine small intestine</td>
<td>Pericardium, cardiac tissue</td>
</tr>
<tr>
<td>Perimount®</td>
<td>Bovine pericardium</td>
<td>Valve replacement</td>
</tr>
<tr>
<td>Hancock® II, Mosaic®, Freestyle®</td>
<td>Porcine heart valve</td>
<td>Valve replacement</td>
</tr>
<tr>
<td>Prima™ Plus</td>
<td>Porcine heart valve</td>
<td>Valve replacement</td>
</tr>
<tr>
<td>Epic™, SJM Biocor®</td>
<td>Porcine heart valve</td>
<td>Valve replacement</td>
</tr>
</tbody>
</table>

1.3 Synthetic Hydrogels and Stem Cell Encapsulation in Tissue Engineering

1.3.1 Synthetic Hydrogels

Hydrogels are hydrophilic materials, composed of one or more monomers, that have the ability to retain relatively large amounts of water without dissolving.58 Their largely aqueous makeup is considered to be integral to their capacity to be biocompatible.59 Hydrogels can be constructed using either synthetic or naturally-derived materials and can be polymerized via chemical or physical crosslinking.60-63 Commonly used synthetic monomers for hydrogel synthesis include polyethylene glycol (PEG),64 polyvinyl alcohol (PVA),65 and polyacrylamide (PAM).66 These hydrogels
can be engineered to be permeable to small molecules, such as gases, low molecular weight metabolites, and ions.67 This allows cells encapsulated in the material to receive nutrients and oxygen, and for removal of waste to take place.68 In addition, due to their large number of polar reactive sites, synthetic hydrogels have the ability to anchor biologically active molecules.69 This versatility has allowed researchers to modulate the adhesion, migration, and proliferation of mesenchymal stem cells cultured on or within synthetic hydrogels for the purpose of tissue regeneration.70,71 Hydrogels created using synthetic polymers also have largely-tunable mechanical characteristics.72,73 This makes them an attractive tool for stem cell culture in that substrate-level elasticity plays a significant role in stem cell fate.74 Therefore, as Engler et. al demonstrated, by modifying substrate stiffness, it is possible to control mesenchymal stem cell lineage and commitment.74 As such, there has been an increase in research dedicated to the development of synthetic hydrogels as functional 3D stem cell scaffolds due to their ability to be finely adjusted in terms of composition and mechanical behavior.

1.3.2 Stem Cell Encapsulation in Synthetic Hydrogels

In the utilization of hydrogels as platforms for tissue regeneration, there are two common methods of stem cell seeding that are generally employed. One strategy is to seed stem cells on the scaffold surface, allowing them to adhere to the porous structure.75 In this scenario, hydrogels are prefabricated, allowing researchers to use a wider range of polymerization methods, as long as the resultant scaffold is cytocompatible.75 Alternatively, cells may be encapsulated within the hydrogel precursor solution prior to polymerization.76 This approach necessitates that suitable materials and bioorthogonal chemical reactions be used in the polymerization process in order to maintain cellular viability and functionality.77 However, cell encapsulation has several benefits in in vivo applications. First, hydrogel precursor solutions have the potential to be injected into a tissue defect site instead of being cast into a desirable
shape prior to implantation. In addition, as opposed to requiring sutures or glue, an injected solution has the ability to diffuse through nearby tissue for effective adhesion. Optimally, scaffold design should allow for in vivo degradation of the hydrogel to progress at the same rate as tissue regeneration.

Largely due to its relative stability in vivo, PEG has been the most widely used synthetic monomer for hydrogel encapsulation. It also has ability to be easily functionalized, allowing for polymer crosslinking density, and therefore, its mechanical properties, including degradation rate, to finely controlled. From a bioactivity perspective, however, PEG hydrogels are relatively inert. Alone, their inability to mimic bioactive molecules in the native ECM significantly limits their capacity to promote tissue regeneration.

1.4 ECM-based Hybrid Hydrogel Scaffolds

In an effort to construct a 3D scaffold that combines the bioactivity of native tissues with the mechanical adjustability of synthetic hydrogels, researchers have designed hybrid hydrogels to promote stem cell proliferation and differentiation. While some investigations have been directed towards combining natural polymers, such as alginate and collagen, with synthetic fractions, numerous studies have employed synthetic oligopeptides to promote cellular attachment and proliferation. These designer peptides, which often contain the Arginylglycylaspartic acid (RGD) motif, are commonly conjugated to synthetic polymers using bioorthogonal chemical reactions, resulting in a functionalized hydrogel. Although the incorporation of designer peptides into hydrogels offers a convenient means of introducing a level of bioactivity into an otherwise inert scaffold platform, synthetic peptides deliver a relatively limited source of biologic information to adherent cells. As a result, they are unable to fully recapitulate proteins found in the native ECM. In addition, designer peptides can be costly to produce in substantial quantities. Therefore, due to its potential to mimic native ECM, there is an area of research dedicated to the integration of decellularized tissue and its
derivatives into synthetic hydrogels. Notably, Grover, et. al demonstrated that decellularized myocardial matrix could be combined with PEG to promote cellular migration and adhesion.96 Furthermore, Visser et. al showed that a blend of decellularized cartilage, meniscus, and tendon tissue could be functionalized and integrated with a gelatin methacrylamide hydrogel to affect chondrogenic differentiation in encapsulated MSCs.97 These studies indicate that ECM-based hybrid hydrogels have the potential to affect stem cell differentiation, and therefore, tissue regeneration, by means of a biologically complex, mechanically tunable scaffold.

1.5 Future Work and Conclusion

While the field of tissue engineering has seen rapid progression in the evolution of biomaterials, there remains a need for further development of stem cell scaffolds that can accurately recapitulate the structural and physiological aspects of native ECM. The notable advancements in cellular culture, scaffolds, and integration methods reviewed here have the potential to lead to groundbreaking clinical products that promote effective and predictable regeneration of functional tissue.
2. IN VITRO EVALUATION OF HYBRID SYNTHETIC/ADIPOSE-DERIVED ECM HYDROGELS AS A CULTURE PLATFORM FOR HUMAN ADIPOSE-DERIVED STEM CELLS

2.1 Project Purpose

There is a distinct need in the field of tissue engineering for a three-dimensional scaffold that has the ability to maintain the multilineage differentiation capacity of mesenchymal stem cells. Human adipose tissue offers an abundant source of allogenic material that contains ECM proteins, peptides, and glycosaminoglycans. When decellularized and combined with a mechanically tunable polymer, these ECM components offer the potential to recapitulate the stem cell niche in a more cost-effective manner than commonly used synthetic peptides. As described herein, human decellularized adipose tissue were incorporated, at varying concentrations, with a thiol-acrylate fraction that was then polymerized to produce hydrogels via a Michael addition reaction. These hydrogels were characterized based on their ability to support the proliferation, maintain the viability and retain the multipotency of human adipose-derived stem cells (hASCs). It was found that hASCs encapsulated in hydrogels containing high concentrations of adipose-derived ECM (adECM) displayed increased expression of potency markers for a longer duration relative to cells encapsulated in Matrigel® or adECM-free PEG hydrogels. These results indicate that adECM hydrogels hold promise as a cost-effective platform for mesenchymal stem cell multipotency maintenance for tissue engineering applications.

2.2 Introduction

From the time that mesenchymal stem cells were discovered and used clinically, there has been a need to expand cells in a native 3D conformation while also maintaining stem cell potency for future use. Corning® Matrigel® Matrix revolutionized stem cell culture when it was first widely distributed. However, nearly 30 years later, clinicians and scientists still rely on Matrigel® to grow and expand stem cells in 3D. While Matrigel® has paved the way for many discoveries, it is still unclear what signaling molecules are present
within the hydrogel. In addition, Matrigel® cannot be mechanically or functionally tuned, and there is a high batch-to-batch variability.

Hydrogels' high water content in addition to their highly tunable functional groups, degradation schemes, and mechanical properties make them widely used as 3D cellular scaffolds for stem cell potency maintenance and expansion.98,99 Researchers have exploited this versatility, combined with synthetic peptides to develop biomimetic hydrogels that provide microenvironments similar to those observed in native cell niches.100-105 Extensive research has shown that the structure and content of such hydrogels can play a critical role in determining cell fate. Synthetic peptides have a substantial advantage as they are reproducible and can be easily modified for incorporation into hydrogel networks. However, synthetic peptides are very expensive to synthesize and purify, limiting the scope and scale of potential applications. As such, there has been a surge in research dedicated to the development of scaffolds, namely hydrogels, derived from naturally occurring biological materials. There is evidence that suggests that these natural biomaterial-laden scaffolds provide a marked increase in biocompatibility and bioactivity.100,106-109 Incorporation of extracellular matrix (ECM) isolated from decellularized tissues in hydrogels has been of particular interest. Cell adhesion, growth, proliferation, differentiation, and cell fate are heavily dependent on the signaling molecules and immobilized proteins contained within surrounding ECM.110-111 Many researchers have used detergents, enzymes, and mechanical forces to remove cellular material while retaining proteins and bioactive molecules.109,112 Processed ECM can then be incorporated into a synthetic scaffold to create a highly tunable construct with biological components. Such scaffolds have shown success in facilitating the regeneration of functional tissue.113,114

Adipose tissue from lipoaspirate is clinical waste that can be used as allogenic material for the development of ECM-laden constructs. Adipose contains various ECM
components, such as collagen, reticular fibers, elastin fibers, nerve fibers, vascular stroma, lymph nodes, and endocrine and paracrine signaling molecules. Subcutaneous adipose tissue provides a readily accessible source of allogeneic material, as many healthy individuals electively seek out plastic and reconstructive surgical procedures to remove unwanted subcutaneous adipose tissue via liposuction or abdominoplasty. The majority of this human tissue is discarded as medical waste. Our preliminary results have documented the reproducible ability to decellularize and extract human adipose tissue ECM (adECM). An evaluation of adECM revealed its key compositional qualities. Analysis of the peptide fragments by mass spectroscopy identified 77 individual proteins in the adECM. Nearly 50% of these proteins were associated with either the extracellular space or the plasma membrane and included multiple collagen family members. Of the remainder, over 37% had cytoplasmic associations while ~8% were of nuclear origin. Overall, the adECM proteins identified were consistent with earlier proteomic analyses of adipose derived cells and tissues. Therefore, the reliable incorporation of adECM into a synthetic scaffold has the potential to be a more cost effective alternative to current commercial peptide-based products.

In this study, we present the synthesis of a hybrid ECM-laden hydrogel to be used as a 3D scaffold to support human adipose-derived stem cell (hASC) growth and maintain cellular potency. The adECM was incorporated into a synthetic thiol-acrylate polymer fraction and polymerized via a base-catalyzed Michael Addition reaction. Modulation of the reaction conditions affects polymer crosslinking. As such, the Michael Addition provides the ability to tune the mechanical properties of the polymer fraction, the component chiefly responsible for the mechanical properties and the degradation profile of the hydrogel. The adECM, obtained by decellularizing lipoaspirate as described by McIntosh et. al., was utilized for its array of bioactive molecules and availability.
integration of adECM with a synthetic polymer provides a dynamic environment that fosters active cellular responses.

Thiol-acrylate hydrogels laden with varying amounts of adECM were synthesized and compared. The mechanical properties of each hydrogel were characterized via compression testing to determine Young’s modulus. Swelling experiments were conducted to determine swell ratio, cross-link mesh size, and mass loss behavior. Human adipose-derived stem cells (hASCs) were encapsulated within each hydrogel prior to polymerization. Cell viability, proliferation, and morphology were evaluated via CCK-8 metabolic assay, Click-iT®Plus EdU assay, and F-actin staining. Maintenance of cell pluripotency was measured via Q-RT-PCR of the human pluripotency genes SOX2 and NANOG.

2.3 Materials and Methods

2.3.1 Cell Culture

hASCs were isolated from lipoaspirate obtained from LaCell LLC (New Orleans, LA). Extracts of subcutaneous adipose tissue were acquired from three consenting donors undergoing elective plastic surgery under a protocol approved by either the Pennington Biomedical Research Center Review Board (Baton Rouge, LA) or Western Institutional Review Board (Puyallup, WA). hASCs were isolated as previously described.116 hASCs were maintained in T125 flasks at 37°C at 5% CO₂ in a humidified atmosphere. Cells were allowed to grow until 80% confluent. Second passage (P2) cells were used in the extraction, DNA synthesis, and quantitative reverse-transcript PCR (qrt-PCR) studies.

2.3.2 ECM Decellularization

Adipose tissue (~100g) from two consenting donors (LaCell LLC, New Orleans, LA) was placed in 200 mL of 3.4 M sodium chloride (NaCl) buffer for 1 hour. The tissue was then homogenized in solution using an an electric homogenizer (Omni Ultra Shear Small
Fibrous tissue that could not be mechanically homogenized was continuously removed and collected, while all other tissue components were disregarded as waste. 100 mL of 2 M urea buffer was added to the collected fibrous tissue, and the solution was refrigerated for 48 hours. The urea/fibrous tissue solution was then centrifuged three times at 23,000 g at 4 °C for 20 minutes. After each centrifugation, the pelleted tissue was collected and the solvent was discarded as waste. The fibrous pellet was then dialyzed against deionized water for 24 hours before digestion with 0.5% (w/v) pepsin in 0.5 M acetic acid. After completion of the digestion reaction, the pepsin was deactivated by raising the pH of the solution to 9.0 using 1 M sodium hydroxide (NaOH). The solution was then incubated overnight at 37 °C, followed by lowering the pH of the solution to 7.4 with 1 M hydrochloric acid (HCl). The neutralized solution was dialyzed again against deionized water for 24 hours before being flash-frozen and lyophilized to yield a adipose-derived extracellular matrix (adECM) powder. The adECM powder was gas sterilized with ethylene oxide prior to use.

2.3.3 Protein Quantification

The Pierce™ BCA Assay (ThermoFisher Scientific, USA) was used to determine the total protein content in ECM samples. Briefly, diluted aliquots (10 μL) were removed (n=3) from processed ECM and incubated with assay reagents for 30 min at 37 °C in a 96-well plate in accordance with the Pierce™ protocol. The plate was then cooled to room temperature and absorbance was measured at 532 nm using a fluorescence plate reader (Wallac 1420 multilabel HTS counter).

2.3.4 Proteomics Analysis

In order to characterize the adECM, liquid chromatography-mass spectroscopy (LC-MS) was performed. Samples were prepared by combining 15 μL digestion buffer (8
mg/mL ammonium bicarbonate in water), 3 \( \mu L \) reducing reagent (30 mg/mL TCEP), and 12 \( \mu L \) sample solution containing 7 \( \mu g \) lyophilized adECM powder or 12 \( \mu g \) BSA standard protein (total volume 30 \( \mu L \)). The sample was then reduced using TCEP (30 mg/mL) at 50°C for 7 min, and cooled to room temperature, before being centrifuged to collect the sample. 3 ul alkylating reagent (18mg/mL iodoacetamide in digestion buffer) was added to the sample and it was incubated in the dark at room temperature for 20 min. Finally, 3 \( \mu L \) proteomics grade trypsin, activated with ammonium bicarbonate (0.1 \( \mu g/\mu L \)), was added to the sample and it incubated at 37 °C for 3 hours and stored at -20 °C. LC-MS/MS analysis was performed using Thermo Finnigan's ProteomeX workstation LTQ linear ion trap MS (Thermo Electron, USA). 12 mL of sample was injected into a peptide trap cartridge (Agilent, USA). The sample was eluted onto a 10-cm reverse-phase Pico Frit column packed in house with 5 \( \mu m \), 300 Å pore size C18, and then separated on an RP column by gradient elution. The mobile phases were H\(_2\)O (A) and ACN (B), both of which contained 0.1% (v/v) formic acid. The flow rate was maintained at 200 nL/min. The gradient was started at 2% B, reached 60% B in 50 min, 80% B in the next 5 min, and then 100% A in the final 15 min. Data-dependent acquisition mode (m/z 300-1800) was enabled, and each survey MS scan was followed by five MS/MS scans with the 30 s dynamic exclusion option enabled. The spray voltage was 1.9 kV and the temperature of the ion transfer tube was set at 195 °C. The normalized collision energy was set at 35%. Samples were acquired in triplicates.

2.3.5 Fabrication of adECM Hydrogels

Hydrogel reagents were reacted together employing a thiol-ene base-catalyzed Michael addition reaction with a 1:1 functional ratio of acrylate to thiol. adECM proteins in phosphate buffered saline (PBS) were preincubated for 30 minutes with PEG-acrylate monomers at various concentrations (w/v%) (0%, 0.01%, 0.1%, and 1%). During
preincubation, a solution of PBS, ethoxilated-trimethylolpropan tri(3-mercaptopropionate) (ETTMP 1300, and 1 M NaOH were reacted together in a separate tube. After incubation, the PEG-acrylate and adECM solution was mixed with the ETTMP 1300 and NaOH solution. Gelation occurred in less than one minute, and could be controlled by altering the temperature and the amount of NaOH.

2.3.6 Analysis of adECM Distribution

Hydrogels of various concentrations (0%, 0.01%, 0.1%, 1%) were embedded in O.C.T for cryosectioning, and stained with Masson’s Trichrome staining kit using the manufacturer’s protocol. Briefly, the cryosection was stained in working Weigert’s iron hematoxylin solution for 30 s and washed in deionized (DI) water for 5 min. It was then placed in biebrich scarlet–acid fuchsin for 5 min. After being rinsed in DI water, the slides were placed in a phosphomolybdic acid/ phosphotungstic acid solution for 10 min and then in an aniline blue solution for 7 min. After rinsing briefly DI water, the sections were placed in a 1% acetic acid solution for 30 s. Slides were then dehydrated and mounted. Images were taken using a BX51 Olympus microscope. Collagen distribution was measured and analyzed via color counting software (Photoshop CS5, Adobe, USA). Each sample was randomly measured in five different positions and four total samples were included for analysis (n=20).

2.3.7 Mechanical Testing

Hydrogels with protein additives (0%, 0.01%, 0.1%, and 1%) were formed using a 5 mm (height) × 10 mm (diameter) cylindrical mold and allowed to swell overnight before being tested to determine elastic modulus and maximal compressive strength. The results reported (n=5) were taken with 80% strain at a compression rate of 1 mm/min from an Instron Mechanical Test System 5696 using an Instron 2 kN static load cell (Instron Corporation, Canton, MA).
2.3.8 Swelling and Mass Loss Experiments

Four sets of hydrogels with varying adECM content (0%, 0.01%, 0.1%, 1%) (w/v) were polymerized via base-catalyzed Michael addition as described above. Upon formation, hydrogels (135 µL) were incubated at 37 °C in 1 mM PBS, pH 7.4. At 0 h, 12 h, 24 h, 48 h, 72 h, 120 h, and 168 h, hydrogel samples were collected, pat dry, and their mass after swelling (\(M_S\)) was recorded. The hydrogel samples were then lyophilized overnight, and their dry mass was recorded (\(M_D\)). Hydrogel swelling ratio (\(Q_M\)) was then calculated using Eq. 2.1 below:

\[
Q_M = \frac{M_S}{M_D} \tag{Eq. 2.1}
\]

\(Q_M\) was then used to calculate the volume swelling ratio (\(Q_V\)) using Eq. 2.2 below:

\[
Q_V = 1 + \frac{\rho_p}{\rho_s} (Q_M - 1) \tag{Eq. 2.2}
\]

Where \(\rho_p\) is the density of PEG-acrylate (1.12 g/cm\(^3\)) and \(\rho_s\) is the density of the solvent (1.0 g/cm\(^3\)) for PBS.

Mass Loss percentage (\(M. L. \%\)) was determined using the Eq. 2.3 below:

\[
M. L. \% = \frac{M_S - M_D}{M_D} \tag{Eq. 2.3}
\]

2.3.9 Viability Of hASCs After Acute Exposure To Hydrogel Extracts

The cytotoxic effects of scaffold degradation products were evaluated using Cell Counting Kit-\(^{\text{TM}}\) (CCK-8) (Dojindo Molecular Technologies), a commonly used assay to measure cell viability and proliferation. Following scaffold incubation periods of 7 and 14 days, extracts were filtered. hASCs were seeded at a density of 91 cells/mm\(^2\) for 24 hours in stromal media (DMEM-F12, 10% FBS, 1% Penicillin-Streptomycin-Amphotericin B solution). The media was replaced with hydrogel extract. hASCs cultured in stromal
media served as a positive control. After 24 hour incubation at 37 °C and 5% CO₂, 100 µL of 10% CCK-8 solution were added to each well and samples were incubated at 37 °C for 12 hours. The absorbance was measured at 460 nm using a plate reader (Wallac 1420 multilabel HTS counter) and the cell viability was normalized based on the standard curve.

2.3.10 Encapsulation and Culture of hASCs with Synthetic adECM Hydrogel

hASCs were encapsulated in the scaffolds at 1 x10⁵ cells / mL. Hydrogels were formed in a 96-well plate at a volume of 45 µL and were polymerized at 37 °C to ensure cell suspension and survivability. Encapsulated cells were cultured in growth media (Dulbecco's modified Eagle's medium [DMEM/F12], 10% fetal bovine serum [FBS], and 1% Penicillin-Streptomycin-Amphotericin B solution) for up to 14 days with media maintenance performed three times a week.

2.3.11 EdU Staining

Encapsulated hASCs were characterized based nuclear morphology, and DNA synthesis rate. In accordance with iClick EdU Andy Fluor 555 Imaging Kit (Genecopoeia) instructions, the nuceloside analog, EdU (5-ethynyl- 2’-deoxyuridine), was introduced to culture media four days prior to cell fixation. At 7 and 14 days, scaffolds were fixed in 4% paraformaldehyde, rinsed three times with PBS, and permeabilized using .5% Triton X-100 in PBS. Scaffolds were stained following iClick EdU Andy Fluor 555 Imaging Kit protocol instructions. To assess nuclear morphology, scaffolds were stained in a 5 µM DRAQ5 solution (Life Technologies). Scans for DRAQ5 fluorescence (Exₘₐₓ 647, Emₘₐₓ681), and Andy Fluor 555 fluorescence (Exₘₐₓ 555, Emₘₐₓ,565) were performed with a white light laser at 641 nm and 519 nm, respectively, in the sequential scanning mode at 1024 x 1024 pixel resolution using a 20x objective (HC PL APO CS2 20x/ .70 1mm; Leica, Bensheim, Germany). Images were brightened uniformly using Adobe Photoshop CC.
2017. Previously described methods were employed to quantify hASC proliferation rate (n=5). Briefly, the Photoshop thresholding tool was used to obtain a cut-off intensity in images taken at 641 nm (DRAQ5) and 519 nm (Andy Fluor 555). Pixels above the cut-off intensity in groupings with areas greater than 7 μm were considered stained. The number of Edu-stained cells was divided by the number of DRAQ5-stained cells to obtain DNA synthesis rate.

2.3.12 F-Actin Staining

To assess cytoskeletal shape, scaffolds were stained in a 200 nM F-actin 488 solution (Life Technologies). Scaffolds were rinsed before being mounted on PBS on a glass slide and imaged using a Leica TCS SP8 confocal laser scanning microscope. Scans for Alexa 488 fluorescence (Ex\textsubscript{max} 495, Em\textsubscript{max} 518), were performed using a a white laser at 460 nm at 1024 x 1024 pixel resolution using a 20x objective (HC PL APO CS2 20x/ .70 1mm; Leica, Bensheim, Germany).

2.3.13 Quantitative Real-time Polymerase Chain Reaction

RNA was isolated from encapsulated scaffolds as previously described. In addition, RNA was purified using the PureLink\textsuperscript{®} RNA Minin Kit in accordance with manufacturer’s instructions. Total RNA to cDNA EcoDry Premix (ClonTech) was used for cDNA synthesis. qRT-PCR was performed using 2× iTaq SYBR green supermix with ROX (Biorad) and primers for SRY-related HMG-box (SOX2) and human NANOG (hNANOG) to quantify gene expression associated with maintenance of multipotency in hASCs encapsulated into hydrogels and cultured for 7 and 14 days. Reactions were performed with a MJ Mini Thermal Cycler (BioRad). The sequences of PCR primers (forward and backward, 5’–3’) were as follows: SOX2, 5’-TACAGCATGTCTACTCGCAG-3’, and, 5’ GAGGAAGAGGTAACCACAGGG-3’; hNANOG, 5’- ATGCCTCACACGGAGACTGT-3’ and 5’- GGGCTGTCTGAATAAGCA-3’. Samples were normalized (ΔCt) against the housekeeping gene 18S rRNA (forward
and backward, 5'-3'): 5'-AAACGGCTACCACATCCAAG-3' and 5'-CCTCCAATGGATCCTCGTTA-3'. The -ΔΔCt value of SOX2 and hNANOG in encapsulated polymers was calculated using the ΔΔCt method.

2.3.14 Statistical Analysis

All results were expressed as mean ± standard deviation. Data were analyzed with one-way analysis of variance, followed by Tukey's minimum significant difference post hoc test for pairwise comparisons of main effects. For all comparisons, a p-value < 0.05 was considered significant.

2.4 Results

2.4.1 Hydrogel Synthesis

In this study, the effects of adECM hydrogels on encapsulated hASCs were evaluated. A schematic for adECM incorporation and hydrogel polymerization is presented in Scheme 2.1.

**Scheme 2.1 - hASC encapsulation and adECM hydrogel fabrication.**
2.4.2 Liquid Chromatography-Mass Spectroscopy

LC-MS was used to characterize proteins found in adECM. Three distinct proteins were identified (Table 2.1). Collagen, type I, alpha 2 was found in all three of the samples. Collagen, type I, alpha 1 was found in samples 1 and 3. Finally, collagen type III alpha 1 was identified only in sample 3 (Table 2.2). The results are consistent with other studies performed by Edwin C.M. et al.118

2.4.3 adECM Distribution

Masson’s Trichrome staining was performed to investigate the distribution of adECM collagen in all adECM hydrogel compositions. Significantly more collagen, stained blue, was found in the 1% adECM group, compared to the 0.1% adECM hydrogel, the 0.01% adECM hydrogel and the 0% adECM group (Figure 2.1). In all samples, collagen, when present, appeared to be punctate and not uniformly distributed throughout the scaffold (Figure 2.2).

Table 2.1 - Identification of proteins in adECM

<table>
<thead>
<tr>
<th>Protein Name</th>
<th>UniProt accession no.</th>
<th>M.W. (kDa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Collagen alpha-2 (I) chain [Human]</td>
<td>A0A087WTA8</td>
<td>129.1</td>
</tr>
<tr>
<td>Collagen alpha-2 (I) chain [Human]</td>
<td>P02452</td>
<td>138.9</td>
</tr>
<tr>
<td>Collagen alpha-3 (III) chain [Human]</td>
<td>P02461</td>
<td>138.5</td>
</tr>
</tbody>
</table>

Table 2.2 - Distribution of proteins among tested adECM samples

<table>
<thead>
<tr>
<th>Sample</th>
<th>Protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Collagen alpha-2(I) chain [Human]</td>
</tr>
<tr>
<td></td>
<td>Collagen alpha-1(I) chain [Human]</td>
</tr>
<tr>
<td>2</td>
<td>Collagen alpha-2(I) chain [Human]</td>
</tr>
<tr>
<td>3</td>
<td>Collagen alpha-1(I) chain [Human]</td>
</tr>
<tr>
<td></td>
<td>Collagen alpha-1(III) chain [Human]</td>
</tr>
</tbody>
</table>
Figure 2.1 - Collagen content of adECM hydrogels. All samples are statistically significant.

Figure 2.2 - Masson’s Trichrome staining of adECM hydrogels. A, D) 1% adECM, B), E) 0.1% adECM, C), F) 0.01% adECM, G), F), H) 0% adECM. Collagen is stained blue. For A, B, C&G, the scale bar is 1 mm and for D,E, F&H, the scale bar is 200 um.
2.4.4 Mechanical Characterization

Figure 2.3 shows the mechanical strength data for hydrogel scaffolds containing different concentrations of adECM. Increasing adECM concentration, lead to a decrease in Young’s modulus. Hydrogels containing 0.01%, 0.1%, and 1% displayed a decreasing trend in Young’s Modulus.

![Graph showing Young's modulus for different adECM concentrations]

** statistical significance, p < 0.05, * statistically significant than ** values, p < 0.05.

Figure 2.3 - Young’s modulus of PEG hydrogels with varying concentrations of adECM additives.

2.4.5 Swelling and Mass Loss

Scaffolds containing adECM were assessed for changes in swelling, mesh size, and mass-loss during incubation. Hydrogel swell ratio over the course of 168 hours is displayed in Figure 2.4. Scaffolds containing 1% adECM showed increased swelling size between 120 and 168 hours compared to all other scaffold samples. There were no significant differences in swell ratio noted among scaffolds containing 0%, 0.01%, and 0.1% adECM across all recorded time points. Scaffold mass loss was evaluated up to 168 hours (Figure 2.5). From 12 to 120 hours, hydrogels containing 1% adECM exhibited
mass loss to the greatest extent compared to all other scaffold samples. At 168 hours, hydrogels containing 1% adECM showed increased mass loss compared to scaffold groups containing 0% and 0.1% adECM. At 24 and 48 hours, hydrogels containing 0.1% adECM displayed greater mass loss compared to samples containing 0.01% adECM, however, at 168 hours, scaffolds containing 0.01% adECM showed increased mass loss compared to 0.1% adECM samples.

Figure 2.4 - Swelling ratio for PEG hydrogels with varying concentrations of adECM additives expressed over an incubation period of 168 hours. Error bars shown. 1% adECM hydrogel was found to have a significantly higher swell ratio at 168 hours than the rest of the samples.

Figure 2.5 - Mass loss of PEG hydrogels containing varying concentrations of adECM additives expressed as percent lost over an incubation time of 168 hours. 1% adECM hydrogels displayed significantly increased mass loss compared to all other samples at 12, 24, 48, 72, and 120 hours. At 168 hours, 1% adECM hydrogels displayed significantly increased mass loss compared to 0.1% and 0% adECM hydrogel samples.
2.4.6 hASC Viability After Exposure to Scaffold Extracts

Viability of hASCs following exposure to hydrogel extracts is shown in Figure 2.6. Compared to cells cultured in stromal media, hASCs exposed to extracts of scaffolds containing 0%, 0.01%, 0.1%, and 1% adECM displayed no significant difference in viability at both 7-day and 14-day time points.

![Graph showing cell viability](image)

Figure 2.6 - Cell viability for PEG extracts with varying concentrations of adECM additives. Extracts were taken after either 7 or 14 days of incubation. Values were normalized to a live control of hASCs cultured in stromal media.

2.4.7 hASC Morphology and DNA Synthesis

Corresponding confocal imaging of hASCs (Figure 2.7 and Figure 2.8) showed cells encapsulated in Matrigel® to have a greater number of protrusions than those cultured in PEG-based scaffolds at 7 days. At 14 days, hASCs cultured in Matrigel® displayed more aligned, stretched and organized development of F-actin in comparison to PEG-based scaffolds. Qualitatively, no significant cellular morphological changes were appreciated within adECM hydrogel groups between 7 and 14 days. Image analysis using DRAQ5
and EdU staining indicated significant cellular proliferation rates for only Matrigel® between 0 and 7 days (214% ± 34%) and 7 and 14 days (288% ± 57%).

Figure 2.7 - Images acquired at 7 days using Leica SP8. Scale bars = 50 µm. hASCs encapsulated in Matrigel® samples stained with DRAQ5 (Blue) (A) and F-Actin (Green) (A'') exhibited a greater number of protrusions compared to cells in 0% adECM (B,B''), 0.01% adECM (C,C''), 0.1% adECM (D,D''), 1% adECM (E,E'') hydrogel samples. hASCs in Matrigel® stained with Edu (Red) (A') displayed a proliferation rate of (214% ± 34%) between 0 and 7 days. PEG-hydrogel samples did not display significant proliferation rates (B'-E').
Figure 2.8 - Images acquired at 14 days using Leica SP8. Scale bars = 50 µm. hASCs encapsulated in Matrigel® samples stained with DRAQ5 (Blue) (F) and F-Actin (Green) (F'') demonstrated more aligned, stretched and organized development of F-actin compared to cells in 0% adECM (G,G''), 0.01% adECM (H,H''), 0.1% adECM (I,I''), 1% adECM (J,J'') hydrogel samples. hASCs in Matrigel® stained with Edu (Red) (F') displayed a proliferation rate of (288% ± 57%) between 0 and 14 days. PEG-hydrogel samples did not display significant proliferation rates (G'-J').

2.4.8 Quantitative Reverse-transcript PCR of Human Pluripotency Markers

Analysis of gene expression markers of pluripotency indicated that 0.1% and 1% adECM groups had significantly higher levels of both NANOG (Figure 2.9) and SOX2
(Figure 2.10) gene expression compared to Matrigel® and 0% adECM samples at both 7 and 14 days. At both time points, there was no significant difference in *NANOG* or *SOX2* expression between the 0.1% adECM and 1% adECM groups.

Figure 2.9 - Relative expression levels of *NANOG* in Matrigel® and PEG hydrogels with adECM additives after 7 and 14 days in culture.

Figure 2.10 - Relative expression levels of *SOX2* in Matrigel® and PEG hydrogels with adECM additives after 7 and 14 days in culture.
2.5 Discussion

hASCs were used to evaluate the impact of different adECM compositions in PEG-acrylate hydrogels on cell behavior. Corning® Matrigel® was used as a positive control because of its wide use in 3D cell culture.

To address the need for a mechanically tunable, cost-effective 3D scaffold that supports stem cell viability and maintains cellular potency, adipose was decellularized and incorporated with a thiol-acrylate polymer fraction that was then polymerized to produce a hydrogel. Scaffolds of various adECM concentrations were evaluated. ECM additives beyond 1% of total volume did not disperse homogeneously, leading to an unstable hydrogel. Therefore, experiments were designed to test the physical and physicochemical properties of PEG hydrogels with adECM concentrations (w/v) of 0%, 0.01%, 0.10%, or 1%.

Hydrogel swelling is dependent upon the polymer network structure, cross-linking density, and hydrophilicity. The equilibrium degree of swelling was experimentally determined for each hydrogel. Initial mechanical studies examined the swelling, mass loss, and porosity of the hydrogel scaffolds. Results indicated that the elastic modulus of PEG hydrogels with adECM concentrations of 0.01% and 0.10% was not significantly different than that of hydrogels containing 0% adECM. Hydrogels containing 1% adECM showed significantly a lower Young’s modulus than all other experimental PEG hydrogels. Similarly, 1% adECM hydrogels displayed significantly more rapid rates of mass loss and greater degrees of swelling. We hypothesize that this phenomenon could be caused by a greater degree of steric hindrance during polymerization, resulting in less efficient crosslinking in hydrogels with higher adECM concentrations. However, 1% adECM hydrogels showed approximately a one hundred-fold increase in elastic modulus compared to Matrigel® (450 Pa). In addition, the degrees of stiffness of the PEG-
based hydrogels evaluated in this study were, overall, greater than those of many other reported PEG-based hydrogels.121-126

After mechanical characterization, subsequent studies focused on assessing hASC viability, proliferation, morphology and differentiation capacity upon encapsulation in hydrogels containing varying amounts of adECM. When exposed to hydrogel extracts, hASCs exhibited no loss of viability in comparison to growth media controls. These results are in agreement with previous findings from PEG based hydrogel extracts studies where mouse fibroblasts (Balb/3T3)) were treated with extracts from PEG-vinyl Sulfone hydrogels.126 Characterization utilizing EdU staining assay indicated decreased DNA synthesis in adECM hydrogels relative to Matrigel® controls at both 7 and 14 days. In addition, qualitative examination of F-actin and DRAQ5 staining results suggested little difference in cellular morphology among PEG hydrogels, but substantial difference at 14 days between Matrigel® and PEG scaffolds. Previous studies have shown that cells proliferate more rapidly in hydrogels containing immobilized matrix proteins, such as RGD. The effects of the adECM additives may have been greater if the matrix proteins were bound. In addition, the study found that greater hydrogel stiffness amplified the effects of the membrane proteins.127 Therefore, the lower adECM concentrations may result in higher cell proliferation due to the effects of matrix stiffness on ECM additives.

Finally, RT-qPCR results indicated that cells encapsulated in all PEG hydrogel samples initially exhibited significantly higher NANO and SOX2 expression compared to Matrigel® samples, and that hydrogels containing higher concentrations of adECM maintained increased expression of both genes at 14 days. Yu et al. demonstrated that hASCs expanded on decellularized adipose tissue microcarriers maintained their trilineage differentiation capacity to a greater extent than those cultured on commercially-sourced Cultispher-S microcarriers, suggesting that adipose-derived ECM scaffolds may have the ability to conserve multipotency in hASCs to a larger degree than
commonly used commercial scaffold platforms. Further studies must be performed in order to further confirm the ability of adECM hydrogels to maintain multipotency in hASCs.

Our work highlights that the addition of adECM to otherwise biologically inactive scaffolds can significantly alter the potency potential of encapsulated hASCs. adECM hydrogels have the capability to be further developed for use in multipotency maintenance in stem cells. Tuning the mechanical characteristics, adECM content, and seeding density of the platform has the potential to lead to the enhancement of cellular expansion and multilineage differentiation capacity.

2.6 Conclusion

Over the course of this study, we decellularized and processed adipose-derived ECM in a form usable for a number of tissue engineering applications. adECM was incorporated in varying concentrations into a PEG-acrylate hydrogel. Analysis suggested that, like Matrigel®, adECM-composite hydrogels provide a microenvironment that is able to sustain cell viability. However, the physicochemical and mechanical properties of PEG-acrylate hydrogels can be easily manipulated and finely tuned to satisfy the needs of project-specific applications. Overall, this technology can be versatile for the growth and expansion of mesenchymal stem cells. In addition, adECM can be processed from abundant lipoaspirates, which are often discarded as medical waste, providing a low cost source of bioactive material for use in scaffold fabrication. Further studies need to be conducted to compare cell behavior in the presence of synthetic peptides. We would hypothesize that signaling molecules and membrane proteins present in ECM would provide a more finely-tuned cell niche than synthetic peptides alone.

2.7 Acknowledgements

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3. PRELIMINARY EVALUATION OF HYBRID SYNTHETIC/THIOLATED ADIPOSE-
DERIVED ECM HYDROGELS

3.1 Project Purpose

Inspired by the findings that adipose-derived ECM can be converted into a
cytocompatible hydrogel after combination with a synthetic thiol-acrylate fraction, efforts
were conducted in order to improve the performance of the hybrid synthetic/adipose-
derived ECM hydrogel platform. In this study, adipose-derived ECM was thiolated prior
to hydrogel polymerization via a ring opening polymerization in order to promote more
uniform dispersion and greater concentration of adECM proteins. adECM was
incorporated, at varying concentrations, with a synthetic thiol-acrylate fraction that was
then polymerized to produce hydrogels via a Michael addition reaction, as described in
Chapter 2 of this thesis. Hydrogels were characterized based on their stiffness and ability
to maintain the viability human adipose-derived stem cells (hASCs). It was found that
hASCs seeded on hydrogels containing higher concentrations of thiolated adECM (tadECM) demonstrated decreased viability compared to tadECM-free hydrogels. These
results indicate that tadECM hydrogels may need to be further improved as stem cell
scaffolds, possibly by increasing tadECM functionality.

3.2 Introduction

The ability to obtain uniformity in scaffold structure has been demonstrated to greatly
improve stem cell spatial distribution, growth and proliferation. Moreover, it can lead to a
more committed differentiation pathway. Decellularized adipose-derived extracellular
matrix has been found to induce adipogenesis in hASCs both \textit{in vitro} and \textit{in vivo}. These scaffolds have been shown to have high concentrations of ECM proteins, however, they are relatively mechanically unstable compared to synthetic polymer
platforms.

In this preliminary study, we present a strategy for increasing the ECM content in
synthetic ECM/PEG hydrogel by using adipose-derived ECM that has been thiolated via
a ring opening polymerization. Thiolation efficiency was quantified prior to the synthesis
of three hydrogels containing various thiolated adECM (tadECM) compositions. The mechanical properties of hydrogels with different ECM concentrations were characterized. In addition, viability of hASCs seeded on the hydrogels was assessed.

3.3 Materials and Methods

3.3.1 ECM Decellularization

Adipose tissue was decellularized as previously described in Chapter 2 of this thesis, briefly, (~100g) was placed in 200 mL of 3.4 M sodium chloride (NaCl) buffer for 1 hour. The tissue was then homogenized using an electric homogenizer (Omni Ultra Shear Small Volume). Fibrous tissue that could not be mechanically homogenized removed. 100 mL of 2 M urea buffer was then added to the collected fibrous tissue, and the solution was refrigerated for 48 hours. The fibrous tissue was centrifuged three times at 23,000 g at 4 °C for 20 minutes. The fibrous pellet was then dialyzed against deionized water for 24 hours before digestion with 0.5% (w/v) pepsin in 0.5 M acetic acid. After completion of the digestion reaction, the pepsin was deactivated by raising the pH of the solution to 9.0 using 1 M sodium hydroxide (NaOH). The solution was then incubated overnight at 37 °C, followed by lowering the pH of the solution to 7.4 with 1 M hydrochloric acid (HCl). The neutralized solution was dialyzed again against deionized water for 24 hours before being flash-frozen and lyophilized to yield a decellularized, adipose-derived extracellular matrix (adECM) powder.

3.3.2 Protein Quantification

The Pierce™ BCA Assay (ThermoFisher Scientific, USA) was used, as described in Chapter 2 of this thesis, to determine the total protein content in ECM samples. Diluted aliquots of processed ECM (10 μL) were removed (n=3) and incubated with assay reagents for 30 min at 37 °C in a 96-well plate in accordance with the Pierce™ protocol. The plate was then cooled to room temperature and absorbance was measured at 532 nm using a fluorescence plate reader (Wallac 1420 multilabel HTS counter).
3.3.3 ECM Thiolation

adECM was thiolated as previously described. Briefly, adECM (80 mg) was immersed in 20 mL EtOH: PBS 1:1 (v/v) solution containing 0.033 M γ-thiobutyrolactone at room temperature for 24 h. Then, the pH of the solution was lowered to 5 for 10 min using acetic acid (Sigma). The solution was dialyzed against deionized water overnight, then lyophilized. Thiolation efficiency was calculated using the DTNB colorimetric assay (Sigma). The thiolated adipose-derived ECM (tadECM) powder was sterilized with ethylene oxide prior to use in vitro.

3.3.4 Fabrication of tadECM Hydrogels

Hydrogel reagents were reacted together employing a thiol-ene base-catalyzed Michael addition reaction with a 1:1 functional group ratio of acrylate to thiol. tadECM proteins in phosphate buffered saline (PBS) were preincubated for 30 minutes with PEG-acrylate monomers. During preincubation, a solution of PBS, ethoxilated-trimethylolpropan tri(3-mercaptopropionate) (ETTMP 1300, and 1 M NaOH were reacted together in a separate tube. After incubation, the PEG-acrylate and adECM solution was mixed with the ETTMP 1300 and NaOH solution. Hydrogels were synthesized at 1: 1: 0, 1: 0.25: 0.75, 1: 0.5: 0.5 (PEG-acrylate: ETTMP: tadECM) by molar functionality. Gelation occurred in less than one minute, and could be controlled by adjusting the temperature and amount of NaOH.

3.3.5 Mechanical Testing

Hydrogels with protein additives (1: 1: 0, 1: 0.25: 0.75, 1: 0.5: 0.5) were formed using a 5 mm (height) × 10 mm (diameter) cylindrical mold and allowed to swell overnight before being tested to determine elastic modulus and maximal compressive strength. The results reported (n=5) were taken with 80% strain at a compression rate of 1 mm/min from an Instron Mechanical Test System 5696 using an Instron 2 kN static load cell (Instron Corporation, Canton, MA).
3.3.6 Seeding and Culture of hASCs with Synthetic adECM Hydrogel

Hydrogels were formed in a 2 mm (height) x 5 mm (diameter) cylindrical mold before being transferred to a 48-well plate and allowed to swell in PBS overnight. Cells were isolated and cultured as described in Chapter 2 of this thesis. Second passage (P2) hASCs were seeded on the hydrogels at a density of 25,000 cells / scaffold. Cells were cultured in growth media (Dulbecco's modified Eagle's medium [DMEM/F12], 10% fetal bovine serum [FBS], and 1% Penicillin-Streptomycin-Amphotericin B solution) for 7 days with media maintenance performed three times a week.

3.3.7 Live/Dead® Staining

At 7 days, cell viability was assessed using the Live/Dead® viability/cytotoxicity kit (Molecular Probes, Eugene, OR). Each specimen was incubated in a 0.2% ethidium homodimer, 0.05% calcein am (v/v) solution in PBS (-Ca, -Mg), protected from light, for 45 min. Cells were visualized via fluorescence microscopy (Zeiss Stereo Lumar) in combination with a digital camera (Hamamatsu Orca ER cooled CCD).

3.3.8 Statistical Analysis

All results were expressed as mean ± standard deviation. Data were analyzed with one-way analysis of variance, followed by Tukey's minimum significant difference post hoc test for pairwise comparisons of main effects. For all comparisons, a p-value < 0.05 was considered significant.

3.4 Results

3.4.1 adECM Thiolation and Hydrogel Synthesis

In this study, the effects of tadECM hydrogels on hASCs were evaluated. A schematic for adECM thioaltion and hydrogel polymerization is presented in Scheme 3.1 and Scheme 3.2, respectively. tadECM thiolation effiency, as evaluated using the DTNB assay, was reported to be 248.7 µmol / g ± 1.6 µmol / g. For comparison, unmodified adECM was found to have a thiol concentration of 104.2 µmol / g ± .6 µmol / g.
Scheme 3.1 - adECM thiolation via ring opening polymerization.

Scheme 3.2 - Synthesis of tadECM hydrogel using tadECM, 4-arm PEG acrylate, and ETTMP. Reaction was catalyzed using sodium hydroxide.

3.4.2 Mechanical Characterization

Figure 3.1 shows the mechanical strength for hydrogel scaffolds containing different concentrations of tadECM. There was no significant difference in Young’s modulus
between hydrogel the 1: 1: 0 and 1: 0.75: 0.25 formulation groups. A significant difference in Young’s modulus was observed between the 1: 0.5: 0.5 hydrogel formulation group and the 1: 1: 0 and 1: 0.25: 0.75 groups.

** statistical significance, p < 0.05.

Figure 3.1 - Young’s modulus of PEG hydrogels with varying concentrations of tadECM additives.

3.4.3 hASC Viability

Figure 3.2 shows hASCs stained with the Live/Dead® staining kit at 7 days. Qualitatively, hASCs seeded on 1: 1: 0 hydrogels exhibited greater viability at 7 days than cells seeded on either 1: 0.25: 0.75 or 1: 0.5: 0.5 scaffolds. In addition, cells appeared to remain adhered to the surface of the 1: 1: 0 formulation hydrogel compared to either the 1: 0.25: 0.75 or 1: 0.5: 0.5 groups.
3.5 Discussion

adECM was thiolated prior to incorporation with a PEG-acrylate/ETTMP polymer fraction to produce a hydrogel. tadECM thiolation was found to be 248.7 µmol / g ± 1.6 µmol / g. This degree of substitution is comparable to the results of Xu et. al, who demonstrated an experimental collagen thiolation efficiency of 297 µmol / g ± 1.6 µmol / g.134

Mechanical characterization of 1:1:0, 1:0.5:0.5, and 1:0.25:0.75 tadECM hydrogel groups demonstrated that a decrease in Young’s modulus correlated with an increase in tadECM content and a decrease in ETTMP concentration. This may due to a lower conversion rate for hydrogel groups with high concentrations of tadECM. Similarly, Artzi et. al found that incomplete monomer conversion was responsible for decreased mechanical strength.135

Finally, Live/Dead® staining of tadECM hydrogels revealed that decreased cell viability correlated with increased tadCM content and decreased ETTMP concentration. These results could be caused by unreacted acrylate groups, which have been found to be relatively cytotoxic in vitro.136

Overall, the results of this preliminary study indicated that more complete thiol-acrylate conversion in hydrogel synthesis should be obtained in order to improve both the mechanical and cytocompatibility properties of the tadECM scaffold. This may be
addressed thiolating adECM to a greater degree by, first, increasing the amount of lysines present in the protein structure. Xu et. al was able to accomplish this goal by subjecting collagen to a carboxylation reaction, followed by an amidation reaction with 2-mercaptoethylamine hydrochloride (MEA) and a EDC-NHS (EDC: 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride; NHS: N-hydroxysuccinimide) condensation reaction.

3.6 Conclusion

In this study, we successfully thiolated adECM and synthesized a hybrid synthetic/tadECM hydrogel. However, mechanical and cytocompatibility studies revealed that reduced Young’s modulus and cell viability correlated with increased tadECM content. As these results may be caused by incomplete thiol-acryalte conversion, future work will focus on improving tadECM functionality. We would hypothesize that increasing the number of tadECM thiol functional groups would lead to improved outcomes.

3.7 Acknowledgements

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4. SUMMARY, CONCLUSIONS, AND RECOMMENDATIONS

4.1 Summary

ECM-based hybrid hydrogels are currently being used in the field of tissue engineering to promote stem cell proliferation and differentiation. In these studies, hybrid synthetic/adipose-derived ECM scaffolds were characterized based on their mechanical properties and ability to support hASC proliferation, viability and multipotency preservation.

Inspired by the findings that adipose-derived ECM can be converted into a cytocompatible hydrogel after combination with a synthetic fraction, efforts were conducted in order to improve the performance of the hybrid synthetic/adipose-derived ECM hydrogel platform. ECM was thiolated prior to hydrogel synthesis in order to promote more uniform dispersion. Thiolated adipose-derived ECM hydrogels were characterized based on their elasticity and ability to maintain hASC viability.

4.2 Conclusions

hASCs encapsulated in hybrid synthetic/adipose-derived ECM hydrogels containing high concentrations of ECM demonstrated greater expression of human potency markers compared to cells encapsulated in ECM-free synthetic hydrogels or in Matrigel®. These results indicate that adECM hydrogels hold promise as a cost-effective platform for hASC multipotency maintenance in tissue engineering applications.

It was found that hASCs seeded on synthetic hybrid hydrogels containing higher concentrations of thiolated adECM (tadECM) demonstrated decreased viability compared to tadECM-free hydrogels. As these results may be caused by incomplete thiol-acrylate conversion, increasing the thiol concentration of the tadECM prior to hydrogel synthesis may lead to improved outcomes.
4.3 Recommendations

1. Further validation of the multipotency maintenance capability adECM hydrogels is needed to evaluate their potential as stem cells scaffolds.

2. Studies investigating the ability of hybrid adECM hydrogels to affect hASC trilineage differentiation are needed in order to assess their capacity to facilitate tissue regeneration. Additives, such as β-TCP, hydroxyapatite, TGF-β3, or BMP-6, may assist in promoting a specific stem cell lineage.

3. Enhanced adECM dispersion in the hydrogels may improve stem cell spatial distribution and proliferation. It may also lead to a more committed differentiation pathway. This may be achieved by increasing the thiol concentration of the adECM.
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