Fabrication And Characterization Of Thiol-Acrylate Based Polymer for Bone Tissue Engineering Application

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FABRICATION AND CHARACTERIZATION OF THIOL-ACRYLATE BASED POLYMER FOR BONE TISSUE ENGINEERING APPLICATION

A Thesis

Submitted to the Graduate Faculty of the
Louisiana State University and
Agricultural and Mechanical College
in partial fulfillment of the
requirements for the degree of
Master of Science in Mechanical Engineering

in

The Department of Mechanical & Industrial Engineering

by

Anoosha Forghani
B.S., Isfahan University of Technology, 2011
May 2016
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ABSTRACT

Thiol-acrylate materials have been demonstrated to have therapeutic potential as biocompatible scaffolds for bone tissue regeneration due to their osteoconductivity, biodegradability, and well-suited mechanical properties. This study connects the mechanical properties and stability of thiol-acrylate polymer with cell adhesion and proliferation of human adipose derived stromal cells. The polymer presented in this study, trimethylolpropane ethoxylate triacrylate-co-trimethylolpropane tris (3-mercaptopropionate) (TMPeTA-co-TMPTMP), was synthesized by an amine-catalyzed Michael addition reaction. Physical, mechanical, and chemical characterizations were performed on the polymeric scaffold, followed by preliminary in vitro cytocompatibility tests. Live/dead staining assays showed significant differences in cell adhesion for TMPeTA (692 and 912 MW). Collectively, these results highlight the potential for these thiol-acrylate based polymers to be a versatile, biocompatible scaffold for bone tissue engineering applications.
INTRODUCTION

Specific polymer characteristics affect cell adhesion in vitro and subsequently tissue formation in vivo such as wettability, mechanical integrity, and surface charge [1, 2]. The importance of hydrophilicity of the polymer greatly enhances the cells function by aiding in the extracellular protein adsorption [3]. Furthermore, the surface charge plays a significant role showing the interaction between charged species within the extracellular matrix and charged molecules on the polymer [1, 4]. The crosslinking density of a polymeric substrate plays an important role in cell behavior illustrated by a change in morphology [5, 6]. Cells only attach to relatively stiff, highly crosslinked substrates by transmembrane receptors (integrins), which connect the cytoskeleton of the cells to the substrate [7, 8].

Challenges of cell adhesion involve adsorption of three protein classes, ECM, transmembrane, and cell adhesion receptors/molecules that all must communicate successfully with the substrate for attachment. Surfaces that lack the ability to adsorb these proteins, such as PEG (polyethylene glycol), lead to weak interactions between the substrate and the cells adhering causing the cells not to adhere to the substrate [9, 10]. With these challenges of attaching cells to polymeric surfaces, the use of adhesive proteins such as RGD or collagen have been used to aid in the adhesion of cells [11-14].

Base catalyzed Michael addition for polymerization of thiol-acrylate polymers has been posited as a biocompatible method for in situ formation of degradable synthetic bone grafts. Anion can add onto the double bond from the acrylate present forming a crosslinked polymer network [12, 14]. Thiol-acrylate polymers contribute to the properties needed for cell attachment by promoting cell adhesion without the addition of these cell adhesive proteins or peptides. Pentaerythritol triacrylate-co-trimethylolpropane (PETA) scaffolds, which were shown to be fabricated through thiol-acrylate chemistry, displayed its potential for bone augments and grafts [15, 16]. The thiol-acrylate polymers
described in this study are synthesized by a nucleophile-initiated Michael addition reaction that precedes by a step-growth polymerization by first forming a tertiary amine catalyst which deprotonates a thiol. The thiolate anion can add onto the double bond from the acrylate present forming a crosslinked polymer network.

The main purpose of this study is to demonstrate the effect of amine content, MW of the monomer and average functionality has on cell adhesion. Several compositions of polymer were synthesized based on varying monomer and base catalyst content. Initial characterization studies were performed involving mechanical, mass loss, and contact angle profiles. Live/dead staining and Picogreen quantification of DNA was used to study the attachment and proliferation of human adipose-derived adult stem cells (hASCs) onto the polymer matrix.
1. LITERATURE REVIEW

1.1 Stem cells in bone regeneration

Bone marrow stem cells (BMSCs) and Adipose derived stem cells (hASCs) are the two main sources of human cells. They both have multipotential differentiation capacity and are used in laboratories; however BMSCs have more invasive harvest procedures. HASCs are alternative sources for BMSCs. In addition to being less invasive, they are capable of differentiating into multiple lineages, including osteogenic lineages (figure 1) [17]. The maximum volume of human marrow derived in each surgery with local anesthesia cannot go beyond 40 ml which contains about $1.2 \times 10^9$ nucleated cells. In contrast, harvesting adipose tissue under the same surgical condition provides about 200 ml and contains $2 \times 10^8$ nucleated cells per 100 ml of lipoaspirate. Thus the number of stem cell derived from lipoaspirate will typically have an excess of $1 \times 10^6$ stem cells, which is 40 times more than cells in marrow [18].

HASCs can be isolated from fat taken from liposuction (figure 2). The adipose cells are washed, filtered, and centrifuged, and plated. In order to have successful osteogenesis and bone regeneration, it is necessary to have high quality cells with an increased capacity to proliferate and differentiate. Proliferation rates and differentiation determine the tissue engineering potential of stem cells. The proliferation rate is the speed at which the cells replicate themselves. Differentiation is the process by which the ability of an unspecialized cell changes to a specialized cell, such as a muscle, nerve, or bone cell (figure 1) [19]. These two parameters are critical, because if cells are not proliferating and differentiating into the desired cell, the engineered tissue will fail in serving the function of the host tissue [19].

When cells go through a longer expansion time and higher passaging number they will lose their proliferation and differentiation capacity. Stem cells have the ability to undergo
multilineage differentiation. Differentiated stem cells of specific lineage pathways can be transplanted to autologous or allogeneic hosts without the risk of immunorejection [20, 21].

Figure 1 lineage-specific differentiation of pluripotent Adipose derived stem cells (hASCs). Reprinted with the permission from [18].

1.2 Tissue Engineering

Trauma, disease, tumor resection, and accidents can sometimes lead to bone defects and fractures that require some type of replacement or bone grafting [22, 23]. Traditional methods to approach bone defects include autografting, bone from the patient, and allografting, bone from a donor.
Figure 2 Processing of isolation of adipose-derived stem cells. Reprinted with the permission from [20].

Although autografts are the “gold standard” for healing bone defects, their disadvantages include longer anesthesia, blood loss, risk of nerve injury, limitation of supply, and donor site morbidity. Perhaps most importantly, the limited volume of bone grafts causes looking for alternative techniques [23]. Allografts (from human to human) are another commonly used grafts which have their own limitations such as the risks of disease transmission to the host body and immunologic rejection [23]. Bone tissue engineering (BTE) is a promising technique for healing bone defects using stem cells overcomes the disadvantages of traditional grafting techniques [24]. Recruiting a range of biomaterials such
as polymers, ceramic and composites as carriers for stem cell delivery is promising new orthopaedic treatment technique to fill bone defects. Recent investigates focus on replacing nonporous, bioinert materials with more porous, bioactive and osteoinducive scaffolds [21]. There are different techniques such as cell based matrices and cell–matrix composites used to restore the function of damaged tissue [22]. The three-dimensional structure of scaffolds supports cells to adhere to the structure, proliferate, differentiate and integrate into surrounding bone tissue. Bone is composed of about 60% inorganic hydroxyapatite and 30% organic matrix which is mainly (about 95%) consists of collagen type I (Figure 3) [25]. An ideal scaffold is biocompatible, biodegradable, porous, and provides adequate mechanical support to the injured tissue [26]. The three dimensional structure of scaffolds allow it to integrate into surrounding tissue and eventually be replaced by new formed tissue. The scaffolds also should be able to transfer cells and growth factor to the defect and encourage damaged tissue to grow [26]. Cells will not be able to migrate into the deeper layers if the pores are too small. However, pores that are too large can decrease the strength and mechanical properties of the scaffold. Thus the range of 200-900 micrometers in size is optimal for cells to migrate to the structure and the nutrients and waste to pass through [26]. A number of biomaterials such as porous ceramics of hydroxyapatite and β-tricalcium phosphate seeded with MSCs, have been successfully implanted into animals in vivo [25].

### 1.3 Biocompatibility

First step of evaluating biocompatibility of a material is in vitro cytotoxicity tests. The evaluation is based on if the cell stays healthy in direct contact with the material [27]. If the material is not biocompatible the shape of the cells will change and they won't stay healthy. In bone tissue engineering there also should be a match between mechanical properties of the implanted material and the surrounding tissue. Any mismatch in rigidity between the biomaterial and the tissue results in unsuccessful implantation [27].
1.4 Current biomaterials used in hASC bone tissue engineering strategies

1.4.1 Bioactive inorganic materials

A wide range of bioactive inorganic materials, similar to the inorganic composition of bone, are of clinical interest in the treatment of bone defects. These materials include tricalcium phosphate, HA, and bioactive glasses, along with their combinations [28,
29]. Tricalcium phosphates resorb faster than HA and have less mechanical properties compared to HA. A bioactive hydroxycarbonated apatite layer rapidly forms on bioactive glasses when submerged in simulated biological fluid which means this material is extremely bioactive (figure 4). This formed apatite layer improves the bonding between surrounding bone tissue and implant which has been proven in vitro by supporting adhesion and proliferation of osteoblasts [25, 29, 30]. HA composition and structure is very close that of natural bone thus is a good candidate for bone tissue engineering [31]. HA and ceramics in general are too brittle so they cannot be good candidate by themselves for load-bearing applications [25].

![Figure 4](image.png)

Figure 4 Hydroxyapatite formed on the surface of bioactive glass with cauliflower morphology foam after immersion in simulated body fluid (SBF) for 28 days. Reprinted with the permission from [28].

1.4.2 Polymers

Bioactive and biodegradable polymers have attracted increasing attention for their use in tissue engineering scaffolds. Some natural polymers that are being used in bone tissue engineering include fibrin, collagen, hyaluronic acid, alginate and chitosan [32]. Synthetic
polymers have the benefit of controlling the chemistry of polymers and material composition (table 1.1). Polymers have some problems if they are using by themselves when it comes to bone tissue engineering such as high degradation rate in vivo. Polymeric scaffolds even with higher compressive strength degrade fast in physiological condition[32]. They also have low cell seeding efficiency specially in 3D form. Produced monomers during degradation of synthetic polymers, such as polylactic acid (PLA), polyglycolic acid (PGA), and polycaprolactone (PCL) are removed by the blood. Polymers in general don’t have high compressive strength [32].

Table 1.1 – A table of the Properties and application of synthetic polymer materials

[33]:

<table>
<thead>
<tr>
<th>Polymer</th>
<th>Tensile/ Compressive strength</th>
<th>Modulus (Gpa)</th>
<th>Degradation time (Weeks)</th>
<th>Degradation product</th>
<th>Applications</th>
</tr>
</thead>
<tbody>
<tr>
<td>Polyglycolid</td>
<td>-</td>
<td>7.0</td>
<td>4–16</td>
<td>Glycolic acid</td>
<td>Suture anchors, meniscus repair, medical devices,</td>
</tr>
<tr>
<td>Poly (L-lactide)</td>
<td>40–20</td>
<td>2.7</td>
<td>12–18</td>
<td>L-lactic acid</td>
<td>Fracture fixation, interference screws, suture anchors,</td>
</tr>
<tr>
<td>Poly (L-lactide-co–D,L glycolide)75/25</td>
<td>-</td>
<td>1.9</td>
<td>4–5</td>
<td>D,L-lactic acid</td>
<td>Orthopedic Implants, coatings</td>
</tr>
<tr>
<td>Poly (L-lactide-co–D,L glycolide)10/90</td>
<td>-</td>
<td>-</td>
<td>12–15</td>
<td>D,L-lactic acid and glycolic acid</td>
<td>-</td>
</tr>
<tr>
<td>Poly (D, L lactide) –</td>
<td>-</td>
<td>-</td>
<td>11–15</td>
<td>D,L-lactic acid</td>
<td>Orthopedic implants, drug delivery</td>
</tr>
<tr>
<td>Poly (D, L-lactideco-glycolide)m75/25</td>
<td>-</td>
<td>2.0</td>
<td>4–5</td>
<td>-</td>
<td>Plates, mesh, screws, tack, drug delivery</td>
</tr>
</tbody>
</table>
Table 1.1 continued

<table>
<thead>
<tr>
<th>Polymer</th>
<th>Tensile/Compressive strength</th>
<th>Modulus (Gpa)</th>
<th>Degradation time (Weeks)</th>
<th>Degradation product</th>
<th>Applications</th>
</tr>
</thead>
<tbody>
<tr>
<td>Poly (D, L-lactideco-glycolide) 50/50</td>
<td>-</td>
<td>2.0</td>
<td>1–2</td>
<td>D,L-lactic acid and glycolic acid</td>
<td>Drug delivery, Fixation and orthopedics implant, adhesion barriers</td>
</tr>
<tr>
<td>PHA and blends</td>
<td>20–43</td>
<td>-</td>
<td>Bulk</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Poly caprolactone</td>
<td>-</td>
<td>0.4</td>
<td>[24]</td>
<td>Caproic acid</td>
<td>Suture coating, dental orthopedic implants, Orthopedic implants</td>
</tr>
<tr>
<td>Polyoorthoester</td>
<td>4–16</td>
<td>4–16</td>
<td>Surface</td>
<td></td>
<td>Orthopedic implants, foam coatings, drug delivery</td>
</tr>
</tbody>
</table>

1.4.3 Hydrogels

Hydrogels such as polyethylene glycol, alginate-based are also commonly used in the field of tissue engineering [25]. They can be used as space filler agents and also drug and cell delivery vehicles. Some hydrogels such as alginate are able to crosslink in situ which means that they can be delivered via minimally invasive surgery [25]. Hydrogels have viscoelastic material properties which makes them applicable for soft tissues, such as cartilage. Hydrogels are good candidate for bioactive molecule and cell delivery [25].


1.4.4 Composite materials

Bone is a composite made of inorganic and organic phases. As a result, composites of the ceramic polymer can mimic the real bone. Composite materials have improved physical, biological, and mechanical properties of tissue engineering techniques. Adding polymer to bioceramic scaffolds decreases the brittleness, controlling the degradability. Similarly, incorporating the inorganic phase such as HA to the polymers improves the mechanical properties of the scaffold [34]. Marra et al showed in their study that blending PLGA with HA improves mechanical properties and also leads to osteogenic behavior of composite. They noticed a significant difference in mechanical properties particularly tensile strength between HA and non HA samples which could be due to interfacial bonding between HA and polymer [35].

Based on recent studies nano sized inorganic components are more bioactive when in nano scale than a micro-sized ones [25]. Nano sized HA has better properties compared to micro sized HA which is due to smaller grain size and larger surface to volume ratio. This larger surface area to volume leads to better sinterability, improved bioactivity and enhanced densification which can improve mechanical properties [30]. Ducheyne et al claimed that formation of apatite layer or bioactive behavior of ceramics depends on their structures [36]. Nano scale HA also have releasing rate of calcium ions close to that of bone which is significantly in higher rate in comparison with micro scale HA [37].

1.5 Thio-Acrylate based polymers

Thiol-ene chemistry has been of a great interest because of advantageous properties such as simple reactions, rapid polymerization and good compressive strength for tissue engineering applications. Thiol-acrylate polymers fabricated through photopolymerization are used in biomedical applications [38]. Base catalyzed Michael addition for the polymerization of thiol-acrylate polymers (figure 5) has been posited as a biocompatible method for in situ
formation of degradable synthetic bone grafts [12, 16, 38]. Thiol-acrylate polymers promote cell adhesion without the addition of adhesive proteins or peptides. Pentaerythritol triacrylate-co-trimethylolpropane (PETA) scaffolds, which were shown to fabricate through thiol-acrylate chemistry, display a potential for bone augments and grafts. There is an emerging interest in degradable thiol acrylate photopolymers as biomaterials.

Figure 5 Schematic representation of in situ tertiary amine catalyzed anionic step growth polymerization mechanism. Reprinted with the permission from [38].
1.6 Cell-material interaction

Adhesion of the cells to polymer substrates is of the utmost importance in tissue engineering which can affect cell proliferation, differentiation and migration [39]. Many researchers studied the interaction of cells with polymers or other types of biomaterial substrates to improve the biocompatibility of engineered materials used as substitute tissue [40]. When scaffolds implanted into the defect, interaction between the cells and scaffold plays an important role to adhesion of the surrounding tissue and growth of the tissue cells into the scaffold. Cell –substrate adhesion happened when receptor of the cells and ligand of the surface interact [41].

Specific polymer characteristics affect in vitro cell adhesion and in vivo tissue formation. These characteristics include wettability, mechanical integrity, stiffness and surface charge. There are no general principles indicate the cellular behavior on a biomaterial. Therefore, in order to study cellular behavior on a substrate, adhesion, morphology, proliferation, cytotoxicity of the cells are required to be assessed and analyzed [42, 43]. The hydrophilic nature of the polymer greatly enhances the cells function by aiding in the extracellular protein adsorption. Hydrophilic substrates support the adhesion of cells, whereas hydrophobic do not promote cell attachment [39]. There are three main physiochemical cues categories which affect cell behavior: topographical, mechanical and chemical [44]. There are many engineered and fabricated materials for tissue engineering applications that are biocompatible and have fairly good mechanical properties, such as compressive strength. However, not all of them have adequate tissue cell-substrate interaction, which can lead to inflammation in surrounding tissue and implant rejection. Polymeric biomaterials can be improved by modifying the surface in a way to enhance interaction between cells and synthetic substrates. Proteins such as RGD or collagen have been used to aid in the adhesion to polymeric surfaces [45].
Substrate stiffness play a significant role in the anchoring of stem cells to biocompatible materials for tissue engineering by sending mechanical feedback to the cells [7]. The substrate causes the cells to generate mechanical forces, thus it is required for the substrate to be able to withstand these forces for further spreading and proliferation of cells. As a result, cells form a delicate cytoskeleton on lower moduli substrates due to weaker forces that are exerted from the cells [5]. Adhesion of cells on a stiff substrate withstands pulling noted by the morphology and shape of the cells as shown in figure 6. The cell morphology is rounded on soft substrates and spindle shaped on stiff substrates. Weak cytoskeletons cannot support the extent of tissue regeneration needed for bone tissue [5]. Based on recent studies stiffness of a substrate is one of the key factors that affect adhesion of the cells to the substrate. The environment that cells are in conjunction with in vivo is soft unlike the hard and stiff substrates that they are usually attached to in vitro. In order to mimic the in vivo situation researchers studied adhesion of the cells to a polymer gel with tunable stiffness. These substrates are coated with specific ECM proteins which improve cell attachment. Integrins are molecules which connect extracellular matrix to the interacellular cytoskeleton and are main molecules in focal adhesion point (figure 6) [7].

Focal adhesions form when cell bind and adhere to a substrate. These focal adhesions need mechanical forces to mature and spread over the substrate. These mechanical forces can be generated by the cytoskeleton part of the cells. Hard and stiff substrates experience large forces generated by the cells. On these substrates mature focal adhesion forms with an organized cytoskeleton spread over the cells [46]. A soft substrate cannot withstand the exerted force from the cell. Therefore, organized and developed cytoskeleton is not formed on soft substrates [7].
Figure 6 Schematic representation of an attached cell to a substrate. Reprinted with the permission from [7].

Stiffness of the substrate affects the cytoskeletal organization and subsequently morphology of the cell. Based on different studies cells spread more on stiffer substrates while they stay rounded on less stiff materials as can be seen in figure 7 [7]. These changes in morphology of the cells affect cell fate including differentiation pathway. Furthermore, stiffness of the substrate and adhesion of the cells to the substrate strongly affects viability of the cells. For instance, in a study which compared behavior of osteoblasts on substrates with different stiffness, cells showed higher proliferation rate and more deposition of mineral on stiffer surfaces [7].

Figure 7 Cultured fibroblasts on A) soft and B) stiff polyacrylamide Gel substrates coated with collagen I with rounded and spindle shape morphology respectively. Reprinted with the permission from [7].
2. MATERIALS AND METHODS

2.1 Preparation of thiol-acrylate composites

All chemicals were used as received. Poly (ethylene glycol) diacrylate (PEGDA, Mn 700), TMPeTA (692 & 912), trimethylolpropane tris(3-mercaptopropianate) (TMPTMP) were obtained from Sigma Aldrich. Diethylamine (DEA) was obtained with 99% purity from AGROS Organics. The catalyst DEA was added to TMPeTA/PBS with increasing % relative to acrylate functionality forming a stock solution. The polymer was prepared by adding TMPTMP to TMPeTA/DEA stock in a 1:1 molar functionality. Eight different sample groups were fabricated for the following characterizations with 692 and 912 TMPeTA with 2.8, 5.0, 10, 16.1% DEA. A PEGDA polymer sample was fabricated as described above by adding 5% DEA to PEGDA forming a stock solution which was added to TMPTMP at a 1:1 ratio.

2.2 Mechanical testing

Cylindrical solid and foam scaffolds with dimensions of 45mm (diameter) x 25mm (height) were tested to determine maximal compressive strength and bulk modulus. Scaffolds were subjected to compression testing at 90% strain. A universal testing machine (Instron Model 5696, Canton, MA, USA) was used at an extension rate of 0.5 mm/min [47].

2.3 Contact angle measurement

Contact angles were determined using VCA Surface Analysis System with Optima XE software for the 692 and 912 TMPeTA fabricated with 2.8, 5.0, 10, 16.1% DEA relative to acrylate functionality. Nanopure water (5ul) was dispensed automatically and allowed to equilibrate for 30 seconds on three separate locations of each polymer sample.
2.4 hASC isolation and culture

Subcutaneous adipose tissue liposuction extracts were acquired from three donors under an approved IRB protocol (LSU#E9239). The procedure involving the hASC isolation is described elsewhere [48]. “Passage 0” refers to the primary cell cultures initial passage and is denoted as p0. The hASCs were trypsinized, split, and plated at a density of 5000 cells/cm² (“Passage 1”) for expansion on T125 flasks to attain 80% confluency. For all cell based tests, passage 2 was used.

2.5 Cell seeding on solid constructs

Prior to polymerization, the monomers were sterilized by filtering through a 0.45μm nylon syringe filter (Celltreat). After sample preparation according to section 2.1, the 8 sample groups were immersed in stromal media (Dulbecco’s modified Eagle’s medium (DMEM), 10% FBS (Fetal Bovine Serum), 1% triple antibiotic solution) for 24 hours. HASCs were added to each sample with the concentration of 50000 cells/well then incubated at 37 °C for 7 days followed by changing media every 3 days.

2.6 Live/Dead staining

Live/dead staining (Cell viability®, Invitrogen – using a Leica TCS SP2 spectral Confocal)) were performed to assess viability of hASCs on the solid constructs 1, 4 and 7 days after seeding. 300μl of PBS containing 4 μM EthD-1 and 2 μM Calcein-AM (Invitrogen) was added to each sample followed by incubation at room temperature for 10 min. The samples were then imaged using a fluorescent microscope (Zeiss SteREO Lumar.V12 fluorescence stereomicroscope), to detect live(green) and dead(red) cells on the samples.
2.7 Picogreen assay

Total DNA content was used to determine the proliferation of cells on each sample. In order to do that, all the samples were lysed using proteinase with the concentration of 0.5mg/ml overnight at 56°C. DNA quantification was done by mixing 50 µl of Proteinase K solution and 50 µl of Picogreen dye solution ( Invitrogen™Quant-iT™ PicoGreen™ dsDNA Assay Kit) in 96 well plates[38]. 100 µl of dye were used as a background and was subtracted from the numbers. Samples were excited at 480 nm and total DNA concentration was compared to a standard curve generated from serial dilutions of hASC in order to calculate the number of the cells in each well.

2.8 Mass loss

The 692 and 912 TMPeTA with 2.8, 5.0, 10, 16.1% DEA polymer samples were fabricated as noted above and punched into (10mm x 10mm) sized constructs. The samples were freeze-dried for 24 hours, and then submerged in F12 media for 7 days at 37°C. The samples were freeze-dried post the 7 day soaking time to remove any absorbed moisture.
3. RESULTS AND DISCUSSION

3.1 Material characterization

Thiol acrylate polymers synthesized via Michael addition offer a polymer that supports the adhesion of stem cells to its surface without the alteration or functionalization with sticky peptides. As shown in the mechanism (figure 8), the scheme begins with the formation of a tertiary amine catalyst by the Michael addition of the amine to the alkene group found within an acrylate. This tertiary amine acts as a strong base deprotonating the thiol which starts the polymerization reaction. As the concentrations of the newly formed tertiary amine catalyst increases, so do the rate of the reactions, which allows for tunable cure times depending on the application [49]. These anionic step-growth polymerization thiol-acrylate reactions lack a termination step, which reduce the concentration of unreacted monomers after the polymerization reaction is quenched. This property is important to the biocompatibility of a substrate, but characteristics including hydrophilicity, crosslinking density, and chemical composition have greater influence on the adhesion and differentiation of the cells.

3.2 Live/Dead staining

Attachment of hASCs to TMPeTA-co-TMPTMP substrates was verified using fluorescent microscopy imaging at 1, 4, and 7. Live/dead assay showed the viability of the live cells by fluorescing green and the dead by fluorescing red. The adhesion of the hASCs on the surface of the samples was observed by the spindle shape morphology. Controls containing no cells were included as a means of comparison. As shown in figure 9, cells successfully attached to the surface of the TMPeTA(692) 2.8%-16.1% DEA polymer at day 1 post cell-seeding and remained attached throughout the 7 day study.
Figure 8 The reaction scheme for the base-catalyzed Michael addition thiol acrylate reaction. The first line denotes the synthesis of the in situ amine catalyst.

The live/dead stain images showed no attachment on TMPeTA(912)-co-TMPTMP samples (2.8-16.1% DEA) at day 4 and 7. No attachment was observed on the PEGDA-co-TMPTMP substrate 24 hr after seeding (figure 10). Therefore no further viability testing was performed on PEGDA due to the initial poor results.

3.3 Picogreen assay

Cell proliferation studies were performed at different time points (day 1, 4, 7) for a total of 7 days. The picogreen results for all 8 groups have been shown in Figure 11. For the 4 TMPeTA(692) samples, an increase in cell number was indicated from day 1 to 7 after seeding which is in agreement with the positive control.
In contrast, TMPeTA(912) samples revealed a steady decrease in attached cells at day 4 and 7 confirming that cell number was reduced between day 1 and 4. Analysis of DNA values showed that TMPeTA polymers synthesized with the lower average molecular weight, 692, are more suitable substrates for cellular proliferation.

Figure 9 Live/dead fluorescence imaging of hASCs on TMPeTA(692) at day 1,4,7 and TMPeTA(912) at day 1.

Figure 10 Live/dead fluorescence imaging of hASCs on PEGDA(700) at day 1.
Live/dead results are in agreement with picogreen (figure 11) which showed a significant drop in the number of spindle shape cells for TMPeTA(912)-co-TMPTMP while showing a proliferation and spreading of cells for the TMPeTA(692)-co-TMPTMP samples (figure 9). The extent of crosslinking density a substrate possesses plays a significant role in the anchoring of stem cells to biocompatible materials for tissue engineering [7] by sending mechanical feedback to the cells. The substrate causes the cells to generate mechanical forces thus it is required for the substrate to be able to withstand these forces for further spreading and proliferation of cells.

![Figure 11 DNA content TMPeTA(692)-TMPTMP samples(2.8%, 5%,10% and 16.1%DEA), and TMPeTA(912)-TMPTMP( 2.8%, 5%, 10% and 16.1%DEA).](image)

Therefore, lower crosslinked substrates have a delicate cytoskeleton formed from the weaker forces that are exerted from the cells. Adhesion of cells on a highly crosslinked substrates withstands more pulling noted by the morphology and shape of the cells as shown in figure 9.
Cell morphology is rounded on soft substrates and more spindle shape on highly cross-linked substrates. HASCs were unable to stay attached after day 1 on the TMPeTA(912) correlating to its inability to form a mature cytoskeleton. These weak cytoskeletons can’t support the extent of tissue regeneration needed for bone tissue.

3.4 Mechanical properties

Since Young’s modulus and crosslinking density are related, the compression modulus was determined for each of the polymers to verify the correlation between the crosslinking of the polymer network and cell attachment. Figure 12 shows a decrease in modulus with increasing amine for both TMPeTA(692) and TMPeTA(912). The varying amine content did not have an effect on cell proliferation for the TMPeTA(692) and (912) samples over the 7 day study. Figure 12, also illustrates that the TMPeTA(692) possessed an overall higher modulus compared to TMPeTA(912) due to TMPeTA(692) having a shorter backbone compared to TMPeTA(912). The shorter backbone yields a more tightly crosslinked network which may be responsible for the increase in cell adhesion on TMPeTA(692) compared to TMPeTA(912) (Figure 9) [50][51]. The di-functional monomer, PEGDA(700), used in the synthesis of the PEGDA-co-TMPTMP polymer described earlier, did not support appear to support cell attachment as no cells were adhered after 24 hours (Figure 10). This correlates with crosslink density as PEGDA (700)-co-TMPTMP is a less crosslinked polymer compared to the tri-functional TMPeTAs containing system. This data is consistent with previous studies wherein the higher the average functionality of a polymer, the higher the overall crosslinking density of the network [51].
A previously studied thiol-acrylate system, PETA-co-TMPTMP, was synthesized via a Michael addition reaction and supports cell adhesion by SEM and proliferation over a 7 day study. PETA-co-TMPTMP not only had an average functionality of 3, it does not have a repeating group. This yielded an even tighter crosslinked polymer compared to both TMPeTA and PEGDA containing polymers.

### 3.5 Contact angle measurement

Wettability is another factor that affects cell adhesion on polymeric substrates. It was reported that a moderate wettability ranging from 20-40 degrees is desired to promote the adhesion of cells, where super hydrophobic/hydrophilic substrates do not support cell adhesion well [1, 52]. Watchem et al. studied the effect of wettability on polymeric substrates used in tissue engineering, such as PMMA (poly(methyl methacrylate)), PLLA(poly-L-lactic acid) and TCPS (tissue-culture polystyrene), and observed increasing cell adhesion with increasing of contact angles [52].
Figure 13 Contact Angles of the TMPeTA(692) and (912) with varying DEA(%) determined by dispensing a 5ul drop of nanopure water.

Figure 13 displays the initial contact angles for TMPeTA(692) and TMPeTA(912) polymers ranging between 28-45 degrees with an increase in hydrophilicity with increasing amine content. This is likely a result of the amine catalyst formed in the first step of this polymerization increasing the polarity of the overall polymer. Another factor contributing to its hydrophilicity is the ethoxylated repeating groups contained within the monomer, TMPeTA [53]. Hydrophilic surfaces such as tissue culture polystyrene absorb more proteins such as fibronectin than hydrophobic materials. However, decreased cell adhesion polymer surfaces that exhibit contact angles below 30 degrees show a, which supports that the TMPeTA (912) may be too hydrophilic for sustained cell attachment[54].
Surface charge also contributes to cell adhesion as a result of the interaction of charged proteins such as extracellular matrix and the substrate. Many commercially available culture flasks and dishes used for cell proliferation experiments are coated using poly-l-lysine, which also contains a charged amine functional group [55]. The chemical composition of the TMPeTA polymers includes the incorporation of the amine catalyst, shown in the first step of figure 8, which is integrated into the polymer network. The tertiary amine catalyst formed is positively charged at physiological pH causing an overall positive charge to the polymer. This increase in overall charge with respect to amine content may explain in part why the 16.1%DEA TMPeTA(692) polymer promotes cell attachment/proliferation while the 2.8%DEA TMPeTA(912) does not promote attachment despite both polymers yielding similar contact angles and modulus.

3.6 Mass loss

The degradation of well-studied polymers such as polycaprolactone(PCL) and poly-lactic-co-glycolic acid copolymers(PLGA) have been shown to affect the cell adhesion/proliferation of stem cells. The disruption of the crosslinked network in ester containing monomers (TMPeTA, PLGA, and PCL) has been shown due to the hydrolysis of the ester bonds in acidic or basic environments[56]. The change in mass over 7 days was measured for each %DEA TMPeTA(692)-co-TMPTMP and TMPeTA(912)-co-TMPTMP as noted in figure 14 to study how mass loss may affect their ability to adsorb proteins needed for cell adhesion. Despite the 16.1%DEA 692 polymer mass loss of ~10%, it was able to sustain attachment/proliferation. This is due to the overall increase in charge per surface area from the in situ catalyst found on the smaller monomer (692) compared the 912 monomer.
Figure 14  Mass loss over 7 days in DMEM-F12 media at 37°C for the TMPETA (692 & 912) polymers.
4. SUMMARY, CONCLUSION AND FUTURE WORKS

4.1 Summary

Biomaterials are divided into three main categories: ceramics, polymers and composites. Bioceramics are good osteoinducers however they are too brittle and don't have adequate mechanical stability. Polymers on the other hand are not as brittle and can modify the brittleness of the ceramic. Any new developed biomaterial should be able to support cell or surrounding tissue adhesion in order to integrate to the damaged tissue and induce defect healing. In this research the novel thiol-acrylate polymer (TMPeTA-TMPTMP) was developed as a potential biomaterial. This polymer has the advantage of easy and rapid polymerization process over the other polymeric biomaterials. This research showed that TMPeTA supports adipose derived stem cell adhesion without any surface modification. The cells showed viability up to day 7 after initial seeding.

4.2 Conclusion

The mechanism in which hASCs attach to polymeric substrates is complicated involving many factors. The TMPeTA(692) displayed better cell attachment/proliferation versus the TMPeTA(912) samples due to it possessing higher crosslinking density, moderate wettability, and lower overall mass loss. The in situ amine catalyst was also a contributing factor especially in terms of the 16.1%DEA TMPeTA(692) sample. It exhibited mass loss comparable to 912 samples, but the higher charge density aided the 16.1%DEA TMPeTA(692) to promote cell attachment/proliferation. Overall TMPeTA (692) supported cell adhesion without any modification which was proved by live/dead staining. Picogreen assay which is a DNA counting assay showed an increase in number of the cells up to day 7 for TMPeTA(692). This polymer also has hydrophilicity behaviour that can improve it's adhesion to the surrounding tissue in vivo when implanted into the defect.
4.3 Future works

1. Performing nano indentation to find the stiffness of the surface of the all 8 groups and study the relation between cell adhesion and stiffness using focal adhesion staining.

2. Study the viability of the material in a longer period of time (up to 21) and running 21 day osteogenic study in vitro.

3. In order to better understand the biocompatibility, mechanical strength, and osteogenic potential of the polymer an in vivo study within small animals can be done.
REFERENCES


VITA

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