Acellular and Radically Polymerized Biodegradable Materials to Control Tissue Interactions After Myocardial Infarction

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Acellular and Radically Polymerized Biodegradable Materials to Control Tissue Interactions After Myocardial Infarction

Abstract
Left ventricular remodeling following myocardial infarction (MI) often induces congestive heart failure, which has limited treatment options. Advances in tissue engineering and polymer chemistry, however have allowed for the development of alternative treatment strategies. Specifically, the formation of biodegradable polymers into complex scaffolds or injectable materials is being evaluated. The underlying hypothesis for this work is that fibrous scaffolds with controlled properties and structures, as well as injectable hydrogels with tunable properties, can be optimized to attenuate this post-MI remodeling response. To this end, four specific aims have been developed to test this global hypothesis.

First, a collection of biodegradable elastomers based on poly(glycerol sebacate) were synthesized, introducing the reactive acrylate group to capitalize on free radical polymerization. By varying the synthetic components, the structure-property relationships associated with changes in molecular weight and % acrylation were identified. The second Aim focused on processing these materials into fibrous scaffolds via electrospinning. Differences in mechanics and mass loss based on % acrylation were translated to the scaffolds. Cellular infiltration, matrix elaboration, and organization throughout the scaffolds were improved with the inclusion of a sacrificial fiber population and fiber alignment during processing.

The third Aim centered on the development of hyaluronic acid (HA)-based, redox-initiated hydrogels, modified with different amounts of reactive methacrylate groups (MeHA), for injectable applications. An increase in modulus was observed for both increasing % methacrylation, and initiator concentration. The final Aim evaluated the impact of the hydrogel modulus on infarct size, LV geometry, and function upon injection in an ovine model. Only treatment with the higher modulus hydrogel had a statistically smaller infarct size than the control. Furthermore, reductions in LV volumes and improvements in function in function were observed with this treatment.

The work presented in this thesis represents advancement in the field of biomaterials towards the goal of developing alternative treatments to limit LV remodeling. The use of acellular scaffolds and injectable materials with tunable properties can provide insight into the impact of different treatment paradigms, enabling develop of further therapies and improved patient care.

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ACELLULAR AND RADICALLY POLYMERIZED BIODEGRADABLE
MATERIALS TO CONTROL TISSUE INTERACTIONS AFTER MYOCARDIAL
INFARCTION

Jamie L. Ifkovits

A Dissertation In Bioengineering

Presented to the Faculties of the University of Pennsylvania
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ABSTRACT
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Jamie L. Ifkovits
Jason A. Burdick, Ph.D.

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1.1 Introduction

Cardiovascular disease (CVD) has been the leading cause of death in the United States since 1900. It is estimated that 71.3 million people presently suffer from CVD, with approximately 10% of those individuals suffering from myocardial infarction (MI) (1, 2). It has been observed that the geometry of the heart changes from its normal prolate ellipse to a larger, more spherical shape post-MI. This is associated with an increase in left ventricle (LV) size and change in geometry, thus inducing mechanical burdens for the injured heart (3-6) as shown by an increase in end-diastolic wall stress (5, 7). Myocardial hypertrophy and/or ventricular dilation may result from this increase in ventricular loading (8). Congestive heart failure (CHF) develops as a result of these alterations in ventricular geometry and mechanics, which ultimately lead to substantially compromised global LV (3-5, 9-12).

At a histological level, an increase in the activation of matrix metalloproteinases following MI leads to a substantial increase in collagen degradation. Since cardiomyocytes are tethered together using collagen, this can lead to continuous stretch and slippage of the myocytes, which contributes to infarct expansion (13). Previous experimental studies involving sonomicrometry array localization (14), echocardiography (7, 15), and magnetic resonance imaging (16, 17) have demonstrated that adverse post-infarction LV remodeling and infarct expansion are associated with stretching and decreased contractile function in the neighboring normally perfused borderzone (BZ) myocardium. Additionally, while this hypercontractility is initially isolated to a region immediately adjacent to the infarct margin, the process extends with time to involve myocardium increasingly remote from the infarct region in a process known as borderzone expansion (18).
The limited success or availability of current clinical care of patients suffering heart disease, such as pharmacological treatment, interventional therapy with pacemakers, and heart transplantations, has fueled the need for the development of alternative treatment strategies (19). The field of myocardial tissue engineering seeks to improve the regional and global function of the heart, such that progression to CHF and death does not occur (19, 20).

Classic tissue engineering strategies employ the use of cells, soluble factors, and scaffolds to develop a successful construct (19). Moreover, the development of a successful construct should meet the following criteria: 1) composed of a suitable matrix, 2) able to maintain the mechanical loading behavior of the heart, 3) able to be electrically stimulated or demonstrate contractile activity, 4) able to maintain viability of the total heart cell population (21). This chapter will address each aspect of the classic tissue engineering triad as it relates to myocardial tissue engineering, with a particular emphasis on the development and use of scaffolds. Additionally, the concept of the use of acellular and injectable materials and their ability to improve heart function after infarct will be covered.

1.2 Cell Source

One of the challenges for myocardial tissue engineering the variety of cell types that comprise the heart including: atrial and ventricular cardiomyocytes, pacemaker cells, smooth muscle cells, endothelial cells, valvular cells, and endocardial cells (22). Since all of these cells play an essential role in maintaining the viability and function of the heart, the choice of cell source for a tissue-engineered construct can be difficult. Furthermore, the human left ventricle has ~5 billion cardiomyocytes and approximately 25% of which are lost as a result of ischemic injury (23). Thus, generating enough cells for regeneration of the heart is a tremendous hurdle (21, 24).

The ideal cell source should be non-immunogenic, easy to harvest, demonstrate controlled proliferation, and the capacity to integrate, both electrically and mechanically with the native tissue (25). Although no currently known cell type meets all of these criteria, several cell
types have been evaluated for engineering myocardium both in vitro and in vivo. The cell types that have been investigated can be broken down into three general groups: 1) somatic muscle cells, 2) cells involved in angiogenesis, and 3) stem cell derived (19-21, 25).

Somatic muscle cells include cardiomyocytes as well as skeletal myoblasts. Cardiomyocytes meet many of the criteria of the ideal cell source, specifically due to their capability to electrically and mechanically integrate with the native tissue (26). Use of these cells in vivo, both through intramyocardial injections (27-29), as well as graft-based delivery (30-33) has demonstrated some functional improvement and decrease in remodeling. However, cardiomyocytes can be difficult to isolate, sensitive to ischemia, and have limited proliferative capabilities. Moreover, there is the possibility of immune rejection, due to the fact that the cells must come from another source (19, 25). Although these cells may not be best for in vivo application, their use in vitro can still provide valuable insight.

Skeletal myoblasts are an attractive cell source for myocardial tissue engineering due to their proliferative capabilities and ability to withstand ischemia better than other cell types, as well as their ability to be harvested from autologous sources (19, 25). Their use in animal models in vivo has demonstrated the ability to engraft (34-36) and reduce remodeling (34, 36-38). However, studies have demonstrated the lack of electrical integration with the host myocardium leading to problems associated with cardiac arrhythmias (36, 39).

Cells involved in angiogenesis, such as fibroblasts and endothelial cells, have been investigated for myocardial tissue engineering due to the dense vascular networks found in the heart. Mice treated with the placement of an epicardial patch containing human dermal fibroblasts demonstrated an increase in cardiac function post-infarct compared to untreated animals (40). Also, rats that were treated with endothelial precursor cells demonstrated less ventricular scarring and an increased capillary density compared to non-treated control animals (41). Moreover, recent in vitro studies have demonstrated the increased survival of cardiomyocytes in scaffold-free grafts with the inclusion of these cell types (33), demonstrating their importance for tissue regeneration.
Due to their ability to differentiate into multiple lineages, stem cells are an attractive source for a wide variety of tissue engineering applications. Bone marrow-derived mesenchymal stem cells (42-45), hematopoietic stem cells (46, 47), and adipose-derived stem cells (48) are all types of adult stem cells that have been evaluated for cardiac regeneration. The reader is directed to review articles, which discuss the use of these cells in further detail (19, 49-51). In general, application of stem cells *in vivo* has led to improved function and reduced remodeling compared to untreated control animals (42-48). However, issues with increased fibrosis (52) and calcification (53), as well as the uncertainty regarding the capability of these cells to transdifferentiate into cardiomyocytes (46, 47) need to be addressed. Further optimization of the delivery route, dosage, and timing of delivery also need to be completed prior to moving forward with clinical studies.

Embryonic stem cells (ESCs) also represent a potential cell source for myocardial tissue engineering. They are pluripotent, robust, and have the potential for genetic modification. Again, the reader is directed to excellent review articles, which discuss the use of ESCs in myocardial regeneration in more detail (19, 49-51). *In vitro* differentiation of ESCs into cardiomyocytes with the same cell surface receptors as found *in vivo* was first observed in 1991 (54). Furthermore, the capability of ESCs to be delivered and integrate into the myocardium was demonstrated in 1996 (55). Since then, numerous studies have demonstrated integration and improvement in function with the delivery of ESCs to the injured heart (56-61). One downfall to the use of ESCs for regeneration is the possibility of teratoma formation within the heart. One study reported that 21% of the animals treated with ESCs developed teratomas within two weeks of transplantation (57). Aside from ethical issues, teratoma formation and immunorejection issues need to be better understood before widespread use of ESCs occurs.

The recent observation of resident cardiac progenitor cells provides promise to circumvent some of the aforementioned issues with other cell sources for myocardial regeneration. Currently, four adult cardiac stem cell populations have been isolated: 1) Lin⁻/c-Kit⁺, 2) Sca-1⁺, 3) Abcg2⁺, and 4) isl1⁺ (50, 51). Treatment with isolated Lin⁻/c-Kit⁺ cells in rats post-infarct resulted in an improvement of cardiac function as well as the formation of both new
cardiomyocytes and vasculature compared to non-treated control animals (62). Although much remains unknown about these cells, it is clear that any differentiation that may occur in vivo as a result of injury does not translate functionally. Much work needs to be completed to better understand these populations of cells in hope of utilizing them for regeneration in the future.

1.3 Soluble Factors

In general, the use of the aforementioned types of cells has led to some level of functional improvement and reduction of the remodeling response as a result of ischemic injury. However, the mechanism of action of these responses is poorly understood. Due to the lack of true integration with the host tissue (i.e., gap junction formation and contractile activity) that is also often observed, it has been suggested that any observed functional improvement might be due to indirect endocrine or paracrine effects such as an increase in angiogenesis and elasticity, as well as a reduction in cardiomyocyte apoptosis (19, 45, 63, 64). If this is the case, the mechanisms behind this improvement needs to be elucidated because the release of soluble factors alone as opposed to cellular therapy may lead to better and faster translation to the clinic.

An increase in vascularity and perfusion in the infarct and borderzone regions is critical for improvement of cardiac function post-MI (45, 64, 65). Neovascularization takes place through many stages, including angiogenesis and arteriogenesis. Angiogenesis results in capillary formation, whereas arteriogenesis results in maturation of vessels. Vascular endothelial growth factor (VEGF) is a potent inducer of angiogenesis, while platelet derived growth factor (PDGF) is involved in arteriogenesis (64-67). An improvement in blood vessel number and maturation with local and controlled release from biodegradable polymers with distinct release kinetics has been observed (66). Furthermore, subsequent release of these growth factors from alginate hydrogels injected into rat models of MI confirmed these observations (68).

Fibroblast growth factor (FGF) is also a pro-angiogenic factor that is less studied, but appears to act similarly to VEGF (64). Delivery of FGF has demonstrated mixed success in vivo. In one study, there was no effect on canine coronary arteries following recombinant FGF-1 administration (69). However, delivery of FGF-2 into the myocardium of patients undergoing
elective coronary bypass grafting demonstrated an increase in vascularity in the ventricle, an increased ejection fraction, and an overall improved prognosis compared to patients not exposed to FGF-2 at a three year follow-up (70).

Granulocyte colony-stimulating factor (G-CSF) plays a role in proliferation and differentiation and has been shown to affect the release of hematopoietic stem cells into the peripheral blood (25, 71). Studies have demonstrated the improvement in vascularity and cardiac function following delivery of G-CSF in animal models (71-74). Delivery of G-CSF was evaluated in clinical trials and did lead to enhanced neovascularization. Unfortunately, there was no improvement in cardiac function compared to patients treated with a placebo (75).

Erythropoietin (EPO) is another cytokine that has been evaluated for myocardial treatment. It is important for erythrocyte survival and differentiation (25). Administration of EPO for 5 days after infarct reduced cardiomyocyte death by ~50% in a rat model (76). Recently, a decrease in infarct size and an increase in capillary density were observed following treatment with EPO compared to saline treated controls in a rat infarct model. Furthermore, an increase in CD34* and c-Kit* cells were observed in the EPO treated animals (77).

Stromal derived growth factor (SDF-1) is a chemokine that is involved in homing of various stem cells for remodeling and repair (78). The specific involvement in the recruitment of stem cells to the heart only following injury was described in 2004 (79). Recently, SDF-1 was delivered to the heart following covalent attachment to a PEGylated fibrin patch and demonstrated improvement in LV function, as well as an increase in c-Kit* cells compared to the control group (78).

Insulin-like growth factor (IGF-1) has been observed to be cardioprotective and lead to reduced cardiomyocyte apoptosis when administered in a murine model of ischemia (80). Peptide nanofibers with encapsulated IGF-1 showed a reduction in cardiomyocyte apoptosis in vitro as well as an improvement in cardiac function when delivered with cardiomyocytes to injured myocardium (27). Similar results were recently observed with delivery of cardiac progenitor cells and IGF-1 encapsulated in peptide nanofibers (81).

1.4 Scaffolds/Biomaterials
In general, scaffolds in tissue engineering play a key role in guiding the growth and organization of the nascent tissue. Similar to the native extracellular matrix (ECM), scaffolds should be able to facilitate similar crosstalk interactions that occur with the surrounding cells. Moreover, it should be non-toxic, non-immunogenic, and biocompatible. Also, the mechanics and degradation rate should be tuned as desired for a particular application. The size and architecture of the scaffold should be taken into account such that diffusion of nutrients and waste can take place and vascular network formation is possible (19, 21, 25).

The native ECM in the heart is a unique architecture consisting of reciprocal spirals that meet in a vortex in the apex (82, 83). This asymmetrical helical structure is energy efficient and advantageous for equal distribution of the stresses and strains associated with the pumping action necessary to distribute blood and nutrients throughout the body (82-85). Furthermore, the alignment of the collagen fibers rotate in direction between the epicardium and endocardium transmurally (86). This complex architecture is responsible for the highly non-linear mechanical behavior observed during mechanical testing of myocardium.

Not only is it a challenge to be able to successfully mimic the structure and non-linear elasticity of the myocardium, but also the optimal material properties (i.e., modulus) desired for engineering myocardial tissue are unknown. Uniaxial tensile testing of rat heart tissue in the longitudinal and circumferential directions (with respect to the epicardial surface) reported moduli of 84 ± 8 kPa and 157 ± 14 kPa, respectively (87). However, recent computer simulations have suggested that increasing the stiffness of the infarct region leads to less infarct expansion and a reduction in the remodeling response (88, 89).

There are two main approaches that have been investigated for improvement of cardiac function and attenuating the maladaptive remodeling response that occurs post-MI: 1) in situ forming, injectable materials and 2) grafts that are wrapped or sutured onto the surface of the heart. In general, some level of functional improvement has been observed in vivo using both of these approaches both with and without cells. However, the underlying mechanisms behind these observations remain unknown and may be due to changes in ventricular geometry and
mechanics associated with the addition of these materials. The rest of this chapter will focus on discussing these two strategies.

1.4.1 Injectable Materials

Injectable materials are attractive for tissue engineering applications due to the minimally invasive nature in which they can be delivered (25, 90, 91). The possibility of delivery via a catheter eliminates the need for open heart surgery and any possible complications of such an intense procedure. This is especially advantageous for older patients, who may not be able to undergo such a procedure.

Christman and colleagues (38) were the first to utilize injectable materials in the heart with the use of fibrin glue that was injected, with and without myoblasts, in a rat model of infarct with reperfusion. They observed that fibrin glue treatment was able to preserve the tissue thickness in the apex and maintain fractional shortening. A secondary study revealed an improvement in cell retention, increased arteriole density, and a reduction in infarct size with fibrin glue treatment, both with and without myoblasts (37). An increase in arteriole density in rats treated with fibrin injections after MI was further confirmed by Huang et al. (92). As a step towards clinical implementation, Martens et al. (93) evaluated catheter-based delivery of fibrin with mesenchymal stem cells to the myocardium in a nude rat model of myocardial infarction.

Alginate has also been studied extensively as a material for intramyocardial injection to limit ventricular remodeling (94-99). Unmodified alginate, as well as alginate that was modified with the RGD peptide sequence in order to facilitate cell adhesion, were evaluated in rat models of MI in two studies (97, 99). In the study by Yu et al. (99) both groups had a significant difference in arteriole density compared to control, however there was no difference between the alginate treatment groups. Tsur-Gang and colleagues observed that the RGD-modified alginate did not attenuate remodeling as well as the unmodified alginate (97).

Two studies also evaluated the impact of the timing of injection on remodeling in rat models of infarct (94, 98). Landa et al. used a biotin labeled alginate, which was delivered to the heart with and without neonatal cardiomyocytes at 7 days and 2 months post-infarct (94). Late administration of the alginate led to an increased scar thickness and a reduced expansion
compared to controls (94). Another study compared administration of alginate and fibrin at 5 weeks post-infarct (98). Both groups treated with biomaterial injections had an increase in wall thickness and arteriole density compared to control animals. However, fibrin had a reduced infarct size and a greater number of arterioles. Also, only alginate was present after five weeks, whereas fibrin was not (98).

Injection of alginate into the LV post-infarct has also been evaluated in large animal studies. The Spinale group injected a fibrin-alginate blends into swine 7 days following infarct and noted a significant reduction in MMP2 in the infarct region of the treatment group, as well as an increase in wall stiffness, which was calculated using pressure measurements (96). The Leor group also used intracoronary injection to deliver alginate in a swine model of infarct (95). A decrease in systolic left ventricle area was observed with alginate treatment; however, this study was completed using early reperfusion (95). It would be a stronger study if a model of permanent occlusion were used, since reperfusion itself can impact remodeling.

The injection of self-assembled peptide-based nanofibers into the myocardium has also been evaluated as a therapy to improve cardiac function after infarct (27, 81, 100, 101). Generally, a growth factor, such as IGF-1 (27, 81) or PDGF (100, 101), is tethered to the nanofibers in vitro prior to delivery. Treatment with IGF-1 tethered nanofibers and either cardiomyocytes (27) or cardiac progenitor cells (81) led to an improvement in cardiac function in rats. Also, administration of nanofibers with tethered PDGF led to an improvement in function compared to administration of nanofibers or PDGF alone (100, 101).

Due to the increased interest in use of injectable biomaterials to limit remodeling and improve cardiac function, several groups have begun to synthesize and characterize alternative materials for this application (102-106). For example, Fujimoto and Wagner have synthesized a biodegradable, temperature responsive hydrogel based on N-isopropylacrylamide, acrylic acid, and hydroxyethyl methacrylate-poly(trimethylene carbonate) (102). Injection of this hydrogel into the infarct region 2 weeks post-infarct demonstrated significant differences in end diastolic area and fractional area change compared to control animals in a rat model. Furthermore, abundant cell infiltration into the hydrogel was observed (102).
Although great strides have been made in the development of injectable materials for cardiac tissue engineering, much work remains to be completed. Further studies are necessary to elucidate the mechanism behind the beneficial responses associated with biomaterial treatment. Work also needs to be done to evaluate the impact of different material properties (e.g., mechanics) on the associated remodeling response. Also, any effects due to the mode of delivery (e.g., catheter-based) and timing of delivery (e.g., 1 week following infarct) need to be understood.

1.4.2 Graft Materials

1.4.2.1 Cell Derived ECM Based Grafts

Cell derived ECM based grafts are obtained by culturing the cell type of interest onto cell culture surfaces which are modified with the thermoresponsive polymer poly(N-isopropylacrylamide) (PNIPAAm). Under normal culture conditions at 37°C, NIPAAm is a hydrophobic polymer capable of supporting cell attachment. However, upon cooling to 32°C the polymer transitions to a hydrophilic, and rapidly swelling substrate, thus releasing any attached cells (19, 90, 107). Often, cells are cultured until a complete monolayer containing many cell-to-cell junctions exists, such that an intact sheet of cells, as well as any elaborated matrix is released instead of individual cells. These sheets can then be stacked into multiple layers and transplanted onto any tissue of interest (19, 84, 90, 107). However, issues with necrosis arise when attempting to stack >6 sheets (~100 μm thick), due to diffusion limitations (90). The reader is directed to an excellent review article regarding cell sheet engineering for cardiac tissue engineering by Masuda et al. (107). Briefly, studies using neonatal rat cardiomyocyte (108) and skeletal myoblast (109) cell sheets that were implanted onto hearts post-infarct (108) demonstrated improve function. Also, a study comparing adipose derived mesenchymal stem cell sheet implantation versus fibroblast cell sheet implantation in a rat model of MI noted an increase in cardiac function and a decrease in wall thinning in the animals treated with the stem cell derived cell sheets as opposed to cell sheets composed of fibroblasts (110).
The Murry group has also used a scaffold-free approach for cardiac repair recently (33). They formed their grafts by combining human ESC derived cardiomyocytes, hESC derived (or human umbilical vein) endothelial cells, and fibroblasts in low adhesion cell culture plates on an orbital shaker. Animals were sacrificed one week following implantation of the grafts onto the surface of the nude rat heart. The vasculature within the grafts anastomosed with the native rat vasculature, suggesting the formation of viable myocardium with integrated vasculature (33).

### 1.4.2.2 In Vitro Engineered Tissue

Several groups have been successful in myocardial tissue engineering in vitro, which has provided insight for further graft development and in vivo studies. The reader is directed to the following studies which describe the importance of mechanical loading (111), electrical stimulation (112), substrate structure (87), and soluble factors (113) on construct performance in vitro. The remainder of this chapter will focus specifically on milestones in the field of in vitro engineered myocardial tissue engineering that were evaluated in vivo.

Li and colleagues used a commercially available Gelfoam, gelatin mesh that was seeded with fetal cardiomyocytes and cultured for 7 days prior to implantation onto the surface of a rat heart 3 weeks post-infarct (114). The graft appeared to be infiltrated with native vasculature. However, the authors reported that graft treated animals did not have significantly better ventricular function at the 5 week time point (114). In contrast, Leor et al. (31) utilized fetal cardiomyocytes that were seeded into porous alginate scaffolds and cultured for 4 days prior to implantation onto the heart 7 days post-MI. At 65 days post-treatment, the scaffold was not visible and graft treated animals had improved vascularity and less dilation of the left ventricle than the untreated control group (31). Another study by this group utilized alginate scaffolds that were seeded with Matrigel containing neonatal cardiomyocytes, IGF-1, VEGF, and SDF. After culturing these constructs in vitro for 48 hours, the grafts were surgically implanted onto the omentum in the abdomen of a rat for 7 days to initiate vascular formation prior to implanting onto the epicardial surface at 7 days post-MI (30). Upon examination at the 4-week time point, the grafts appeared to be structurally and electrically integrated with the native tissue. Moreover, a thicker scar and an increase in cardiac function were evident for graft treated animals. A study by
Tan et al. (115) evaluated the potential of a scaffold derived from small intestinal submucosa and seeded with mesenchymal stem cells for myocardial repair in a rabbit model. An overall improvement in pathology, specifically with an increase in capillary density and contractile function was observed in graft treated animals (115).

Instead of cell seeded-preformed grafts, other groups have evaluated composite mixtures of cells and scaffolds. Zimmerman and Eschenhagen prepared a graft using neonatal rat heart cells that were encapsulated within a collagen matrix and matrigel (32). Complete constructs were constructed by stacking five constructs in a clockwise fashion. In vitro grafts demonstrated relatively strong contractile properties. Grafts were implanted onto the surface of a rat heart 14 days post-infarct and were observed to prevent dilation and to increase wall thickness and fractional area shortening at the 4-week time point. Moreover, a lack of arrhythmias was also observed (32). Kofidis et al. (116) utilized a similar approach and fabricated their grafts by encapsulating human ESCs within collagen. However, instead of suturing the graft to the epicardial surface, grafts were surgically implanted into the wall of the myocardium. The transplanted cells remained viable and an increase in wall thickness and fractional area shortening was observed for graft treated animals (116).

1.4.2.3 Left Ventricular Restraints

In a less traditional type of tissue engineering strategy, researchers have used polymeric meshes, that are wrapped around the heart, in an effort to restore the heart to a normal geometry and therefore reduce the mechanical burden that exists following infarct (3). Kelley and colleagues (117) wrapped a knitted poly(propylene) Marlex mesh around an ovine heart prior to infarction. At 8 weeks post-infarct, all control animals exhibited signs of heart failure, whereas mesh treated sheep did not. Moreover, mesh treatment preserved wall thickness and left ventricle chamber size, whereas a reduction in wall thickness and left ventricle dilation was observed (117). In a study by Bowen et al. (118), a reduction in MMP1 (collagenase) and MMP2 (gelatinase) as well as an increase in collagen was evident in the borderzone region with treatment. A similar study evaluated the effect of stiffening a posterior infarction using a Marlex mesh in sheep. Mesh treated sheep demonstrated less mitral regurgitation as well as a
reduction, although not statistical in end diastolic and end systolic volumes (119). This observation was also made in another study where a compliant Marseline (polyester) mesh was used to attenuate the infarct expansion (120).

Pilla and colleagues (121) made similar observations when using a bidirectional woven mesh, the Acorn Cardiac Support Device (CSD), which was surgically implanted onto the heart one week following infarct in an ovine model. Specifically, end diastolic volumes were significantly decreased and ejection fraction was statistically increased two months after treatment (121). In a later study, a reduction of myocyte length and volume was observed, as well as an increase in collagen within infarct, peri-infarct, and borderzone regions (122). The authors believe that the preservation of the fibrilar collagen in the peri-infarct region is likely involved in the observed reduction in infarct expansion and remodeling (122).

The Acorn CSD has also been evaluated in human patients (123-125). Konertz and colleagues noted a reduction in mitral regurgitation and ventricular dimensions, as well as an improvement in ejection fraction in 27 patients that were treated with the Acorn CSD (123). Bredin et al. (125) recently published a follow-up study where 20 patients were followed over the course of 32 ± 5 months following implantation of the Acorn CSD. Again, a decrease in cardiac dimensions as well as an increase in cardiac function and quality of life was observed. The next steps in evaluating the Acorn CSD is to determine optimal patient selection protocols, as well as the appropriate timing of therapy (125).

A few groups have recently evaluated the use of elastic biodegradable materials as left ventricular restraints (126-128). Elastic materials are attractive for cardiac applications due to their ability to undergo plastic deformation and function within the dynamic mechanical environment found in the heart (19, 128). Also, a preliminary report, which investigated the over-expression of elastin in the infarct region demonstrated that increasing the elasticity in the infarct region leads to an improvement of function due to a decrease in thinning and dilation (129). The Wagner group (126, 127) evaluated the implantation of a biodegradable poly(ester urethane urea) porous graft two weeks post-infarct on remodeling in a rat model. At the 8-week time point it appeared that the graft was completely degraded and the remnant patch area appeared to be
infiltrated with macrophages and fibroblasts. An increase in wall thickness and capillary density, as well as an increase in muscle-like bundles (by alpha smooth muscle staining) were observed in the graft treated animals compared to control (126). Recently, Chen and Harding (128) reported the use of the biodegradable elastomer poly(glycerol sebacate) (PGS) as a graft. However, in this study the PGS film was implanted onto the surface of a non-infarcted heart to evaluate the feasibility and biocompatibility of PGS as a graft. Implantation of the PGS film did not significantly alter cardiac function compared to control animals (128).

Although the use of scaffolds, both acellularly and with cells, has demonstrated an improvement in the remodeling response that occurs post-MI, the need for a major surgical procedure for this approach is a major drawback. Moreover, studies need to be performed to better understand the beneficial response associated with the use of these grafts.

1.5 Conclusions

The use of cells, soluble factors, and scaffolds within the realm of myocardial tissue engineering has been reviewed within this chapter. Implanted cells only minimally affect the global function of the heart due to the lack of structural cues for alignment due to the intense disarray of collagen fibers that exists post-infarct. Due to the poor engraftment and differentiation of the implanted cells in the native myocardium, any observed improvement in function is believed to be due to the change in volume associated with the presence of the cells or the release of paracrine factors. With this in mind, as well as the lack of a functional cell source, acellular approaches for the reduction left ventricular remodeling and improvement in the global and regional function of the heart post-infarct are very attractive. However, much work needs to be performed to fully understand the mechanism of action that exists with the use of either acellular injectable materials or left ventricular restraints. Specifically, the salutary effect of material properties (e.g., mechanics) on the observed remodeling response needs to be elucidated in order to identify the optimal material and restraint techniques (injection versus implantation) prior to clinical translation.
References:


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Chapter 2

Research Overview

2.1 Specific Aims & Hypotheses

Each year millions of Americans suffer from myocardial infarctions (MI). Left ventricular (LV) remodeling post-MI often induces congestive heart failure (CHF), which is life threatening and has few viable treatment options. Early infarct expansion (stretching) and associated LV dilation have been identified as indicators of poor long-term prognosis even in asymptomatic patients. Implantation of physical restraints, as well as injections of various types materials with and without cells have been somewhat successful in the attenuation of this remodeling response. With recent advances in polymer chemistry, scaffold development, and drug delivery capabilities, new strategies for therapeutic development are now possible. Specifically, hydrogels and elastomeric and biodegradable polymers that are non-toxic and biocompatible are being developed as implants for a variety of applications, including cardiac repair. This dissertation describes work towards this goal through two approaches. First, through development of biodegradable elastomers with tunable properties that are similar in mechanical properties to soft tissues, and processing into fibrous scaffolds with controlled architectures. Secondly, through the development of a redox initiated hydrogel system capable of in situ polymerization for injection into damaged myocardium.

Global Hypothesis: We hypothesize that fibrous scaffolds with controlled properties and architectures, as well as injectable hydrogels with tunable properties, can be used to attenuate the maladaptive post-MI remodeling response. Specifically, this work will test four hypotheses that are described below. The overall goal of this work is to gain a better understanding of these novel materials toward their development as a therapy to reduce LV remodeling and the onset of CHF.
Specific Hypothesis 1: Variations in molecular weight and degree of modification will lead to a collection of biodegradable and photocrosslinkable polymers with different moduli and % extension at break.

Specific Aim 1: To synthesize and characterize a tunable collection of photopolymerizable and biodegradable elastomers. Macromers will be synthesized through the introduction of the acrylate functional group into the synthetic scheme of the elastomer poly(glycerol sebacate) (PGS) such that crosslinking through free radical polymerization is possible. The molecular weight and amount of functional group introduced will be altered to investigate the resulting structure/property relationships of the formed networks. Specifically, the reaction behavior, mechanical properties, degradation kinetics, in vitro cellular interactions, and in vivo biocompatibility will be evaluated.

Specific Hypothesis 2: Photocrosslinkable, fibrous scaffolds composed of candidate macromers and processed into defined structures will dictate degree of cellular alignment and matrix elaboration.

Specific Aim 2: To process the biodegradable elastomers into fibrous scaffolds using photopolymerization and to investigate in vitro and in vivo cellular interactions based on material and structure. Candidate macromers from Aim 1 will be combined with carrier polymers, in order to be electrospun into fibrous scaffolds. Scaffolds will be prepared by electrospinning onto a flat plate or rotating mandrel to collect randomly dispersed or aligned fibers, respectively. The mechanical properties in both the fiber and perpendicular directions, as well as cellular interactions will be studied. Furthermore, aligned dual polymer scaffolds will be prepared with the simultaneous electrospinning of the macromer from one jet and a sacrificial polymer from a second jet to control scaffold porosity. The sacrificial fiber will be removed and the resulting effects on architecture, mechanics, and cellular interactions will be evaluated both in vitro and in vivo.
Specific Hypothesis 3: Variations in degree of modification, macromer concentration, and initiator concentration will lead to redox-initiated hydrogels with different moduli and gelation behavior.

Specific Aim 3: To develop crosslinkable hyaluronic acid based hydrogels for injection in vivo. A previously established methacrylated hyaluronic acid hydrogel system will be further developed and optimized to polymerize via redox initiation, a technique that is compatible for injections in vivo. The bulk properties and delivery and penetration upon injection into explanted tissue will be used to identify specific formulations that met criteria for our proposed application. Ultimately, a dual-barrel syringe system that can be used to inject the macromers and initiators in vivo will be identified.

Specific Hypothesis 4: Variations in the modulus of the hydrogels will influence infarct size, left ventricular geometry, and cardiac function.

Specific Aim 4: To assess the functional and geometric sequelae of intramyocardial infarct stiffening using injectable hydrogels. After identifying the macromer formulations and an injection process, a well-characterized ovine model of post-MI remodeling will be used to assess the impact of the injected hydrogels (with a range of mechanics). Global and regional LV function and geometry will be assessed by means of state-of-the-art three-dimensional echocardiography at baseline, immediately following anteroapical MI, immediately following macromer injection, and at multiple long-term follow up intervals. End diastolic volume, end systolic volume, cardiac output, ejection fraction, and infarct area will be compared between the cohorts as a measure of remodeling.

2.2 Research Overview

The motivation for developing alternative materials and processing capabilities to elicit a desired response, specifically in the myocardium (i.e., matrix organization) has already been discussed in Chapter 1. Millions of Americans suffer from myocardial infarction (MI) each year and alternative treatment strategies need to be developed in order to prevent the onset of congestive heart failure, which often develops as a result of the adverse remodeling response
that occurs post-MI. Chapter 3 will review the types of crosslinking strategies utilized to form the polymer networks described in this dissertation. Finally, this work will focus on either 1.) development of acrylated poly(glycerol sebacate) scaffolds with controlled architectures and 2.) development of injectable hyaluronic acid hydrogels with controlled mechanics and gelation.

Chapter 4 describes the synthesis and characterization of a collection of hydrolytically degradable acrylated poly(glycerol sebacate) (PGS) macromers and their crosslinking into networks. The structure property relationships associated with changing the molecular weight or number of acrylate functional groups introduced onto the macromer will be investigated. Furthermore, the degradation behavior, cytotoxicity, and biocompatibility of one of the candidates will be evaluated. The influence of variables associated with electrospinning fibrous scaffolds, as well as carrier polymer and macromer used, on scaffold properties will be described in Chapter 5. Chapter 6 describe the additional processing capabilities of fiber alignment and multi-polymer electrospinning and their control over scaffold architecture. Finally, the resulting influence of scaffold architecture on cellular infiltration and matrix elaboration will be described.

In a slight shift, the influence of injectable biomaterials on the left ventricular response that occurs post-MI will be more directly assessed. Chapter 7 describes the optimization of redox initiation to crosslink methacrylated hyaluronic acid (MeHA) macromers. The influence of macromer methacrylate substitution, concentration, and initiator concentration will be assessed and used to select two candidate macromers for in vivo applications. The salutary effects of intramyocardial injection of two MeHA hydrogels with varying mechanics on remodeling will be investigated in Chapter 8. Global left ventricular geometry and function, as well as infarct area will be used to evaluate the remodeling response.

Finally, Chapter 9 discusses the overall conclusions and impact of this work. Furthermore, any encountered limitations, as well as possible future directions will be described and elaborated upon.
Chapter 3

Overview of Free Radical Polymerization in Biomaterial Processing


3.1 Introduction

Polymeric materials are being widely used and continuously developed for a variety of biomedical applications. The past few decades have seen an increase in the development of degradable biomaterials for applications such as tissue engineering and drug/molecule delivery (1-3). In tissue engineering, polymeric materials can provide scaffolding for the controlled development and evolution of 3-dimensional tissues (1, 4). When designed to be degradable, the materials are eventually replaced by the growing tissue (1, 4). In molecule delivery, degradable polymers are used to entrap various molecules that are released as the polymer degrades or through diffusion mechanisms. If growth factors are delivered, this approach can lead to alterations in cellular differentiation and the type and quality of tissue that forms (4).

Numerous clinical applications benefit from the ability to in situ form biomaterials. For example, poly(methyl methacrylate) (PMMA) bone cements are commonly used to secure various implant prostheses in orthopaedics (5-7). In these applications, methyl methacrylate monomer is mixed with PMMA to form a viscous solution that can be injected in vivo and polymerized via redox or thermal initiation. In dentistry, dimethacrylate monomers with ceramic fillers are polymerized in tooth caries via a photoinitiated polymerization to in situ form composite restorations (8). These materials have excellent properties with respect to mechanics, but are nondegradable.
With advances in synthetic chemistry, novel multifunctional monomers and macromers have been synthesized that form degradable polymers via free radical initiated polymerizations, potentially as injectable biomaterials. Free radical polymerization is a type of chain polymerization and can be accomplished through the use of thermal, redox, or photoinitiated initiation (9). This chapter will review information on the general polymerization behavior for all three types of initiation and degradation mechanisms and outline the various monomers and macromers that have been synthesized for the production of degradable biomaterials for applications in tissue regeneration, with a focus on redox and photopolymerization.

3.2 General Network Formation and Degradation

In general, the polymerization of multifunctional monomers is a complex process. The reaction mechanism (i.e., initiation, propagation, and termination) for a radical chain polymerization is outlined in Figure 3.1 (10). The initiation phase involves the production of radicals through a variety of means, including thermal, redox, and photoinitiation mechanisms. These specific mechanisms will be discussed below, along with their respective kinetics. In general, initiation involves two steps: 1) production of the initiator or primary radical and 2.) reaction of the initiator or primary radical with the monomers or macromers that are present. Propagation is the growth of activated chain radicals by successive addition of monomer molecules to form long kinetic chains. Termination is the conclusion phase of the polymerization and can occur through a combination reaction where two radicals react with each other. Although the majority of termination occurs via this bimolecular combination reaction, it can also occur via disproportionation where a hydrogen atom that is adjacent to the radical center is transferred to another radical center resulting in one saturated molecule and one unsaturated molecule. The rate of termination ($R_t$) is a bimolecular reaction and depends on the concentration of radicals in the system, whereas the rate of polymerization ($R_p$) is a measure of the rate at which double bonds are consumed during the polymerization. Since the bulk of this occurs in the propagation steps, $R_p$ can be approximated as a second order reaction that depends on both the double bond
and radical concentration. If one assumes pseudo-steady state on the radical concentration (i.e., \( R_i = R_t \)), \( R_p \) becomes a function of \( R_i \), the monomer concentration \([M]\), and the propagation \((k_p)\) and termination \((k_t)\) kinetic constants (Figure 3.1). While relatively simple in form, the polymerization behavior is quite complex, because \( k_p \) and \( k_t \) are highly dependent on the conversion and evolving network structure (9).

![Reaction mechanism for free radical polymerizations and the corresponding rate equations, assuming pseudo-steady state conditions. \([I]\) is initiator concentration, \( f \) is initiator efficiency, \( I_a \) is the intensity of absorbed light, and \( \Phi \) is the number of propagating chains initiated per light photon absorbed.](image)

**Figure 3.1.** Reaction mechanism for free radical polymerizations and the corresponding rate equations, assuming pseudo-steady state conditions. \([I]\) is initiator concentration, \( f \) is initiator efficiency, \( I_a \) is the intensity of absorbed light, and \( \Phi \) is the number of propagating chains initiated per light photon absorbed.

A typical plot of \( R_p \) versus time for a multifunctional monomer homopolymerization is shown in Figure 3.2, along with the integrated rate curve, which gives double bond conversion. The magnitude of the peak maximum and the time to reach this maximum rate are altered by changes in initiation conditions (e.g., initiator concentration) (11, 12). From the onset of the reaction, the polymerization rate increases with conversion, in a region termed autoacceleration (13, 14). During this time, the 3-D network structure is evolving, and the mobility of terminating macroradicals decreases, and consequently, the rate of termination decreases. This mobility
restriction on termination leads to a build-up in the concentration of radicals in the system, and the rate of polymerization increases. Of further note, at some point during the period of autoacceleration, termination is controlled by a reaction diffusion mechanism as radicals terminate by reacting through unreacted double bonds present in the system (15, 16). After the polymerization rate reaches a maximum, autodeceleration occurs as propagating species are now diffusion controlled (11, 13). Understanding this complex interplay of reaction and diffusion controlled mechanisms is critical for the design of in situ forming biomaterials. For example, while autoacceleration helps decrease the total polymerization time, autodeceleration limits the maximum conversion.

![Polymerization Rate and Conversion](image)

**Figure 3.2.** General polymerization rate and conversion during the radical polymerization of multifunctional monomers.

In fact, with highly crosslinked glassy networks, a double bond conversion of one is almost never reached due to severe restrictions on the mobility of the reacting molecules. Unreacted monomer can have significant effects on both the mechanics and biocompatibility of the resultant network with severe implications on biological applications. For instance, low conversions can decrease the mechanical properties of the biomaterial and unreacted and potentially toxic monomer can leach from the network and have detrimental effects on the surrounding tissue. Thus, attaining conversions approaching 100% is very important and can be
problematic. However, the formation of hydrogel networks in an aqueous environment can occur with high conversions due to the high mobility of reacting species during gel formation (17).

3.2.1 Thermal Initiation

Thermal initiation is the most common type of free radical polymerization for both commercial applications and theoretical studies. However, its use for biomaterials and tissue engineering applications is limited due to the high temperatures that are typically necessary to provide the 100-170 kJ mol$^{-1}$ bond dissociation energy required for radical formation. The reaction kinetics for thermal initiation is shown in Figure 3.1 and the activation temperature for each system is dependent on the initiator used. Acyl peroxides, alkyl peroxides, hydroperoxides, peresters, and azo compounds are generally used as thermal initiators based on the fact that these groups contain O-O, S-S, or N-O bonds based on their dissociation kinetics (9, 18).

3.2.2 Redox Initiation

In general, redox initiation takes place through direct transfer of an electron from an oxidant to a reductant. The concentration of each of these molecules dictates the reaction rate. This type of radical initiation is beneficial due to the reduced activation energy required, allowing for polymerization to occur in a wide range of temperatures and mild conditions compared to thermal initiation (9, 18). Additionally, redox initiation may be used for injectable applications for \textit{in situ} network formation where light penetration is not possible for photopolymerization (19).

In general, there are several types of redox systems that can be used to initiate polymerization. Common redox initiators for tissue engineering applications include: peroxides (e.g., benzoyl peroxide with N,N-dimethyl-p-toluidine (20) and hydrogen peroxide with ferrous salt (21)) and persulfates (e.g., ammonium persulfate with N,N,N$'$,N$''$-tetramethylene diamine (22-25) or ascorbic acid (19)). Recently, investigators have been successful with the use of enzymes as one of the redox components to initiate polymerization (26, 27). The reader is directed to the article by Sarac for an extensive review on types of redox initiators used in a variety of applications (18).
3.2.3 Photoinitiation

During photoinitiation, the primary radical is generated upon exposure of visible or ultraviolet light. The initiator concentration and light intensity dictate the initiation rates and kinetics of this type of chain polymerization. Unlike thermal or redox initiation, photoinitiation can be spatially controlled with the use of photomasks to isolate crosslinking to specific regions of interest (9, 28, 29).

Moreover, temporal control over the photoinitiation process can be used to minimize temperature rises during the exothermic radical polymerization, which is not possible with thermal initiation. Burdick et al. showed that by changing initiation conditions, and specifically, the initiating light intensity, the temperature rise is readily controlled (28). For instance, when a sample was polymerized from room temperature with ultraviolet light, the surface temperature reached a maximum of \( \sim 46^\circ C \) when polymerized with a light intensity of 100 mW/cm\(^2\), whereas a sample polymerized with 25 mW/cm\(^2\) only reached a maximum temperature of \( \sim 33^\circ C \). The control over temperature rise during polymerization, which is not possible with thermal or redox initiations, could also influence the encapsulation of growth factors, since protein stability can decrease with increased temperatures. Another issue that should be addressed in using photopolymerization to form thick biomaterials is the attenuation of the initiating light source due to absorption by the initiator molecules. However, this can be overcome using systems such as photobleaching initiators, where the initiator radicals absorb light at a different wavelength than the initiator molecules (28), or dual initiators, where a photoinitiation mechanism raises the sample temperature high enough to initiate thermally. This process allows for high conversions in areas where light does not reach.

3.2.4 Polymerization in the Presence of Cells

The delivery of cells to damaged tissues is often one of the primary goals of tissue engineering. With redox and photopolymerization, it may be necessary to polymerize networks directly in the presence of cells. Due to the reaction conditions, potentially harmful initiator molecules, radicals, and light will be present during this polymerization process, and thus, it is
necessary to determine whether cells remain viable after encapsulation. Cell encapsulation is only possible with certain types of macromers, and specifically, with water soluble macromers that form highly hydrated polymers upon polymerization. Thus, many of the materials addressed in this review are not candidates for cell encapsulation and delivery due to their hydrophobicity, and consequently, diffusion limitations to entrapped cells.

To address toxicity during polymerization, several groups have investigated initiation conditions that lead to viable cells upon polymerization (19, 21, 27, 30, 31). One specific initiating system that uses the water-soluble photoinitiator Irgacure 2959 (I2959, 2-hydroxy-1-[4-(hydroxyethoxy) phenyl]-2-methyl-1-propanone) and low-intensity ultraviolet light, has been widely used for the encapsulation of multiple cell types in photopolymerizable hydrogels (31-33). However, there are several examples where a loss of cell viability has been attributed to the specific initiation system used (19), and thus, it is essential to pick the appropriate conditions for cell encapsulation to promote cell viability.

3.2.5 General Degradation Behavior of Crosslinked Polymers

As detailed throughout this review, numerous monomers and macromers have been synthesized that are polymerizable using free radical initiation into networks that are degradable. Several general possibilities for the formed network structures are illustrated in Figure 3.3. In the first example (Figure 3.3A), a polymer or oligomer is end-capped first with degradable units and then photoreactive groups (e.g., acrylates or methacrylates). Upon exposure to light and in the presence of a photoinitiator, this tetrafunctional macromer forms a network with degradable units found in the crosslinks. The hydrolytically or enzymatically degradable units are then cleaved in the presence of an aqueous environment or an enzyme, respectively. This leaves degradation products of the original core molecule, the degradable units, and kinetic chains formed during the free radical polymerization of the reactive groups (e.g., poly(acrylic acid) for acrylates).

In the next example (Figure 3.3B), the reactive groups are found along the polymer backbone linked through degradable units. These pendant reactive groups then polymerize to form a network that eventually degrades into the starting polymer, the degradable units, and kinetic chains. If the number of pendent reactive groups is limited to two, the behavior reduces to
the behavior shown in Figure 3.3A. Additionally, the reactive group may be found along the polymer backbone as shown in Figure 3.3C. These networks degrade into the kinetic chains and segments of the starting polymer, depending on the nature and location of the degradable groups. Finally, the pendent reactive groups may be found along a polymer chain that is in itself degradable (Figure 3.3D). In this situation, the network cleaves along the backbone and releases kinetic chains that likely incorporate segments of the starting polymer. Although additional examples can be suggested, these comprise the majority of systems that are covered throughout the review.
Figure 3.3. Schematic of structures, network formation, and degradation for various free radical (photo) polymerizable monomers and macromers. Reactive groups are represented by =, whereas hydrolytically degradable groups are represented by ///\ . Networks are produced using free-radical polymerization of reactive groups (e.g., acrylates/methacrylates) into kinetic chains (dashed lines) that are degraded via hydrolysis or enzymatic breakdown.

As stated in these examples, one of the primary degradation products is the kinetic chains that are formed through the free-radical polymerization of the reactive groups. These
kinetic chains are typically poly(methacrylic acid) (PMAA) or poly(acrylic acid) (PAA) if the reactive groups are methacrylates or acrylates, respectively. Methods to understand and control the kinetic chain lengths are important for biomaterials applications since the compatibility of water soluble polymers is influenced by their molecular weight (34). Furthermore, the kinetic chains will influence the final polymer structure, which can influence certain material properties.

As a first approximation, the kinetic chain length is controlled by varying the rate of polymerization relative to the rate of chain terminating events. If one assumes that \( R_i \) is equal to \( R_t \), then \( R_i \) (a function of the initiator concentration and initiating light intensity) can be used to control the distribution of kinetic chain lengths. Burkoth et al. (35) utilized matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) to analyze the degradation products of crosslinked polyanhydrides and Burdick et al. (36, 37) used gel permeation chromatography to assess kinetic chain lengths. These studies determined that network conversion, the rate of initiation, and sample depth heavily influence kinetic chain length distributions (36, 37).

It is difficult to predict network physical properties and degradation behavior based entirely on macromer structure (e.g., molecular weight, branching), because factors such as backbone chemistry and hydrophobicity play a role in these properties. However, an increase in the polymer crosslinking density, due to an increase in macromer concentration or a decrease in macromer molecular weight, typically leads to enhanced mechanical properties and an increase in the time for network degradation, due to a decrease in water diffusion and an increase in the number of bonds that must be cleaved to break the network into water-soluble components. Likewise, an increase in polymer crosslinking density can be used to slow the delivery of entrapped drugs or growth factors because diffusion becomes more limited. These factors are also dependent on whether the system is a highly swollen hydrogel or a highly crosslinked network.
3.3 Synthetic Polymers

3.3.1 Crosslinkable Polyanhydrides

Polyanhydrides were originally modified with crosslinkable methacrylate groups by Anseth et al. (38) to produce polymer networks with controlled degradation and mechanics with degradation, and had the added potential of network formation directly in the body. The general structures for selected dimethacrylated anhydride monomers are shown in Figure 3.4. In general, the core of the molecule consists of hydrophobic repeating units, such as sebacic acid (SA), carboxyphenoxy propane (CPP), or carboxyphenoxy hexane (CPH), but other anhydride monomers, including methacrylated tricarballylic acid (MTCA, trimethacrylated) and methacrylated pyromellitylimidoalanine (MPMA-ala, amino acid containing) have been synthesized to impart increased crosslinking density and a biologically recognized component, respectively (39).

![Figure 3.4](image.png)

**Figure 3.4.** Photocrosslinkable polyanhydrides, typically synthesized via the reaction of acids with methacrylic anhydride. The “R” groups allow for tailoring of the hydrophobicity of the crosslinks, and consequently, polymer degradation.

Photocrosslinkable polyanhydrides have been extensively characterized with respect to reaction behavior and material properties. Even with the very mild initiation conditions (e.g., with
0.1 wt% initiator and 7 mW/cm² ultraviolet light, polymerization occurs within 3 minutes), polymerization times are well within acceptable clinical timescales, allowing for network formation intra-operatively (11). In general, the densely crosslinked networks formed from multifunctional anhydride monomers degrade by a surface erosion mechanism, with mass loss only at the surface erosion zones on the exposed areas of the polymer. These erosion zones allow only minimal penetration of water into the polymer, so hydrolysis occurs exclusively near the polymer surface and is dictated by the polymer chemistry. For example, a disk (~1.7 mm thickness) composed entirely of MSA degrades in ~3 days, whereas a disk composed entirely of the more hydrophobic MCPH takes over 1 year to completely degrade (40). Since erosion only occurs at the polymer surface, structural integrity is maintained for longer degradation periods than in polymers that degrade throughout their bulk. For example, over 90% of the tensile modulus of poly(MSA) and poly(MCPH) networks are maintained for up to 40% mass loss (40). In bulk eroding systems, polymer chains are cleaved relatively homogeneously through the network, and mechanical properties can plummet even when very little mass loss has occurred (41, 42).

Photocrosslinkable polyanhydrides have been explored for several applications. Since they are injectable, they can be formed directly in a bone defect through a free radical polymerization and, with time, degrade as new bone fills the defect and replaces the degrading polymer. Histological sections shown in Figure 3.5 illustrate the dramatic difference in the filling of defects between a prepolymerized polymer implant and an in situ forming implant. In Figure 3.5A, good contact between the polymer implant and the surrounding bone is seen; however in Figure 3.5B, the presence of a large gap in the medullary canal of the rat tibia indicates a lack of complete defect filling by the polymer plug. This may be one of the most important benefits of the photocurable polyanhydrides, since good contact between the biomaterial and bone tissue is essential in tissue regeneration and in maintaining good mechanical properties with the polymer implant. Several studies have also shown that polyanhydrides are biocompatible when degraded in vivo (43-46). The controlled degradation and polymerization behavior also make crosslinked polyanhydrides excellent candidates for the delivery of molecules for applications in not only orthopaedics, but also for in situ forming and drug releasing materials (43-48). Owens et al.(47)
developed a compressed antisolvent precipitation and photopolymerization (CAPP) process, which enables photopolymerization of multifunctional monomers to form degradable crosslinked particles and is applicable to forming microparticles from photocrosslinked polyanhydrides.

Figure 3.5. Histological sections of in vivo photopolymerized (A) and prefabricated (B) polyanhydride implants in a tibial defect in rats. These images illustrate the filling of the defect that is possible with injectable and photopolymerizable materials.

3.3.2 Crosslinkable Poly(ethylene oxide)

Poly(ethylene glycol) (PEG), a water soluble polymer, has a long history of use in biomaterials. This is primarily due to the extreme hydrophilicity of PEG, which decreases the adsorption of proteins and can be used to alter the interaction of materials with tissues and cells. Additionally, the end groups on PEG are easily modified through a variety of synthetic reactions. For instance, the reaction of PEG with acryloyl chloride or methacryloyl chloride in the presence of triethylamine is a simple technique for adding reactive vinyl groups (49). Photopolymerizable PEG hydrogels have been used for numerous applications including as membranes for the encapsulation of islets of Langerhans (49-51), as barriers to reduce intimal thickening after balloon angioplasty,(52) as matrices for chondrocyte encapsulation in cartilage regeneration (53-
for osteoblast encapsulation in bone regeneration (58), for the encapsulation of mesenchymal stem cells (59), and for the delivery of nitric oxide to reduce platelet adhesion and smooth muscle cell proliferation (60). Additionally, Elbert and Hubbell (61) used diacrylated PEG macromers to fabricate hydrogels incorporating various peptides by first reacting the macromer with a cysteine-containing peptide via a conjugate addition reaction and subsequent photopolymerization into a network.

Hubbell and coworkers (62) added oligomers of α-hydroxy acids between the PEG and photoreactive groups to produce degradable macromers. The general synthesis scheme for this reaction is illustrated in Figure 3.6. PEG was first mixed with dl-lactide (or glycolide) in the presence of stannous octoate and reacted under vacuum. In the second step, the intermediate product was redissolved in dichloromethane and reacted on ice with triethylamine and acryloyl chloride. The final product was obtained by filtration and subsequent precipitation in dry diethyl ether. Metters et al. (63) followed up this study by characterizing the swelling and mechanical behavior of the PEG-b-PLA hydrogels with degradation. The study showed that the volumetric swelling ratio of the networks exponentially increased, while the compressive modulus exponentially decreased with degradation and that the behavior is modulated by altering the crosslinking density (i.e., initial macromer concentration) of the hydrogels. A statistical kinetic model predicting the degradation behavior of the hydrogels was also developed (64, 65) that accurately predicts the cleavage of crosslinks in the PEG-b-PLA networks. A second generation model incorporating network non-idealities was also introduced (66).

For tissue engineering applications, the acrylated PEG-b-PLA macromers have been primarily explored for the regeneration of cartilage tissue. In this work, hydrogels were fabricated from non-degradable PEG macromers, degradable PEG-b-PLA macromers, and copolymers of these two macromers (67-70). The overall conclusion of the work is that the copolymers support the most ideal cartilaginous tissue formation. Specifically, the neocartilage formed in the non-degrading hydrogels was not as spatially distributed and did not maintain the proper phenotype as the cartilage formed in the copolymer gels. Additionally, the crosslinking density of the hydrogels influenced the amount, type, and distribution of cartilage tissue that was engineered.
The histological images in Figure 3.6 show the distribution of one extracellular matrix component (i.e., collagen) produced by osteoblasts photoencapsulated in degradable PEG hydrogels fabricated from macromers with various ratios of lactic acid and caprolactone as the degradable component (previously unpublished). The lactic acid crosslinks are more rapidly hydrolyzed from the networks than the caprolactone crosslinks, and thus, can be used to temporally control the network degradation. After 12 weeks of implantation subcutaneously in the dorsum of nude mice, the histology indicates that the initial hydrogel chemistry plays a large role in controlling extracellular matrix distribution. For the hydrogel comprised entirely of the more slowly degrading macromer, there is little distribution of collagen, whereas for the 50:50 ratio of macromers, there is even distribution of collagen matrix throughout the construct.

![Synthesis of photopolymerizable and hydrolytically degradable PEG macromers.](image)

**Figure 3.6.** Left) Synthesis of photopolymerizable and hydrolytically degradable PEG macromers. Right) Collagen staining for osteoblasts photoencapsulated in degradable PEG hydrogels, where the degradable unit is a slowly degrading caprolactone (A) or a 50:50 mixture of macromers containing either caprolactone or lactic acid (B). These alterations in degradation can be used to control the distribution of extracellular matrix by entrapped cells.
Degradable PEG hydrogels have also been extensively investigated as matrices for drug delivery applications. West and Hubbell photoencapsulated model drugs of various molecular weights in the hydrogels and showed that release profiles were altered by changing the drug molecular weight, the number of degradable units in the macromer, and the PEG molecular weight (71, 72). Burdick et al. (73) used PEG hydrogels for the delivery of osteoinductive growth factors to stimulate osteoblast function and mineralized tissue formation. This work illustrated the activity of growth factors released from the hydrogels through the stimulation of ectopic bone formation subcutaneously in rats. Finally, Quick and Anseth photoencapsulated DNA in PEG hydrogels and used several techniques to preserve the integrity of the DNA during the encapsulation process (73-75). Various models (76, 77) have been developed that theoretically predict release behavior from the photopolymerizable and degradable PEG hydrogels.

Other PEG-based synthetic macromers have also been developed. Wang et al. synthesized PEG hydrogels that contain phosphate groups (78). The PhosPEG-dMA macromers were water-soluble and formed hydrogels upon exposure to ultraviolet light in the presence of a photoinitiator. The hydrogels lost mass continuously over a 9-week period and supported the encapsulation of viable human mesenchymal stem cells. Finally, Li and colleagues (79) synthesized a new biodegradable and photocrosslinkable macromer with a polyphosphoester (PPE) backbone containing PEG spacers followed by acrylate groups. Properties such as the swelling ratio and mass loss were found to decrease as the amount of macromer increased. No cytotoxicity was observed when bone marrow stem cells isolated from goats (GMSCs) were cultured in media containing macromer up to concentrations of 10 mg/ml. Furthermore, it was shown that GMSCs retained their viability when encapsulated in these photopolymerized gels (79).

As an alternative to hydrolytically degradable hydrogels, West and Hubbell (80) developed networks that were proteolytically degradable by enzymes present near a cell surface during cell migration. The hydrogels were either collagenase (APGL) or plasmin (VRN) sensitive, depending on the peptide incorporated. The synthesized hydrogels degraded specifically in the presence of the various proteases. Mann et al. (81) used an alternate procedure to synthesize
proteolytically degradable hydrogels. Adhesive peptides were grafted to the hydrogels and the 
hydrogels supported the viability, proliferation, and production of extracellular matrix components 
by encapsulated smooth muscle cells. This work was continued by Gobin and West (82) to 
control the migration of fibroblasts through collagenase and plasmin degradable hydrogels. Their 
work illustrated the importance of the hydrogel design (i.e., tethered ligand density) on cell 
migration and showed that both proteolytically degradable sites and adhesive tethers were 
necessary for cell migration.

Finally, PEG-based macromers have been used to fabricate complex 3-D structures, 
taking advantage of the spatial resolution that is afforded during photoinitiated polymerizations. 
Although non-degradable PEG was used, Tsang et al. (83) and Arcaute et al. (84) developed 
systems of photopatterning and stereolithography to fabricate complex structures that incorporate 
living cells. These techniques are useful in the development of multicellular scaffolds with 
complex architecture for the engineering of a variety of tissues. 

3.3.2 Crosslinkable Poly(propylene fumarates)

Poly(propylene fumarate)s (PPFs) are linear polyesters consisting of repeat units with 
multiple ester groups and unsaturated carbon-carbon double bonds. The general structures of 
several PPFs are shown in Figure 3.7. Networks are formed by covalent crosslinking through the 
carbon-carbon double bonds using free radical polymerization and hydrolyze into primarily 
fumaric acid and propylene glycol, which are cleared from the body in the metabolic pathway (85-
94). Fisher et al. were the first to investigate the photoinitiated crosslinking mechanism of PPF 
networks with variations in initiator concentrations (88). This work demonstrates the importance 
of initiator type and concentration on network properties such as sol fraction and tensile modulus 
(88). Timmer et al. also investigated the importance of initiator type by comparing the mechanical 
properties and average molecular weight between crosslinks ($M_c$) of PPF-based networks formed 
via thermal- and photoinitiation (95). In general, networks formed via photoinitiation had greater 
compressive moduli and strength, reaction conversions, and crosslinking densities than networks 
formed by thermal initiation (95).
PPF-based networks are attractive for orthopaedic applications due to their high compressive strengths (95, 96). Various techniques such as polymerization in silicone molds (96), stereolithography (97), and porogen leaching (86, 87, 89, 92, 94) have been used to develop PPF scaffolds for implantation. Scaffolds implanted into rabbit cranial defects demonstrated bone ingrowth (89, 92), especially when the scaffolds were coated with transforming growth factor-$\beta_1$ (TGF-$\beta_1$) (92). Various copolymers containing PPF-based macromers have also been developed. For example, oligo(poly(ethylene glycol) fumarate) (OPF) macromers, containing alternating blocks of fumaric acid and poly(ethylene glycol) (PEG) (Figure 3.7) were developed by Mikos and others (98-100). Photocrosslinked hydrogels based on these macromers exhibit a range of bulk properties, such as mesh size and percent elongation at fracture, depending on the molecular weight of the PEG block (100). Further functionalization of these networks was demonstrated by OPFs modified by the addition of GRGD groups and crosslinked with PPF to form biodegradable scaffolds (98). In addition, networks with compressive and diametrical tensile strengths ranging from 1.8-146.0 MPa and 2.5-9.3 MPa, respectively, have been prepared by the crosslinking of methacrylated propylene fumarate and acrylated poly($\varepsilon$-caprolactone) (PCL) oligomers (101). Moreover, multiblock copolymers consisting of PPF and PCL, as well as PPF, PCL, and PEG, have developed for tissue engineering applications by Yaszemski and coworkers (93, 102).

![Figure 3.7](image_url)  
**Figure 3.7.** Examples of photocrosslinkable poly(propylene fumarates) for generating highly crosslinked networks (A) and hydrogels (B).
3.3.3 Crosslinkable Poly(α-hydroxy esters)

Poly(α-hydroxy esters), such as poly(d,l-lactic acid) (PLA), poly(glycolic acid) (PGA) and poly(ε-caprolactone) (PCL) are among the most thoroughly investigated synthetic biomaterials (62, 103-109). However, poly(α-hydroxy esters) are highly crystalline polyesters that lack modifiable side groups, which could be used to facilitate better material-cell interactions (104-109). Several investigators have combined degradable PLA and photopolymerization technology to manipulate the backbone with amino acids to allow for further functionalization (108, 109). PLA films have also been surface grafted with poly(acrylic acid) and poly(acrylamide) using photopolymerization in an attempt to generate a bioactive surface with rapid degradation (107).

Various copolymers containing blocks of poly(α-hydroxy esters) and other synthetic polymers have been prepared. For example, PCL-b-PEG-b-PCL networks have been prepared and exhibit an increase in the compressive modulus and degradation rate when compared to PCL alone (110). Networks based on adipic acid, 4-hydroxycinnamic acid, and PCL diols with elastomeric properties have also been prepared (111, 112). PCL has also been combined with trimethylene carbonate and the reaction behavior and bulk properties have been characterized (113-121). Furthermore, star-PCL-b-PLA macromers have been synthesized (122, 123). These networks show dependence in physical properties and degradation depending on the length of the copolymer block (122, 123). Han and colleagues prepared lactide-based PEG networks emanating from glycerol centers (104-106). The degradation rates of these networks were controlled by the ratio of the lactide and PEG incorporated (104, 106).

Diethylene glycol has also been used as an initiator for the ring opening polymerization of d,l-lactide and ε-caprolactone, which were subsequently methacrylated to allow for crosslinking (12, 124-126). Alterations in the oligomer chemistry led to changes in mechanical properties and degradation behavior. In particular, a decrease in bulk eroding mass loss, as well as an increase in protein adsorption, osteoblast viability, and other indications of bone formation corresponded to an increase in network hydrophobicity (12, 124-126). Porous scaffolds of one of the more hydrophobic oligomers were prepared using salt leaching and implanted into critical-sized cranial
defects in a murine model and demonstrated the potential of this material as an *in situ* synthetic graft for large bone defects (127). Similar triblock polymers were synthesized and prepared as scaffolds using micro-patterning (128).

### 3.3.4 Crosslinkable Poly(vinyl alcohol)

Poly(vinyl alcohol) (PVA) has been used in a variety of biomedical applications, such as contact lenses, tendon repair, drug delivery and as scaffolds for a wide variety of tissue engineering applications including bone, cartilage, and heart valves (129, 130). Hydrogels prepared from this synthetic polymer are of particular interest due to its biocompatibility, high water content, tissue-like elasticity, and ease of fabrication and sterilization (17, 130-133). Perhaps the most attractive feature of using PVA is the abundance of pendant hydroxy groups within the repeating unit, which allow for various chemical modifications such as the introduction of methacrylate groups or biological molecules like fibronectin (17, 129-137).

Previously, PVA hydrogels have been crosslinked using chemical means such as aldehydes (136) and physical mechanisms such as repeated freeze-thaw cycles to induce crystallinity (133, 136). Limitations to these methods include toxicity of the aldehydes and stability of physical crosslinks. Muhlebach and colleagues were the first to use photopolymerization to crosslink PVA by modification of the aforementioned pendant hydroxy groups with acrylic acid and methacrylic acid for contact lens applications (133, 138). Several other groups have been successful in introducing various functional groups for polymerization by reaction with various reagents such as glycidyl acrylate (135) methacrylamidoacetaldehyde dimethyl acetal (129), and 2-isocyanatoethyl methacrylate (17). A wide range of mechanical properties and degradation times are obtainable by varying the concentration of reactive groups and number and type of degradable groups (129, 131, 136). These hydrogels degrade via hydrolysis of ester bonds until reverse gelation occurs when there is no longer an infinite network present, but only branched, soluble chains (132). Nuttelman and coworkers engineered a hydrogel scaffold by preparing PLA capped with hydroxyethyl methacrylate (HEMA), which was subsequently grafted onto PVA (133). This copolymer demonstrated control over degradation and increased cell adhesion based on the number of lactide repeat units per side chain (133).
Copolymers of PVA and PEG as well as PVA and chondroitin sulfate have been created and used to encapsulate chondrocytes for cartilage tissue engineering applications (17, 137).

3.3.5 Crosslinkable Poly(β-amino ester)s

Poly(β-amino esters) are a class of materials that were originally developed as nonviral gene delivery vehicles because of a reduced toxicity when compared to other cationic polymers used for the same application (139, 140). Anderson and colleagues synthesized and characterized a large library of poly(β-amino esters) with acrylate end groups using various amines and diacrylates and a simple synthetic process with no byproducts (141). The general structure of these macromers is shown in Figure 3.8. The various reagents were chosen to introduce chemical diversity and variations in hydrophobicity within the macromer library and an excess of diacrylates (i.e., diacrylate:amine > 1) was used to ensure reactive end groups. The macromers can be photopolymerized into networks with hydrolysis resulting in small molecule bis(β-amino acid)s, diols, and poly(acrylic acid) kinetic chains as degradation products (141). The macromers formed polymers that exhibited a wide range of degradation behaviors and times (an example of one time point is shown in Figure 3.8), and it was observed that mass loss was slower when more hydrophobic amines were incorporated into the macromers. In addition, a wide range of mechanical properties (E = 4-350 MPa) was obtained through variations in macromers. It is important to note that the stiffness of these materials did not necessarily correlate to degradation time. Therefore, materials exhibiting the optimal mechanical properties and degradation rates can be prepared for a variety of tissue engineering applications (141). Brey and coworkers further illustrated the ability of using macromer molecular weight and branching to control the network properties and cellular interactions (142, 143).

3.3.6 Other Synthetic and Crosslinkable Polymers

Other systems of synthetic polymers have been prepared using various modifications and processing techniques. For example, Grinstaff and coworkers synthesized photocrosslinkable dendrimers based on PEG, glycerol, and succinic acid (144-147). These systems have been applied as scaffolds for cartilage tissue engineering and corneal tissue repair (144, 145, 147).
Thiol-acrylate photopolymers, which are capable of photopolymerization with or without the addition of photoinitiators, have been polymerized by a mixed-mode chain and step growth mechanism between the diacrylate (e.g. PEG-PLA) monomer and a multifunctional thiol (e.g. pentaerythritol tetrakis(3-mercaptopropionate)). A wide range of bulk properties and degradation rates are offered through the versatility of the chemistry involved in this system (148-150). Furthermore, one significant benefit of these networks is that curing depths may exceed 10 cm with initiator free systems (148).

Figure 3.8. The general chemical structure of poly(β-amino ester)s and an example of mass loss at one time point (99 days) where R₁ depends on the diacrylate (A-J) used and R₂ depends on the amine (1-12) used. These results illustrate the diversity available in the macromer library.
3.4 Natural Polymers

3.3.1 Free radical initiated crosslinkable collagen and gelatin

Gelatin, or denatured collagen, has several desirable properties for use as a biomaterial including its biological interaction capabilities and numerous groups for modification (151). Previously, chemical crosslinking was initiated using various bifunctional reagents such as glutaraldehyde, but these gels resulted in cases of local cytotoxicity and calicification (151, 152). Van Den Bulcke and coworkers derivatized gelatin by reacting it with methacrylic anhydride to introduce photocrosslinkable moieties (151). They noted that the overall strength of the gel was controlled by the degree of substitution of the gelatin and the storage conditions (151). Chaikof and coworkers also used methacrylic anhydride to incorporate an acrylate moiety onto the lysine and hydroxylysine residues of the collagen backbone (153) (Figure 3.9). Photocrosslinking of the macromer using visible light irradiation and in the presence of rat aortic smooth muscle cells (RASMC) was used to prepare gels (153). A significant increase in the mechanical integrity of the construct was observed, however it was still sufficiently less than the tensile strength required for in vivo applications, such as vascular grafts. Notably, they showed that the triple helical conformation, as well as cell viability were maintained during the crosslinking process (153).

In addition to reaction with methacrylic anhydride, collagen has been derivatized using an EDC/NHS conjugation method to add photosensitive cinnamate moieties (152). This method produced photocrosslinked gels with mechanical properties comparable to those crosslinked by glutaraldehyde (152). Moreover, collagen and gelatin have been reacted with other reagents to facilitate light induced chemical crosslinking. For example, gelatin has been modified with benzophenone and xanthene dyes and has been reacted in the presence of PEG diacrylate for the production of tissue adhesives (154). Gelatin has also been derivatized with styrene groups by reaction with 4-vinylbenzoic acid. This modified gelatin has also been reacted with PEG diacrylate and investigated as a tissue adhesive glue for arterial repair (155). The release rate of albumin from these gels is indirectly related to the gelatin concentration and degree of derivatization and the adhesivity of the gel was greater than that of fibrin glue (156). Styrene-
derivatized gelatin has also been investigated for applications as delivery vehicles (157-159), nerve guides (160, 161), as well as carriers for chondrocyte transplantation (162).

3.3.2 Crosslinkable polysaccharides

3.3.2.1 Hyaluronic Acid

Hyaluronic acid (HA) is a naturally derived nonimmunogenic, nonadhesive glycosaminoglycan composed of D-glucuronic acid and N-acetyl-D-glucosamine and is found in many connective tissues (163-171). HA undergoes enzymatic degradation (163, 165-169, 171, 172) and plays roles in the promotion of cell motility and proliferation (163, 165, 169, 172), wound healing (163, 166, 167, 172), angiogenesis (166-168), and the reduction of long-term inflammation (163, 166-168). It is attractive for biomaterial applications because it can be modified with various functional groups such that covalent crosslinking reactions are possible (32, 33, 163-176). Smeds et al. modified HA for photopolymerization with the addition of a methacrylate group by reaction with methacrylic anhydride (Figure 3.9) (171). They investigated the influence of the degree of methacrylation on bulk mechanical properties such as swelling, compression, and creep compliance. They demonstrated that the hydrogels prepared from methacrylated HA (MeHA) are capable of swelling up to 14 times their dry weight and are stronger and more resilient than corresponding alginate gels (170).

Due to its role in cardiac morphogenesis, MeHA has been studied as a scaffold for heart valve tissue engineering (172, 174). Masters and co-workers investigated the spreading and proliferation of valvular interstitial cells (VICs) on MeHA based gels (172). VICs encapsulated within the gels remained viable and the scaffold degradation products increased VIC proliferation (172). Burdick et al. systematically studied the role of HA molecular weight, degree of methacrylation, and macromer concentration on the bulk properties of the resulting MeHA gels (177). Volumetric swelling ratios varied from approximately 42 to 8 and the compressive modulus ranged from approximately 2 to over 100 kPa when the macromer concentration was varied from 2 to 20%. It was noted that the viability of fibroblasts seeded within these gels decreased as the macromer concentration increased. Neocartilage was observed in gels seeded with swine
auricular chondrocytes (163). Further study of this system for cartilage engineering applications demonstrated optimal neocartilage production in hydrogels fabricated with 2 wt% of 50 kDa MeHA macromer and early passage chondrocytes (32, 33). MeHA has also been investigated for corneal regeneration (175) and for micromolding of cellular microarrays (165, 173).

HA was also modified with N-3-aminopropyl methacrylamide (169) and glycidyl methacrylate (GMHA) (164, 166-168). GMHA was modified with acrylated forms of PEG and EDC/NHS was used to conjugate the model peptide hexaglycine to GMHA (166). Stable hydrogels with high peptide conjugation efficiencies (up to 80%) and defined physiochemical properties were formed by controlling the reactant ratios (166). GMHA was also partially oxidized to reduce chain rigidity and examined for vocal fold regeneration (164). Moreover, protein release from GMHA scaffolds has been investigated in an effort to create a combinatorial scaffold and delivery device (168).

3.3.2.2 Dextran

Dextran is a biodegradable bacterial polysaccharide consisting of $\alpha$-1,6 D-glucopyranosyl residues with approximately 5-10% $\alpha$-1,3 linked side chains (178-180). It has previously been studied for delivery of pharmaceutical drugs, peptides, and proteins (178, 180, 181). Repeat units in dextran have three free hydroxy groups that allow for further functionalization with various reactive moieties and allow for covalent crosslinking (179-181). For example, dextran has been modified by reaction with methacrylic anhydride to incorporate an acrylate functional group onto one of the hydroxy groups (Figure 3.9) (178, 182). A wide range of swelling properties can be obtained by altering the degree of methacrylate substitution. Hydrogels based on this system were loaded with different drugs, such as doxorubicin and their release profiles were investigated. It was observed that a higher degree of substitution led to a delay in drug release time and the quantity of drug release also decreased as the molecular weight of the drug increased (182). Hydrolytic degradation of dextran-based hydrogels was induced by the addition of PLA subunits onto the backbone (180, 181, 183). This system was further modified by reacting dextran with allyl isocyanate and PLA such that a wider range of physical properties could be obtained due to
the increased degree of substitution (180, 183). Drug release from these gels was found to be dependent on the rate and formulation of the 3D porous scaffold in the gel, the hydrolytic degradation of PLA, and the hydrophobic interaction of PLA and the drug (183).

3.3.2.3 Chitosan

Chitosan is a partially deacetylated form of chitin, a natural linear homopolymer of β-1,4-linked N-acetyl-D-glucosamine (184-186). Useful advantages of chitosan include its hemostatic activity (184), immunological activity, and ability to accelerate wound healing (184, 186). It has been modified by reaction with N,N,N’,N’-tetramethylethylenediamine, EDC, and 4-O-β-D-galactopyranosyl-(1,4)-D-gluconic acid to introduce azide and lactose moieties for photocrosslinking (Figure 3.9). This modified chitosan (AZ-CH-LA) has primarily been investigated as a tissue adhesive (184, 186, 187) and it has been shown that AZ-CH-LA demonstrates binding strength stronger than fibrin glue and is effective at sealing punctures in thoracic aorta and lungs in rabbit models (187). Azide modified chitosans have also been investigated as matrices for drug delivery (185, 188) and as a cell-seeded material for micromolding (189) and photolithography (190). Moreover, chitosan has also been modified by reaction with 4-vinylbenzoic acid, which has further been used to create tubular structures upon irradiation with visible light (191).
Figure 3.9. Examples of various photopolymerizable natural polymers including (A) collagen, (B) hyaluronic acid, (C) chitosan, (D) dextran, and (E) chondroitin sulfate.

3.3.2.4 Chondroitin Sulfate

Chondroitin sulfate is an enzymatically degradable proteoglycan composed of repeating units of glucuronic acid and N-acetylgalactosamine with a sulfate and carboxyl group located on each disaccharide unit (192-194). It is one of the major components of native cartilage tissue aggrecans and is capable of absorbing large quantities of water, and is thus, regarded as being responsible for the great compressive strength of the tissue (194). Chondroitin sulfate has been modified for photocrosslinking (Figure 3.9) by reaction with glycidyl methacrylate to form GMA-CS (192, 194) and an inversely proportional relationship between degree of methacrylate
substitution, as well as crosslinking density and water content, was found (194). GMA-CS was combined with PEO diacrylate to form hydrogels, which had increased water content when compared to hydrogels composed of PEO diacrylate alone. The viability of chondrocytes loaded into these gels was also maintained (192). Similar results were obtained with chondroitin sulfate modified with methacrylic anhydride and copolymerized with PEG(193) and PVA.(17)

3.5 Conclusions

In summary, a wide range of different precursor molecules are being developed that form networks via radical polymerizations for tissue engineering and molecule delivery applications. The diversity in polymer properties, ranging from highly crosslinked and hydrophobic networks to loosely crosslinked, swollen hydrophilic hydrogels, expands the applicability of these biomaterials. As more knowledge and understanding of biological events such as tissue healing and cell-material interactions becomes available, the materials used in these applications will become smarter and more complex. Particularly, there is increased interest in designing biological function into polymers, such as enzymatically degrading crosslinks and adhesion sites, to better control the interactions between cells and materials. Also, as advances are made in the area of polymer synthesis, it is becoming easier to tailor polymer properties and structure as desired. Novel materials may be able to open up avenues for unique material behavior such as externally triggered properties (e.g., temperature induced), shape-memory polymers, and the patterning of structures 3-dimensionally. The next decade is bound to produce previously unimaginable biomaterials that can help to overcome our shortage of tissue available for transplantation to patients.
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methacrylate and chondroitin sulfate: Mechanism of ring-opening-transesterification 
Chapter 4

Synthesis and Characterization of Acrylated Poly(Glycerol Sebacate)


4.1 Introduction

The well-known tissue engineering paradigm accounts for the importance of scaffolds, cells, and growth factors and combinations of these components for the successful design and integration of constructs into living systems to enhance tissue regeneration (1). It is generally believed that cells either delivered or from surrounding tissues receive necessary cues from their microenvironment, which consists of both matrix (e.g., mechanics, chemistry) and soluble factors (2, 3). With this in mind, the chemical and physical properties of scaffolds are of vital importance in controlling cellular behaviors (e.g., differentiation and matrix production) and in the overall success of the construct (2, 4-9).

Scaffolds may be comprised of natural enzymatically degradable biopolymers (e.g., hyaluronic acid) or synthetic polymers (e.g., polyurethanes), which are typically biodegradable, depending on the desired application and in vivo environment (10, 11). One advantage to using synthetic polymers is the ability to tailor scaffold mechanical properties and degradation kinetics through chemistry and processing (2, 7, 12). Synthetic materials frequently have moduli on the order of MPa and GPa (7, 8, 11, 13), whereas the elastic modulus of many tissues is on the order of Pa and kPa (8, 13). While hydrogels may have moduli on the order of many native tissues (14), they are by definition hydrophilic materials, which generally implies minimal protein adsorption, and consequently minimal cell attachment compared to their hydrophobic polymeric counterparts (15). Furthermore, many tissues exhibit elasticity such that they can function and
recover in the mechanically dynamic environment that exists in the body (6, 9, 13, 16). Therefore, investigators have been motivated to synthesize and develop novel materials that better mimic the stiffness and elasticity of native tissues (2, 9, 10, 13, 16-18).

Wang and colleagues (16) synthesized a tough biodegradable elastomer, poly(glycerol sebacate) (PGS), that has potential for the engineering of soft tissues due to its mechanical properties and biocompatibility (13, 16, 19). However, the curing of PGS requires high temperature and vacuum conditions (16, 19), which makes processing into complex scaffolds difficult and *in vivo* crosslinking impossible. Recently, Nijst et al. (18) reported the modification of the free hydroxy groups on the PGS condensation product with acrylate functional groups to form an acrylated PGS (Acr-PGS) macromer. The introduction of the acrylate functionality introduces control over the crosslinking and thus expands upon the current processing options of this elastomer. We report here the modification of PGS precursors with the acrylate functionality, but investigate the role of both molecular weight and degree of acrylate substitution on the formed network properties. Since crosslinking of the vinylic bonds on the Acr-PGS can occur via both redox and photo-initiated free radical polymerizations (20), we also explore these different mechanisms for enhanced processing of the networks. Additionally, these networks could be crosslinked using Michael-Type addition reactions with the addition of a multifunctional nucleophile (e.g., di-thiol), but that approach is not shown here.

The overall objective of this work is to develop radically polymerizable macromers that form biodegradable elastomeric networks upon crosslinking. To accomplish this, a range of Acr-PGS macromers were synthesized and their network properties (e.g., degradation and mechanics) were characterized upon crosslinking. The reaction behaviors for both redox and photo-initiated polymerizations and the tissue response to these biodegradable elastomers, including injectable formulations, were also investigated.
4.2 Materials and Methodology

4.2.1 Acr-PGS Macromer Synthesis and Characterization

All reagents were used as received from Sigma-Aldrich (St. Louis, MO) unless otherwise noted. The condensation product was formed via the condensation reaction of equimolar amounts of glycerol (ThermoFisher Scientific, Waltham, MA) and sebacic acid. The reagents were combined and stirred at 120°C under a nitrogen atmosphere for approximately 2 hours and then a vacuum of 19 mbar was applied for various amounts of time (26 to 63 hours) to obtain condensation products of varying molecular weights. For acrylation, the condensation product was dissolved in methylene chloride (ThermoFisher Scientific) containing triethylamine (TEA, equimolar to acryloyl chloride) and 500 ppm 4-methoxyphenol (inhibitor 10 wt% in methylene chloride). Various molar ratios (Table 4.1) of acryloyl chloride (1:10 v/v in methylene chloride) were dripped into the solution. These values were calculated using the estimation that two of the three hydroxy groups present in glycerol reacted with the sebacic acid and provide a range of overall acrylations. An additional 500 ppm 4-methoxyphenol was added to the reaction chamber and a rotary evaporator (40°C, 450 mbar) was used to remove the methylene chloride. Ethyl acetate was added to the reaction flask and the solution was vacuum filtered to remove the TEA salts and washed three times with 10 mM hydrochloric acid (ThermoFisher Scientific). Ethyl acetate was removed via rotovapping (40°C, 99 mbar) to leave a viscous liquid, which was redissolved in methylene chloride and stored at 4°C. The condensation product and macromer molecular weights and chemical structures were verified using gel permeation chromatography (GPC, Waters GPC System, Milford, MA) and \(^1\)H NMR spectroscopy (Bruker Advance 360 MHz, Bruker, Billerica, MA).

4.2.2 Reaction Characterization

Macromers were mixed with 0.5 wt% of the photoinitiator 2,2-dimethoxy-2-phenylacetophenone (DMPA, 10 wt% in methylene chloride) and excess methylene chloride was removed by rotovapping. The reaction behavior was monitored using Real Time Attenuated Total Internal Reflection Fourier Transform Infrared Spectroscopy with a zinc selenium crystal (ATR-
FTIR, Nicolet 6700, ThermoElectron, Waltham, MA). A sample of the macromer with photoinitiator was placed directly on the surface of the crystal, covered with a glass coverslip and monitored in real time with exposure to ultraviolet light (1.5 mW cm\(^{-2}\), 365 nm Omnicure S1000, EXFO, Quebec). In the case of redox initiation, samples were prepared by the addition of 1.0 wt\% benzoyl peroxide (BPO, 10 wt\% in methylene chloride) or N,N-dimethyl-p-toluidine (DMPT) to the macromer/methylene chloride solution. Methylene chloride was removed by rotovapping and the samples were dissolved in 200 proof ethanol and transferred to a dual barrel syringe (PlasPak Industries, Norwich, CT), which was subsequently placed in a 60°C oven overnight to remove any excess ethanol. Samples were ejected from the dual barrel syringe directly onto the surface of the crystal. Reaction conversion was determined by monitoring the change in the vinylic double bond peak (~1635 cm\(^{-1}\)); however, direct quantification was not possible due to overlapping adjacent peaks and an unstable baseline. The gelation times and reaction exotherms were quantified using a slowly stirring stirbar (60 rpm) in a vial post-mixing and a thermocouple temperature probe.

4.2.3 Degradation and Material Property Characterization

For sample fabrication, the macromer/initiator solutions were poured into a 50 × 15 × 1 mm Teflon mold and placed in an oven at 60°C overnight. The construct was then covered with a glass slide and polymerized with exposure to ~10 mW cm\(^{-2}\) 365 nm ultraviolet light (Blak-Ray, Ultraviolet Products, Upland CA) for 10 minutes. Polymer disks (1 mm thick, 5 mm diameter) were punched from the resulting polymer slabs. To monitor in vitro degradation, samples were weighed, submerged in 150 mM NaCl PBS, and placed on an orbital shaker at 37°C. At each time point (2, 4, and 8 weeks) samples (n=3) were removed, lyophilized (Freezone 4.5, Labconco, Kansas City, MO) for 24 hours, and weighed to determine mass loss. For mechanical testing, strips (15 × 5 × 1 mm) were cut from the slabs and tensile testing was conducted on an Instron 5848 mechanical tester (Norwood, MA) with a 50 N load cell at a strain rate of 0.1%/sec.
4.2.4 Indirect Cytotoxicity Evaluation

The cytotoxicity of networks was also assessed. Strips of polymer prepared from Macromer 6 were prepared as previously discussed for mechanical testing, followed by further trimming to form 5 × 5 mm squares. These squares were placed in 200 proof Ethanol overnight and sterilized by placement under the germicidal ultraviolet lamp for 30 minutes followed by rinsing 3x in PBS. The squares were then put on top of the membrane of a transwell insert. This allows for the cells to be exposed to any molecules released from the elastomer without directly contacting it. The transwell inserts were placed in the wells of a 24 well plate (1 insert/well), which contained hMSCs that were plated at a density of 10,000 cells/cm² 24 hours prior to allow for cell attachment before treatment. Viability was assessed at day 1 and day 3 using the MTT assay (n=3, ATCC, Manassas, VA).

4.2.5 In Vivo Tissue Response

Animals were cared for according to a protocol approved by the University of Pennsylvania Institute for Animal and Use Committee. Photopolymerized polymer slabs were prepared as described above. Polymer disks (1 mm thick, 5 mm diameter) were punched and submerged in ethanol. Ethanol was evaporated off and the disks were placed under a germicidal ultraviolet lamp for 30 minutes. Redox initiated macromer solutions were loaded into a sterile dual barrel syringe followed by exposure to the germicidal lamp for 30 minutes. Precrosslinked and preweighed disks (n=4 per timepoint, 4 disks per animal) were implanted subcutaneously into the dorsal pocket of male Sprague-Dawley rats. Redox initiator loaded macromer solutions were also injected (n = 4) into the dorsal pocket of a male Sprague-Dawley rat. The animals were sacrificed at various time points (2, 4, and 8 weeks) and the polymer samples and surrounding tissue was collected and fixed with 10% formalin for 24 hours. Standard hematoxylin and eosin (H&E) staining of paraffin embedded sections was used to investigate the tissue response. Additional samples were removed to monitor in vivo degradation behavior. All tissue was excised from the sample prior to lyophilization to obtain the sample dry weight.
4.3 Results and Discussion

When designing a scaffold for tissue engineering, there are several design criteria to keep in mind. Many believe that it is important to closely match the biomaterial mechanical properties with those of the surrounding native tissue to assist in the gradual transfer of stresses from the implant to the newly formed tissue (6, 16). Specifically, the elasticity of tissues is often overlooked in material design, yet biodegradable elastomers may fill that need. Elastomers are generally defined as lightly crosslinked polymers that easily and quickly undergo large, reversible deformations with complete recovery (20). These important features of elastomers (e.g., PGS) make them attractive materials to alleviate the compliance mismatch problem that often exists with synthetic polymeric implants, particularly in the dynamic environment of the human body (16).

4.3.1 Macromer Synthesis and Characterization

Our approach is to fabricate tissue engineering scaffolds using a modified PGS condensation product (i.e., Acr-PGS) that can be crosslinked under mild and physiologic conditions. The first step in the synthesis of the Acr-PGS macromer is the combination of trifunctional glycerol and difunctional sebacic acid via a polycondensation reaction in a 1:1 molar ratio for varying amounts of time (26 to 63 hours). The starting reagents were chosen because they are naturally present in the body and have been previously approved by the U.S. Food and Drug Administration for medical applications (16). The $M_w$ of the condensation product as defined by GPC increased with reaction time and ranged from $\sim$4.06 to 23.46 kDa, illustrating the tunability of molecular weight. The GPC curves shown in Figure 4.1 are representative of Macromer I ($\sim$4.06 kDa) and Macromer 6 ($\sim$23.46 kDa). The peaks move further to the left on the scale due the differences in elution times of the two different condensation products. As with most condensation reactions, condensation products were polydisperse (2.01 to 4.60) and generally increased with reaction time. The broadening of the peak of Macromer 6 compared to Macromer I in Figure 4.1 also depicts this increase in polydispersity.
Multifunctional Acr-PGS macromers were prepared by reaction of the condensation product with varying amounts of acryloyl chloride. These amounts were defined assuming that two of the three hydroxy groups on the glycerol reacted with sebacic acid during the condensation reaction and were chosen to provide a range of acrylations. The Acr-PGS % acrylations were determined using $^1$H NMR.
Figure 4.1. Acr-PGS Condensation Product Characterization. Acr-PGS condensation product chemical structure (A) and corresponding representative $^1$H NMR spectra (B). Letters correspond to the protons identified in the structure, unlabeled peaks are due to solvent or unreacted glycerol. Representative GPC curves for macromers of varying molecular weight, as seen in Table 4.1.
As seen in the representative $^1$H NMR spectra of an Acr-PGS condensation product shown in Figure 4.2, peaks at ~1.3, 1.6, and 2.3 ppm correspond to the protons in the olefin chain from the sebacic acid and multiplets at ~4.2 and 5.2 ppm correspond to the protons in the glycerol. Figure 5.2 depicts a representative $^1$H NMR spectra of an Acr-PGS macromer. The peaks at ~5.9, 6.1, and 6.3 ppm correspond to the protons in the acrylate functional group. The % acrylation was determined by comparing the actual number of acrylate groups with the theoretical values for 100% incorporation of the acrylate group into the condensation product. The % acrylation values range from ~9.6 to ~88.0% (Table 4.1). These six Acr-PGS macromers represent a range of molecular weights and acrylations, and thus, provide insight into the relationships between macromer structure and network properties.

**Table 4.1.** Summary of Acr-PGS macromers synthesized and investigated.

<table>
<thead>
<tr>
<th>Macromer</th>
<th>$M_n$ (kDa)</th>
<th>$M_w$ (kDa)</th>
<th>% Acrylation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2.01</td>
<td>4.06</td>
<td>88.0</td>
</tr>
<tr>
<td>2</td>
<td>2.01</td>
<td>4.06</td>
<td>21.7</td>
</tr>
<tr>
<td>3</td>
<td>2.01</td>
<td>4.06</td>
<td>9.0</td>
</tr>
<tr>
<td>4</td>
<td>3.40</td>
<td>7.02</td>
<td>21.7</td>
</tr>
<tr>
<td>5</td>
<td>4.91</td>
<td>22.6</td>
<td>17.9</td>
</tr>
<tr>
<td>6</td>
<td>5.33</td>
<td>23.5</td>
<td>9.6</td>
</tr>
</tbody>
</table>

The introduction of the acrylate functional groups was also confirmed using ATR-FTIR by visualization of the characteristic absorption of the acrylate group at ~1635 cm$^{-1}$ (Figure 4.3). In general, the intensity of this adsorption increased as the % acrylation increased. For example, Figure 4.3A displays the characteristic acrylate absorption for Macromer 1 and is representative of high % acrylation (88.0%), whereas Figure 4.3B displays the characteristic acrylate absorption
for Macromer 6 and is representative of a low % acrylation (9.6%). A large difference in the acrylate absorption intensity is observed when comparing the two spectra.

**Figure 4.2.** Acr-PGS macromer structure (A) and representative $^1$H NMR spectra (B). Peak letters correspond to those in the macromer structure above. Unlabeled peaks correspond to protons from initiator, inhibitor, or unreacted glycerol (peak d).

### 4.3.2 Network Formation

The photopolymerization reaction was investigated by introducing 0.5 wt% photoinitiator (DMPA) into the Acr-PGS macromer and monitoring the consumption of the acrylate group peak in real time with exposure to 365 nm ultraviolet light (Figure 4.3A). The maximum conversion occurred after ~ 8 minutes. The large difference in acrylate absorption between the initial and final time points and the near baseline level at the final time point indicate a high level of conversion of the acrylate group to crosslinks. The redox-initiated crosslinking was also monitored by introduction of 1.0 wt% of the bi-component BPO and DMPT initiation system, which is commonly used in bone cements (21-23) (Figure 4.3B). The maximum reaction conversion occurred after ~ 20 minutes. Again, the difference in intensity between the initial and final time points indicates the high conversion values.
Figure 4.3. Consumption of the acrylate peak with time during photopolymerization of Macromer 1 (0.5 wt% DMPA, light intensity ~1.5 mW cm\(^{-2}\)) (A). Consumption of the acrylate peak with time during redox initiated polymerization of Macromer 6 (1.0 wt% BPO and DMPT) (B).

The gelation time was defined as the point when a slowly stirring stirbar was stopped after injection of the macromer into a vial. For this system, gelation occurred at ~5 minutes and a maximum temperature of ~30°C (starting from room temperature) was observed. The maximum conversion and gelation time can be tailored by altering the amount of initiator incorporated into the macromer system, as is the case with similar bi-component initiator systems (24, 25), depending on the application. For example, delivery of this material to the heart through a catheter might require slower gelation than direct injection into a defect. The minimal increase in temperature is also important if this polymer is to be used as an injectable formulation to prevent temperature induced tissue necrosis.
4.3.3 *Network Mechanical Properties*

Polymer slabs for mechanical and degradation analysis were prepared using photopolymerization. Typical stress versus % elongation relationships for networks formed from Acr-PGS macromers are shown in Figure 4.4A. It is important to note that many of the samples broke at the clamp, and thus could lead to lower than actual values for the % strain at break. The Young’s modulus was determined from the slope of the linear portion of the plot (<20% strain) and varied (~0.15 to 30 MPa) depending on the Acr-PGS macromer (Figure 4.4B). The % strain at break also varied (~5 to 200%) depending on the Acr-PGS macromer used for network formation. In general, the Young’s modulus increased as the degree of acrylation increased for a given molecular weight. As expected, the % strain at break increased as the Young’s modulus and % acrylation decreased for a given molecular weight. Furthermore, the Young’s modulus and % strain at break increased with increasing molecular weight for similar degrees of acrylation. As seen in Figure 4.4B, Macromer 3 and 6 have similar Young’s moduli, but their elongation at break varied by almost an order of magnitude. Based on these results, it is important to note that not all of the macromers formed elastomeric networks. Therefore, Macromer 6 was selected for the remaining studies since its mechanical properties are the most elastomeric.

Relationships between macromer structure and network properties can easily be drawn from this data. For instance, increases in the % acrylation lead to increases in the number of crosslinks formed, which is associated with an increase in the modulus of a resulting material and a decrease in the ability to elongate a material before failure. Additionally, more elastomeric-like features are obtained as the molecular weight of the condensation product is increased. This is a clear demonstration that small modifications during synthesis can lead to drastic differences in the properties of the resulting material. Thus, this same backbone chemistry can be used to develop materials suitable for a wide range of applications. For example, a more elastic and softer material (e.g., Young’s modulus ~150 kPa) may be more ideal for cardiac tissue engineering, whereas a less elastic and stiffer material (e.g., Young’s modulus ~30 MPa) may be more ideal for bone tissue engineering. Although not investigated here, dynamic fatigue testing
would be necessary to illustrate the potential of these materials to withstand the dynamic in vivo environment (e.g., beating of the heart).

**Figure 4.4.** Representative tensile stress vs. elongation plots for networks formed from the Acr-PGS macromers with various MWs and acrylations (A). Young's modulus (black) and % strain at break (white) for networks formed from the various synthesized macromers (B). Further information on the macromers can be found in Table 4.1.

4.3.4 *Indirect Cytotoxicity Evaluation*

In order to assess the cytotoxicity of any leachable by-products of the elastomer, Macromer 6 was suspended above hMSCs in culture through a transwell insert. A general morphological assessment on the cells was completed on day 3 and appeared to look similar to the control group (Figure 4.5.A and B). The MTT assay is a fluorescent assay that is a measure
of mitochondrial metabolism and is therefore indirectly correlated to cellular viability. Moreover, there was no significance difference in absorbance between the groups at Day 0 (2 hours after exposure) or Day 3 (Figure 4.5.C). This indicates that the crosslinked elastomeric networks are not cytotoxic in these early \textit{in vitro} environments.

![Image](image.png)

**Figure 4.5.** Control (A) and elastomer exposed (B) hMSCs after 72 hours. MTT data at day 1 and day 3 (C).

4.3.5 \textit{Network Degradation and In Vivo Tissue Response}

The \textit{in vivo} and \textit{in vitro} mass loss of photopolymerized samples reached a maximum of \(~37\%\) and \(~33\%\), respectively, at 8 weeks (Figure 4.6). The \textit{in vivo} mass loss was potentially greater because of its location in a more dynamic environment where there is more fluid exchange to remove any degradation products from the implant region and due to the presence of enzymes compared to the \textit{in vitro} environment. Also, the sample preparation was slightly different due to the sterilization of the \textit{in vivo} samples. Based on this mass loss data, it is suspected that this material would be suitable for a variety of tissue engineering applications. Since redox-initiated samples could not be massed prior to \textit{in vivo} injection, the degradation
profile could not be monitored. However, it is anticipated that it would be comparable to its photoinitiated counterpart if similar conversions are reached.

**Figure 4.6.** *In vivo* (black) and *in vitro* (white) degradation results at 2, 4, and 8 weeks for networks formed from Macromer 6.

After two weeks of implantation, the host reaction to the polymer disks was comprised of granulation tissue, with new blood vessels, loose connective tissue formation and mild chronic inflammation. Macrophages and foreign body giant cells were present at the polymer-tissue interface (Figure 4.7A). At the four and eight week time points, a thin fibrous capsule is present around the implant with minimal associated chronic inflammation. There is no evidence of inflammation or necrosis within the adjacent subcutaneous fibroadipose tissue, skin adnexal structures or deep skeletal muscle (Figure 4.7B,D). This represents a typical host response to a biocompatible material. The reaction to the injected polymer contained slightly more perivascular chronic inflammation in the surrounding host tissue, but without evidence of necrosis or tissue damage (Figure 4.7C). This slight difference in response to the injected polymer may be due to a mild toxicity associated with the initiators or differences in the polymer configuration and surface area as compared to the photoinitiated samples.
Figure 4.7. H&E staining for in vivo tissue response to networks formed from Macromer 6 at 2 weeks (A), 4 weeks (B), 4 weeks via injectable redox initiation (C), and 8 weeks (D). T depicts tissue, P depicts polymer, scale bar = 100 μm.

4.4 Conclusions

In this study, radically polymerized networks with tunable mechanical properties were successfully synthesized and characterized. Notably, an increase in Young’s modulus with increasing acrylation, as well as an increase in the % strain at break with increasing molecular weight were observed, indicating that these properties can be tuned through the design of the macromer and the networks with elastomeric properties can be obtained. The reaction behavior was rapid and reached high conversions with both redox and photoinitiated polymerizations. Cells that were cultured in the presence of the elastomer had a similar morphology and viability (as assessed by the MTT assay) to control samples. The networks also degraded more rapidly in vivo and only mild inflammation was seen, even with injectable formulations. This biodegradable and elastomeric polymer system can be further explored for the engineering of numerous tissues
where elasticity is an important parameter.
References:


5.1 Introduction

The field of tissue engineering is progressing from a focus on structurally simple tissues or those that have significant natural regenerative capabilities to more complex tissues, coincident with the development of more advanced technologies (1, 2). With this progress, there has been a tremendous demand to produce more suitable materials and processing techniques to address the requirements (e.g., mechanical properties) of these more intricate organs and tissues (3). This demand has inspired many investigators to either modify existing synthetic and natural polymers (e.g., the introduction of controlled degradation) (4, 5) or to generate novel synthetic polymers with diverse properties for a wide range of tissue engineering applications (6-12).

As an example, poly(glycerol sebacate) (PGS) was developed to fulfill an unmet material need for the engineering of soft tissues, introducing a polymer with elastic properties that can also degrade and support cellular adhesion and proliferation (6). Recently, the synthetic scheme of PGS was modified to incorporate reactive acrylate groups (Acr-PGS), which introduces the spatial and temporal benefits of radical crosslinking (e.g., photocrosslinking) to overcome the limitation of the high temperature and vacuum required to cure PGS, thus extending its processing capabilities (13-15). The bulk properties of Acr-PGS networks are controlled by
simple adjustments during macromer synthesis (e.g., the macromer molecular weight and %
acrylation), thus rendering the system tunable with respect to mechanics and degradation (13).

Beyond bulk polymer properties, investigators have recently begun to recognize the
importance of the microenvironment’s structure on controlling cellular behavior. For example,
mesenchymal stem cells (MSCs), which are increasingly utilized for tissue engineering
applications, respond very differently to 2-dimensional versus 3-dimensional environments (e.g.,
fibrous structures) (16-18). Within the past decade, electrospinning has become a popular
technique to create fibrous scaffolds that are similar to the size scale and architecture of the
native extracellular matrix (19-22). These scaffolds may be particularly useful for fiber-aligned
tissues (e.g., meniscus, myocardium) and can be aligned for anisotropic properties and cellular
behavior (22, 23).

Several important processing variables (e.g., applied voltage, flow rate) need to be
considered when attempting to electrospin a new polymer (24). For example, the molecular
weight and polydispersity are related to the chain entanglement ratio, which dictates electrospun
fiber formation for a particular polymer (25). For low molecular weight reactive polymers that do
not meet this criteria, the introduction of a carrier polymer (e.g., poly(ethylene oxide), PEO) into
the electrospinning solution can be used for processing. If an initiator is electrospun with a
reactive polymer, the radical polymerization can be performed with introduction of a light source
after fiber formation (13, 21). Gelatin has been successfully blended with various synthetic
polymers, such as PLA (26) and PCL (27, 28) to improve cellular interactions when compared to
fibrous meshes of those polymers alone (26, 28-30), thus making it attractive as a possible carrier
polymer to facilitate the electrospinning of Acr-PGS.

The objective of this work was to synthesize a collection of Acr-PGS macromers that
crosslink into networks with a range of material properties (e.g., mechanics and degradation) and
that can be electrospun into fibrous scaffolds with gelatin as a carrier polymer to facilitate cellular
adhesion. This system was designed for applications that require a fibrous scaffold that is also
biodegradable, supports cellular adhesion, and has tunable properties. To illustrate this, the
scaffold biomechanics, degradation kinetics, and cellular interactions between the bulk polymers
and electrospun scaffolds were investigated. Prior work has demonstrated that bulk polymer networks formed from these macromers elicit a non-toxic response and resulted in only thin fibrous capsules consistent with subcutaneous implantation (13). Therefore, as an initial proof of concept, the capability of utilizing Acr-PGS electrospun scaffolds for a soft tissue application was explored by implanting as a cardiac patch in a rat model of myocardial infarction.

5.2 Materials and Methodology

5.2.1 Acr-PGS Macromer Synthesis

All reagents were purchased from Sigma Chemical Company (St. Louis, MO) and used as received unless noted. Acr-PGS was synthesized as previously described (13). Briefly, the PGS prepolymer was formed by the condensation reaction of equimolar amounts of glycerol (ThermoFisher Scientific, Waltham, MA) and sebacic acid. The reagents were combined at 120°C under nitrogen for 2 hours before a vacuum was applied for 72 hours. The resulting branched prepolymer was directly split into three groups of equal mass. For acrylation, prepolymer was dissolved in methylene chloride (1:10, ThermoFisher Scientific) containing triethylamine (TEA, equimolar to acryloyl chloride) and 500 ppm 4-methoxyphenol (MeHQ). Each prepolymer group (termed Low, Mid, and High) was reacted with a different molar ratio of acryloyl chloride (10, 15, and 30%, respectively, 1:10 v/v in methylene chloride), which was slowly dripped into the solution. These molar ratios were calculated using the estimation that two of the three hydroxy groups present in glycerol reacted with the sebacic acid during the condensation reaction and are meant to provide a range of overall acrylations. An additional 500 ppm MeHQ was added to the reaction flask and a rotary evaporator (40°C, 450 mbar) was used to remove the methylene chloride. Ethyl acetate was added to the reaction flask and the solution was filtered to remove the TEA salts and washed three times with 10 mM hydrochloric acid to remove any remaining salts and unreacted acrylic acid (ThermoFisher Scientific). Ethyl acetate was removed via rotovapping (40°C, 99 mbar) to leave a viscous liquid, which was redissolved in methylene chloride and stored at 4°C. The prepolymer molecular weights were verified using GPC (Waters
GPC System, Milford, MA) and the acrylation was assessed with $^1$H NMR spectroscopy (Bruker Advance 360 MHz, Bruker, Billerica, MA). Macromers were mixed with 0.5 wt% (with respect to the mass of macromer) of the photoinitiator 2,2-dimethoxy-2-acetophenone (DMPA, 10 wt% in methylene chloride).

5.2.2 Carrier Polymer Optimization of Electrospun Acr-PGS Scaffolds

Solutions for electrospinning were prepared by dissolving the macromer/initiator in 1,1,1,3,3,3 hexafluoro-2-propanol (50 wt% HFIP) for electrospinning with gelatin or 90% ethanol (50 wt% EtOH) for electrospinning with poly(ethylene oxide) (PEO). In order to facilitate fiber formation, the macromer/HFIP solution was combined with 10 wt% gelatin B (from bovine skin) in HFIP or 10 wt% PEO (200 kDa, Polysciences, Warminster, PA) (dissolved in a sealed vial at 50°C for 2 hours). Various solutions containing different ratios of Acr-PGS/solvent and carrier polymer/solvent (Table 1) were electrospun at room temperature using a flow rate of 1.5 mL/hr, distance to collection plate of 10 cm (for gelatin carrier polymer) or 15 cm (for PEO carrier polymer), and a +15 kV applied voltage. Scaffolds were crosslinked with exposure to ~10 mW cm$^{-2}$ 365 nm ultraviolet light (Blak-Ray, Ultraviolet Products, Upland, CA) in a nitrogen atmosphere. Scaffolds were gold sputter coated and viewed using scanning electron microscopy (SEM, Penn Regional Nanotech Facility, JEOL 6400 or JEOL 7500 HR-SEM, Tokyo, Japan).

To investigate cellular interactions with the Acr-PGS prepared using the different carrier polymers, samples were prepared by electrospinning each macromer/carrier polymer solution onto glass coverslips (22 × 22 mm, Corning, Lowell, MA) for 40 minutes with the previously mentioned electrospinning parameters. After electrospinning, samples were maintained under vacuum overnight to ensure complete solvent removal, photocrosslinked as above with ultraviolet light (Blak-Ray) in a nitrogen atmosphere, and incubated in PBS overnight. Samples were placed in non-treated wells of a 6-well plate, sterilized, seeded with human mesenchymal stem cells (hMSCs, 300,000 cells scaffold$^{-1}$), and assessed at 24 hours post-seeding for viability and morphology using a commercially available Live/Dead kit (Invitrogen). Based on these results, gelatin was chosen as the carrier polymer and was used for all remaining studies.
Table 5.2. Concentration of the High Acr-PGS macromer and carrier polymers in electrospinning solutions.

<table>
<thead>
<tr>
<th>Acr-PGS:Carrier Polymer ratio (v/v)</th>
<th>Electrospinning Solution Concentrations (wt%)</th>
<th>Scaffold Composition (wt%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Acr-PGS</td>
<td>Carrier Polymer</td>
</tr>
<tr>
<td>30:70</td>
<td>15</td>
<td>7</td>
</tr>
<tr>
<td>40:60</td>
<td>20</td>
<td>6</td>
</tr>
<tr>
<td>50:50</td>
<td>25</td>
<td>5</td>
</tr>
</tbody>
</table>

5.2.3 Fabrication of Electrospun Acr-PGS/Gelatin Scaffolds

Solutions for electrospinning were prepared by dissolving the macromers/initiator found in Table 5.2 in HFIP and combined with gelatin in HFIP as described above. Scaffolds were electrospun for 16 hours using the conditions previously described. After electrospinning, scaffolds were stored under vacuum overnight and crosslinked with exposure to ~10 mW cm\(^{-2}\) 365 nm ultraviolet light (Blak-Ray, Ultraviolet Products, Upland, CA) in a nitrogen atmosphere. Scaffolds were gold sputter coated and viewed using scanning electron microscopy (SEM, Penn Regional Nanotech Facility, JEOL 6400 or JEOL 7500 HR-SEM, Tokyo, Japan). Fiber diameters (n=50) were quantified from the SEM images using NIH ImageJ software.

5.2.4 Network Characterization

For studies performed on bulk materials, macromer/initiator solutions were poured into a 50 × 15 × 1 mm Teflon mold and placed in a vacuum oven at 60°C overnight to remove methylene chloride. The sample was then polymerized with exposure to ~10 mW cm\(^{-2}\) 365 nm ultraviolet light (Blak-Ray) for 10 minutes on each side. Prior work demonstrated maximum acrylate conversion during this time period (13, 21). For in vitro degradation studies, samples (10 × 2 × 1 mm) were immersed in phosphate-buffered saline (PBS) at 37°C with frequent PBS changes. At each time point, samples (n=3) were removed, lyophilized (Freezone 4.5, Labconco, Kansas City, MO) for 24 hours and weighed to determine mass loss. The contact angle of 100 μL of water on the bulk polymer surfaces (n=3) was assessed using the static sessile drop method.
technique (CAM-Plus Micro, Tantec, Fairfield, OH). The amount of unreacted macromer (sol fraction) of the networks was determined as the mass loss following incubation of bulk polymer disks (n=3, 4 mm diameter × 1 mm thick) in methylene chloride for 72 hours, which allowed unreacted macromer to swell free of the network.

Samples for mechanical testing (n=4-6, 15 × 5 × 1 mm for slabs and 25 × 5 × 1 mm for scaffolds) were cut and uniaxial tensile testing was performed on an Instron 5848 mechanical tester (Canton, MA) at a strain rate of 0.1% s⁻¹. For fibrous scaffolds, samples (both dry and after incubation in DI water for 1 hour) were preloaded with 0.5 N at 0.5% strain for 60 seconds, followed by preconditioning with 10 sinusoidal cycles of 0.5% strain at 0.1 Hz prior to testing to failure. The tensile modulus of the construct was calculated from the linear region of the stress-strain curve (0-3%) and initial sample geometry.

5.2.5 Cytotoxicity and Cell Adhesion

To evaluate indirect cytotoxicity of the bulk Acr-PGS materials, polymer disks (4 mm diameter × 1 mm thick) were prepared as described above. Samples were incubated in PBS overnight, sterilized with exposure to a germicidal lamp in a laminar flow hood for 30 minutes per side, and placed in transwell inserts above cultures of human mesenchymal stem cells (hMSCs, Lonza, seeded 24 hours previously at 6,000 cells cm⁻²) maintained in standard growth media consisting of Alpha-MEM (Gibco, Invitrogen, Carlsbad, CA) supplemented with 16.7% fetal bovine serum (Gibco), 1% penicillin (Gibco), and 1% streptomycin (Gibco). Cell proliferation was measured using the AlamarBlue™ (AB, Invitrogen) fluorescence assay. Growth media containing 10% (v/v) AB was added to each well. After a 4-hour incubation period, 100 μL aliquots (n=3/group) of the AB containing media was removed from each well for fluorescence measurement (530 nm excitation, 590 nm emission, Synergy HT, Biotek Winooski, VT). Blank control samples for each group (tissue culture plastic, High, Mid, and Low) in growth media without cells were also treated in order to remove any background fluorescence measurements. Samples were then fed with fresh growth media without AB. For continual assessment, the AB
assay was completed every other day on the same population of cells up to day 3 after exposure to polymer.

For bulk cell adhesion studies, the macromer/initiator solution was dissolved in ethanol (50 wt%) and 35 μL of this solution was added into the wells of a 24 well plate. Ethanol was evaporated off overnight and macromers were cured under a nitrogen purge and ultraviolet light (Blak-Ray) for 10 minutes. Films were incubated in PBS overnight and sterilized with exposure to a germicidal lamp in a laminar flow hood for 30 minutes followed by incubation with growth media for 2 hours prior to seeding. hMSCs were seeded onto the films at 6,000 cells cm⁻² and the AB assay was used to monitor cell proliferation every other day until day 7. At the same time points, cells were rinsed with PBS 3× and fixed in 4% formalin for 10 minutes. The cells were permeabilized with 0.25% Triton X-100 in PBS for 10 minutes, and nonspecific binding sites were blocked with 3% bovine serum albumin and 0.1% Tween-20 in PBS for 15 minutes. Actin stress fibers were stained using FITC-conjugated phalloidin (0.66 μg/mL in blocking solution) for 40 minutes at 37°C. Films were rinsed three times with PBS at each step and images were taken on a fluorescent microscope (Axiovert, Zeiss, Germany) with a digital camera (Axiovision, Zeiss). Images were post-processed using NIH ImageJ software.

To investigate cellular interactions with the Acr-PGS/gelatin fibers, samples were prepared by electrospinning each macromer/gelatin solution onto glass coverslips (22 × 22 mm, Corning, Lowell, MA) for 40 minutes with the previously mentioned electrospinning parameters. To ensure that the scaffolds adhered to the glass, the coverslips were treated with poly(3-trimethoxysilyl)propyl methacrylate (TMSMA) as was previously described (31) prior to electrospinning. Briefly, coverslips were plasma treated for 3 minutes (700 mAmp, Plasma Prep II, SPI Supplies, West Chester, PA), coated with TMSMA, baked at 100°C for 30 minutes, baked at 110°C for 10 minutes, washed with DI water, and dried overnight. After electrospinning, samples were maintained under vacuum overnight to ensure complete solvent removal, photocrosslinked as above with ultraviolet light (Blak-Ray) in a nitrogen atmosphere, and incubated in PBS overnight. Samples were placed in non-treated wells of a 6-well plate,
sterilized, seeded with hMSCs, and assessed for viability and morphology (with addition of DAPI, 2 μg/mL) as described above. Cell proliferation was also evaluated using the AB assay and cells were imaged as above.

5.2.6 In Vivo Assessment

Scaffolds from the High, Mid, and Low groups (~ 7.0 mm²) were evaluated in the rat model of acute myocardial infarction for their ability to attach to the infarct bed and for their biodegradation behavior. All groups were compared at the 2-week time point (n = 3, scaffold thickness 150 μm) and the High group (n = 3, scaffold thickness 300 μm) was additionally evaluated at 4 weeks. Infarcted and untreated (scaffold-free) animals were used as controls. Rowett (mu/rnu) athymic nude rats (body weight of 225-250 grams, Harlan Sprague Dawley, Indianapolis, Indiana) were used in studies approved by the Columbia University Institutional Animal Care and Use Committee. Similar to Fujimoto et al. (32), rats were anesthetized with inhaled isoflurane (2-3%), endotracheally intubated, and mechanically ventilated. The heart was exposed through a left thoracotomy and the pericardium incised. The left anterior descending artery (LAD) was then permanently ligated with a 7-0 prolene suture 2-3 mm below the edge of the left atrium. The scaffold (7.0 mm²) was then sutured directly onto the epicardial surface of the infarct bed using four 7-0 prolene stay sutures into each corner of the scaffold. Scaffolds covered ~20% of the epicardium. Scaffolds were implanted 20 minutes post-ligation and the ribs were reapproximated and the soft tissue closed in two layers. Two or four weeks after patch implantation, rats were sacrificed and intact hearts were collected for histology. Samples were stained using hematoxylin and eosin and the Masson trichrome stain to evaluate the extracellular matrix.

5.2.7 Statistical Analysis

All data is presented as mean ± standard deviation. Comparisons using a Student’s t-test assuming unequal variances was used for data in which the variances between groups was not equal, otherwise, single factor ANOVA with Tukey’s post hoc test was used to determine statistical significance among groups with p < 0.05.
5.3 Results and Discussion

As the field of regenerative medicine advances, there is a need to develop alternative materials and scaffold processing techniques to closely mimic the properties of the surrounding native tissue. Elastomers, such as PGS, are attractive for tissue engineering applications due to their ability to undergo large, reversible deformations with complete recovery (25), and possibly attenuate the compliance mismatch problem that often exists with synthetic polymeric implants in various dynamic environments that exist in the body, such as in the heart (6). To further exploit the potential of the synthetic elastomer PGS, acrylate functional groups (Acr-PGS) have been introduced, such that crosslinking can take place under relatively mild conditions with the use of a photoinitiator and light (13, 15). The specific focus of our study was to establish a process for fabrication of fibrous elastomer scaffolds with a range of mechanical and degradation properties for soft tissue engineering, and to test in a preliminary way the utility of these scaffolds in a heart infarction model. We propose that the wide range of biomechanical properties (tensile Young’s modulus ranging from ~60 kPa to 1 MPa) and degradation rates (~45 to 70% mass loss by 12 weeks) of these scaffolds enables their custom design for a variety of tissue engineering applications.

5.3.1 Macromer Synthesis and Scaffold Fabrication

Acr-PGS was synthesized using a condensation reaction of glycerol with sebacic acid that is further reacted with acryloyl chloride (Figure 5.1). The use of trifunctional glycerol in the condensation reaction introduces the possibility of branching and the resulting fraction of hydroxyl groups converted during acrylation leads to a relatively complex macromer structure. A representative $^1$H NMR spectra example with peak assignments can be found in Chapter 4. The bulk network properties (i.e., mechanics and degradation) of Acr-PGS networks can be tuned for a particular application by altering both the prepolymer molecular weight and the amount of acrylate functional groups introduced into the macromer system (13, 15). In this work, a small collection of Acr-PGS macromers was synthesized with the same molecular weight, but with variable acrylation. Based on previous work, a wider range of bulk network properties was
obtained using a condensation product molecular weight of approximately 20-25 kDa (13), therefore, a batch of PGS condensation product was synthesized with a similar condensation product weight-average molecular weight of 24.24 kDa, as verified by GPC (Table 5.2). The condensation product was then divided and acrylated to various extents (defined as Low, Mid, and High) as determined by $^1$H NMR (Table 5.2).

**Figure 5.1.** Schematic of the electrospinning process. A solution of Acr-PGS macromer, carrier polymer, and the photoinitiator in volatile solvent is electrospun to form a fibrous mat of Acr-PGS and carrier polymer. The scaffold is subsequently exposed to ultraviolet light to form crosslinked fibrous scaffolds.
Table 5.3. Synthesized Acr-PGS macromers with constant molecular weight and variable acrylation.

<table>
<thead>
<tr>
<th>Macromer</th>
<th>$M_n$ (kDa)</th>
<th>$M_w$ (kDa)</th>
<th>% acrylation</th>
</tr>
</thead>
<tbody>
<tr>
<td>High</td>
<td>3.74</td>
<td>24.24</td>
<td>23.5</td>
</tr>
<tr>
<td>Mid</td>
<td>3.74</td>
<td>24.24</td>
<td>14.2</td>
</tr>
<tr>
<td>Low</td>
<td>3.74</td>
<td>24.24</td>
<td>1.04</td>
</tr>
</tbody>
</table>

In order to successfully electrospin Acr-PGS scaffolds, a carrier polymer was needed to facilitate fiber formation, due to the relatively low molecular weight of Acr-PGS compared to commonly electrospun polymers (e.g., PCL, 80 kDa) (19, 23). For example, PEO has been successfully utilized as a carrier polymer to electrospin poly(β-amino ester)s (PBAEs) of relatively low molecular weight (~4 kDa) (21). Once the reactive macromer is crosslinked with ultraviolet light, a semi-interpenetrating network is formed. However, if PEO is used, there is the potential that it will not completely swell from the fibers and will block protein adsorption and as a result, limit cell adhesion (21). With this concern in mind, gelatin was also investigated as a carrier polymer due to its ability to improve cell adhesion in electrospun scaffolds when blended with other synthetic polymers (26-30).
Figure 5.2. Influence of carrier polymer and mass ratio on fiber formation. SEM images of scaffolds with Acr-PGS with the different carrier polymers (PEO: left column, gelatin: right column) concentrations of 83.3% (A, B), 76.9% (C, D), and 68.2% (E, F) by mass in the resulting mat. The High macromer was used for this initial study. Scale bar = 50 µm.

Based on prior work, it is known that the ratio of reactive macromer to carrier polymer in the blend is important in determining the resulting fiber morphology (13, 21). Therefore, the macromer (High Acr-PGS) and the two carrier polymers were combined in various ratios (Table 5.1), electrospun, and the resulting fiber morphologies were investigated using SEM after photocrosslinking (Figure 5.2). We have previously demonstrated that these macromers reach high double bond conversions during photopolymerization, both in the bulk and as scaffolds composed of similar reactive polymers at a depth of 1 mm (13, 21). In general, as the amount of Acr-PGS in the blend was increased, there was an increase in the webbing between the fibers and a decrease in fiber uniformity (Figure 5.2). The mass ratio of 77% Acr-PGS to 23% carrier polymer in the resulting fibrous mesh was selected as the optimal ratio for all other experiments in order to maintain Acr-PGS as the majority of the scaffold for control over the mechanics and degradation of the scaffolds, while providing relatively uniform fibers.
In a pilot study, hMSCs were seeded onto the surface of electrosprun scaffolds in order to evaluate any impact on cellular response that may be due to carrier polymer content. As suspected, the cells appeared to remain rounded on scaffolds that were prepared using PEO as the carrier polymer (Figure 5.3). However, cells appeared to attach and interact with polymers that were prepared using gelatin as the carrier polymer. This is evident in Figure 5.3, which depicts the cells aligning themselves along the fibers (stained red with ethidium homodimer). Based on these results, gelatin was identified as the optimal carrier polymer for all remaining studies.

![Acr-PGS: PEO vs Acr-PGS: Gelatin](image)

**Figure 5.3.** Influence of carrier polymer on cellular interactions. hMSCs were visualized using a Live/Dead kit at 24 hours post-seeding. Cells on remain round on scaffolds with PEO (left), whereas they spread and attach to scaffolds with gelatin as the carrier polymer (right).

In order to ensure that any differences in mechanics, degradation, or cellular responses are due to the Acr-PGS macromer used for scaffold fabrication, the two additional Acr-PGS macromers (Mid and Low) were electrospun with gelatin at this same concentration and evaluated using SEM (Figure 5.4). Fibrous scaffolds with relatively uniform morphology were formed from all macromers without statistical differences in fiber diameter between scaffolds prepared from different macromers.
Figure 5.4. Influence of Acr-PGS acrylation on fiber formation. SEM images of scaffolds fabricated from the three different Acr-PGS macromers; High (A), Mid (B), and Low (C) using gelatin as a carrier polymer and a final mass ratio of 77 wt% Acr-PGS to 23 wt% gelatin. Fiber diameter as a function of the degree of acrylation (D). Scale bar = 10 \( \mu \text{m} \).

5.3.2 Characterization of Network Properties

The bulk polymer network properties for contact angle, sol fraction, as well as mechanics and degradation were characterized. The contact angle of the bulk polymers was calculated using the sessile drop method. There is a decrease in both the contact angle and sol fraction as the % acrylation increases (Table 5.3). The probability of crosslink formation between the kinetic chains decreases as the number of acrylate groups is reduced, thereby increasing the number of soluble oligomers formed.
Table 5.4. Properties of the bulk polymer networks formed from the three different macromers.

<table>
<thead>
<tr>
<th>Bulk Polymer Network</th>
<th>Contact Angle (°)</th>
<th>Sol Fraction (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>High</td>
<td>55.69 ± 4.74</td>
<td>13.34 ± 0.90</td>
</tr>
<tr>
<td>Mid</td>
<td>70.36 ± 3.93</td>
<td>32.00 ± 2.78</td>
</tr>
<tr>
<td>Low</td>
<td>85.62 ± 13.74</td>
<td>51.91 ± 2.81</td>
</tr>
</tbody>
</table>

The mechanical properties of bulk Acr-PGS and Acr-PGS/gelatin fibrous scaffolds both dry and hydrated were evaluated using uniaxial tensile testing (Figure 5.5). As expected, a range of magnitudes in both the Young’s modulus and extension at break were observed as the acrylation concentration varied. For bulk polymer networks, the Young’s moduli (~31 kPa to 6.6 MPa) increased and the extension at break (~110 to 9%) decreased with increasing amounts of acrylation in the Acr-PGS backbone (Figure 5.5). There were statistically significant differences in both of these properties between all acrylation groups. It is important to note that the samples often broke at the clamps and thus may be reported as lower than actual values and increases in observed variability with respect to extension at break.
Figure 5.5. Representative stress versus strain curves for groups prepared from the Mid macromer (A). Tensile properties of bulk polymers, dry scaffolds, and hydrated scaffolds for the High (black), Mid (grey), and Low (white) macromers for Young’s modulus (B) and % extension (C). *p<0.05, **p<0.01.

The fibrous scaffolds were tested in both the dry state and following incubation in water for 1 hour in order to investigate the mechanical properties under more physiological conditions, such as those found in vivo. In the dry state, there is relatively little variation and no statistical
difference in the modulus of the scaffold between acrylation groups. This could be due to the
gelatin, which has been reported to have a modulus of ~500 MPa for similar electrospun scaffolds
tested in the dry state under tension (30), dominating the mechanics in the dry state. This is
evident by a substantial increase in the modulus to ~10 MPa in the fibrous state compared to the
slab form in samples prepared from the various macromers (Figure 5.5). However, when the
samples are hydrated, the degree of acrylation of Acr-PGS appears to have more influence over
the mechanics of the scaffolds, with similar trends in the moduli between the groups as was
observed for the bulk samples (Figure 5.5). Due to premature failure at the grips, substantial
changes in extension with extent of acrylation are not reflected in the collected data. The tested
hydrated scaffolds lost ~9% of their mass (across all groups, data not shown) within the hour of
hydration, presumably due to gelatin and small amounts of unreacted macromer being leached
from the scaffolds. The variability between the dry and hydrated samples is most likely due to
water absorption by the remaining gelatin component, thus forming a resilient, hydrated
composite. These values are lower than those reported in the literature for pure gelatin scaffolds
(29, 30) since the scaffold is composed of only ~23% gelatin. Moreover, formation of scaffolds
consisting of gelatin alone cannot be directly compared to the blended scaffolds, due to the
crosslinking (i.e., with glutaraldehyde) necessary for stabilization. Here, a stable interpenetrating
blended network is obtained, which does not require secondary chemical crosslinking of the
gelatin. This is advantageous because further chemical crosslinking of the gelatin may result in
an increase in the rigidity of the scaffolds and a decrease in the effectiveness of the gelatin with
respect to cell adhesion (29).

The in vitro degradation of the Acr-PGS bulk polymers and scaffolds was monitored over
an 18-week period. Within each formulation (bulk versus fibrous scaffold), the mass loss
correlated with the acrylation with the Low group losing mass faster than the Mid group, which
was faster than the High group. The mass loss in these systems is a combination of the gelatin
release, sol fraction, and hydrolysis of the crosslinked Acr-PGS into its naturally derived
monomers sebacic acid and glycerol, and a relatively low concentration of poly(acrylic acid)
kinetic chains. Since HFIP is the only solvent in which both the macromers and gelatin are
soluble, $^1$H NMR cannot be utilized to determine the relative ratios of components released. It is not surprising that the samples with fewer acrylates degraded more quickly, since there are fewer crosslinks to degrade prior to mass release and there is a higher sol fraction present. Additionally, scaffolds degraded at a faster rate than their respective bulk macromer counterpart with the exception of the bulk Low material, which degraded completely within 12 weeks (Figure 5.6A). An initial increase in the mass loss of the scaffolds is observed up to three weeks, which is most likely due to some loss of the gelatin carrier polymer in the interpenetrating network to the aqueous medium. Therefore, it is impossible to directly compare the degradation rates of the scaffolds and bulk polymers, which do not incorporate gelatin. The scaffolds maintained their fibrous nature with degradation, as observed in the SEM image of the Mid fibrous scaffold after 6 weeks (Figure 5.6B). Note that residual salts from the PBS are also observed.

**Figure 5.6.** *In vitro* mass loss for High (diamond), Mid (square), and Low (circle) groups processed as slabs (filled) and fibrous scaffolds (open) with time (A). SEM images of the Mid scaffold at 6 weeks of degradation in PBS (B). Scale bar = 10 µm.
5.3.3 Cytotoxicity and Cell Adhesion

In order to evaluate the cytotoxicity of the Acr-PGS macromers, hMSCs were cultured in the presence of the bulk Acr-PGS materials, without having direct contact with the polymer. This assay provides information on the potential toxicity of leachable components and degradation products from the samples. The wells were confluent at 3 days and no statistical differences in fluorescence (i.e., metabolic activity) between the cells cultured with or without polymer present were observed (Figure 5.7). Thus, changes in direct cellular interactions may be attributed to non-optimized integrin binding, rather than toxic elements from the systems.

![Figure 5.7](image)

**Figure 5.7.** Metabolic activity/proliferation, as assessed by the AlamarBlue assay, over 3 days of hMSCs exposed to bulk polymer samples formed from each macromer.

When hMSCs were seeded directly on films of the polymers, the metabolic activity of the cells cultured on the polymers was significantly lower than that of cells cultured on tissue culture plastics (Figure 5.8A). However, as evident from actin staining and an increase in fluorescence with culture, cells appear to adhere, spread, and proliferate on films prepared from the High and Mid Acr-PGS macromers (Figure 5.8B). Cellular viability and adhesion is significantly reduced on the Low polymer, where the majority of cells were rounded (Figure 5.8A, B). There are several reasons that cellular interactions may not be optimized for interactions with the Acr-PGS
networks, including non-optimal protein adsorption, mechanics, and the tackiness of the material. In previous studies, greater cell attachment was observed on surfaces with intermediate contact angles versus surfaces with higher or lower contact angles (33-35). For example, polystyrene and polyethylene terephthalate are moderately hydrophobic polymers (contact angles ~60-70°) that support greater cell attachment compared to highly hydrophilic polymers, such as cellulose (contact angle ~18°) or tetrafluoroethylene hexafluoropropylene (contact angle ~102°) (35). The variability in contact angles in our study may contribute to differences in cellular responses; however, the lack of toxicity in the non-contact study indicates that it is not likely due to the release of any cytotoxic components. Thus, the significant difference in metabolic activity between tissue culture plastic and the High and Mid films is most likely due to poor cell attachment initially (Day 1, Figure 5.8A) resulting from suboptimal protein absorption.
Figure 5.8. Bulk cell interactions between hMSCs and Acr-PGS networks. hMSC metabolic activity/proliferation, as measured by fluorescence, over 7 days using the AB assay (A). Fluorescent images of hMSC morphology (actin fiber staining) when cultured on thin films of the Acr-PGS macromers (B). *p<0.05, Scale bar = 100 μm.

However, the cellular viability and morphology was very different on thin scaffolds that were electrospun with gelatin present compared to thin polymer films. Specifically, cell attachment and proliferation were similar for electrospun fibrous scaffolds and glass coverslips (Figure 5.9A), with confluent monolayers found within 7 days post-seeding for all formulations. Cells appear to adhere and spread over the fibers (Figure 5.9B), which are readily visualized since Acr-PGS interacts with the DAPI nuclear stain. Cells were extending pseudopodia and interacting with individual fibers (Figure 5.9C) for scaffolds formed from all three macromers. This
is in contrast to studies in which PEO was used as a carrier polymer with Acr-PGS and cells were observed to remain spherical and not spread along the fibers (Figure 5.2). The increase in cell attachment and proliferation compared to the bulk polymers may result from differences in surface topography or the presence of gelatin as the carrier polymer in this system, therefore eliminating the reliance on protein adsorption from serum to facilitate cell adhesion.
**Figure 5.9.** hMSC interactions with scaffolds prepared with the different Acr-PGS macromers. hMSC metabolic activity/proliferation, measured by fluorescence, on the different scaffolds over 7 days, using the AB assay (A). Fluorescent images of actin stress fibers (green) and electrospun fibers (blue) depicting the morphology of hMSCs cultured on the different scaffolds (B). A magnified image of the inset at Day 1 depicting the direct cellular interactions with the hMSCs and the electrospun fibers (C). *p<0.05, scale bar = 100 μm.
5.3.4 In Vivo Assessment

The initial tissue response and the capacity of the scaffolds to function within a therapeutically relevant scenario were evaluated using a rat model of acute myocardial infarction. This study was designed to investigate the feasibility of applying these scaffolds to the infarct bed, and to obtain preliminary insights into the effects of scaffold properties (stiffness, degradation) on tissue outcomes in this experimental model. Scaffolds from the three different Acr-PGS macromers (High, Mid, Low) were assessed at a 2-week time point; scaffolds from the High group were additionally evaluated at the 4-week time point. The scaffold-free control shows some collagen deposition consistent with normal post MI fibrosis (Figure 5.10A). Notably, the scaffolds from all three groups were entirely degraded over the 2-week period of implantation (Figure 5.10B-D). The rapid degradation compared to the in vitro samples may be due to the inflammation and cellular infiltration in the post-infarct environment, which may have contributed to the increased hydrolysis of the scaffolds. This effect was clearly seen since the scaffolds were very thin (~150 μm compared to 1 mm for in vitro studies).

Interestingly, there was a correlation between the tissue response and the amount of acrylation. The Low acrylation macromer samples showed increased collagen deposition compared to the scaffold-free control (Figure 5.10A). However, there were few inflammatory cells present after 2 weeks. For the Mid macromer group, inflammatory cells were still present, despite the scaffold being mostly degraded. Numerous arterioles were consistent with the early resolution stages of inflammation (Figure 5.10B). A greater amount of inflammation in the infarct area with many granulocytes visible was observed where the High macromer group scaffolds were implanted (Figure 5.10D). In summary, a more resolved inflammatory response with evidence of collagen deposition was observed for Low samples, whereas the Mid and High samples demonstrated an unresolved host response due to the high levels of inflammation. Notably, the rapid degradation that was observed in this environment leads to rapid release of degradation products locally, which explains the tissue response.
Figure 5.10. In vivo tissue response to Acr-PGS scaffolds. The scaffolds were implanted epicardially post LAD ligation for 2 or 4 weeks. Trichrome staining of control (A), Low (B), Mid (C), and High (D) scaffolds at 2 weeks. A thicker High scaffold was implanted and observed at 4 weeks using trichrome staining (E) and H&E (F). The * marks the center of the scaffold, while the arrows delineate the edges, scale bar = 50 μm.

After complete degradation of the 150 μm thick scaffolds was observed at 2 weeks, the study was repeated using 300 μm scaffolds of the High macromer to study a longer term response (4 weeks in vivo) of the host to the material. The scaffolds were still present at 4 weeks, and the cellular infiltration was observed only in the outer portion of the scaffold (Figure 5.10E), which may lead to higher local concentrations of enzymes, and therefore degradation. The center of the scaffold is marked by an asterisk, and the arrows mark the edges (Figure 5.10E and F). The scaffold was not fully degraded and was covered with the host's foreign body capsule. Greater detail of the capsule using H&E staining shows a minimal presence of granulocytes and a resolved inflammatory response with significant fibrosis at the 4-week time point (Figure 5.10F). This pilot study demonstrates the feasibility of suturing electrospun scaffolds composed of Acr-PGS and gelatin onto the epicardial surface of the heart. Moreover, it demonstrates that the host response to the implanted material, in the setting of acute cardiac
infarction, markedly depends on scaffold thickness and degree of acrylation. Future work will involve evaluation of the potential functional improvement (i.e., left ventricle size, hemodynamic data, etc.) of animals with implanted scaffolds.

5.4 Conclusions

In this study, a small collection of biodegradable Acr-PGS macromers that could be crosslinked into networks of varying bulk properties (i.e., mechanics and degradation) was successfully synthesized and characterized. These macromers were combined with gelatin or PEO carrier polymers in order to fabricate electrospun scaffolds. hMSCs were able to attach and interact with the fibers prepared using gelatin, whereas they remained rounded on fibers prepared using PEO. Based on these studies, gelatin was selected as the carrier polymer for the remaining studies. Similar trends were observed for the bulk polymers and scaffolds, in terms of the dependence of their mechanical properties on the degree of acrylation. The rate of mass loss also correlated to the extent of acrylation, such that the more acrylated materials degraded more slowly. Cells attached and remained viable on bulk thin films of the different macromers, however potentially suboptimal protein adsorption decreased their adhesion and metabolism relative to controls on tissue culture plastic. In contrast, cell adhesion and proliferation on electrospun scaffolds was indistinguishable from controls, possibly due to the presence of the gelatin, which decreased the dependence on adsorbed protein from serum and facilitated cell adhesion on the scaffolds. Finally, the scaffolds were implanted into a rat model of acute myocardial infarction to investigate the functional tissue response and possible use of the scaffolds for cardiac patch applications. The scaffolds were capable of being sutured onto the epicardial surface of the heart. Thin (~150 μm) samples degraded entirely in 2 weeks and an increase in the inflammatory response was observed with increasing amounts of macromer acrylation. Thicker (~300 μm) samples were collected at 4 weeks post-implantation and a foreign body capsule was observed, including some cellular infiltration into the scaffolds. Future work will involve further functional assessments of the implanted scaffolds and optimization of properties for a specific application.
References:


Chapter 6

The Influence of Fibrous Photocrosslinked Poly(glycerol sebacate)
Structure and Porosity on Cellular Infiltration and Matrix Organization


6.1 Introduction

Myocardium is composed primarily of highly aligned myocytes flanked by collagen fibrils, which is important for the maintenance of the global ventricular geometry. This anisotropy allows for the energy efficient production of the contractions necessary for delivering nutrients throughout the body (1-4). The organization is maintained by a delicate balance of matrix metalloproteinases (MMPs) and tissue inhibitors of matrix metalloproteinases (TIMPs) and is disrupted as a result of myocardial infarction (MI). The upregulation of MMPs that occurs post-MI initiates substantial collagen degradation leading to infarct expansion and ultimately a disorganized scar structure (2, 3, 5).

Traditional tissue engineering strategies involving the use of implanted cells have been minimally successful in improving cardiac function, potentially due to the lack of structural cues necessary for cellular alignment due to the disorganized scar that exists (6). The use of various restraints, such as polymeric materials that are sutured onto the epicardium (7, 8) or intramyocardial injection of various hydrogels (9-11) have been successful in reducing post-infarct expansion and improving cardiac function. In an effort to understand the influence of infarct restraints on extracellular matrix structure, Blom et al. (7) noted a preservation of fibrillar collagen within the vulnerable peri-infarct region with use of the CorCap Acorn Cardiac Support Device (CSD) post-infarct. Furthermore, significant accumulation of organized matrix surrounding the individual fibers of the CSD was observed. This work suggests that maintenance of the
organized extracellular matrix structure may reduce infarct expansion and contribute to improvement in cardiac function post-MI.

Recent work has also demonstrated the importance of the microenvironment mechanics on cardiomyocyte contraction. Specifically, Engler and co-workers observed a reduction in the beating frequency of cardiomyocytes on stiff polyacrylamide gels (>10 kPa, by AFM) versus soft gels (<10 kPa by AFM) (12). Moreover, a recent study investigated the over-expression of elastin in the infarct region and demonstrated that increasing the elasticity in the infarct leads to an improvement of function due to a decrease in thinning and dilation (13). In Chapter 4, we modified the synthesis of the biodegradable elastomer poly(glycerol sebacate) (PGS) (14) to introduce reactive acrylate groups (Acr-PGS) and in Chapter 5 capitalized on photopolymerization to form fibrous Acr-PGS scaffolds with tunable mechanics and degradation. Thus, the physical characteristics of PGS, such as its elastic behavior, make it attractive as a cardiac graft to potentially reduce infarct expansion and improve regional and global LV function. When an electrospun scaffold was implanted on the surface of an infarcted heart (Chapter 5), it was noted that tissue in-growth occurred primarily in the periphery of the scaffold and not throughout. Thus, it is important to investigate approaches to overcome this limitation and to control directional organization of formed tissues.

Electrospinning is an attractive scaffold processing technique for cardiac applications because of the ability to easily align the fibers in order to mimic the anisotropic nature of the heart tissue (15, 16). Fibers are aligned in the electrospinning process by collection onto a rotating mandrel (17-21). The rate at which the mandrel rotates controls the degree of fiber alignment and thus the anisotropy ratio of the scaffold (19). Alignment of the fibers has been shown to influence cellular interactions by providing structural cues to facilitate orientation of cells along the fiber (18, 22, 23). Furthermore, cells cultured on aligned scaffolds have been shown to produce organized matrix, and thus improve the mechanical properties of the various constructs (18, 24).

One limitation in aligned, electrospun scaffolds is the reduction in porosity of the scaffold due to the dense packing of fibers, which can decrease cellular infiltration into the scaffolds. This is especially an issue for scaffolds composed of fibers < 1 \( \mu \)m in diameter (25) or slowly
degrading polymers (17, 18). Investigators have attempted to improve the porosity of the scaffolds for better cellular infiltration using several means, including using a rotating frame collector for fibers (26), layered hydrosplining, (27) and electrospinning of salt particles into scaffolds, which are subsequently leached from the system (28, 29). Baker and colleagues (17) simultaneously electrospun the slow degrading poly(caprolactone) (PCL) and the water soluble poly(ethylene oxide) (PEO) from two separate jets to create a composite scaffold that showed an increase in porosity when the sacrificial PEO fibers were leached from the system following incubation in aqueous medium. They observed a significant increase in cell infiltration for samples that consisted of < 40% of PEO by mass. However, greater than 60% PEO by mass compromised the structural integrity of the scaffold (17).

The objective of this work was to fabricate and characterize electrospun Acr-PGS scaffolds using control over structure (via fiber alignment) and porosity (via introduction of a sacrificial fiber population) and to evaluate the resulting impact on cellular infiltration and alignment both in vitro and in vivo as a subcutaneous implant. Furthermore, the impact of the structure and porosity on matrix elaboration and organization was assessed. This work serves as an initial step for the development of Acr-PGS cardiac grafts with the eventual goal of using these grafts to understand how scaffold structure, mechanical properties, and degradation kinetics of left ventricular restraints can impact infarct remodeling.

6.2 Materials and Methodology

6.2.1 Acr-PGS Macromer Synthesis

All reagents were purchased from Sigma Chemical Company (St. Louis, MO) and used as received unless noted. Acr-PGS was synthesized as previously described (30). Briefly, the PGS prepolymer was formed by the condensation reaction of equimolar amounts of glycerol (ThermoFisher Scientific, Waltham, MA) and sebacic acid. The reagents were combined at 120°C under nitrogen for 2 hours before a vacuum was applied for 47 hours. For acrylation, the condensation product was dissolved in methylene chloride (1:10, ThermoFisherScientific) containing triethylamine (TEA, equimolar to acryloyl chloride) and 500 ppm 4-methoxyphenol
(MeHQ) and 15% acryloyl chloride (1:10 v/v in methylene chloride), which was slowly dripped into the solution. This molar ratio was calculated using the estimation that two of the three hydroxy groups present in glycerol reacted with the sebacic acid during the condensation reaction. An additional 500 ppm MeHQ was added to the reaction flask and a rotary evaporator (40°C, 450 mbar) was used to remove the methylene chloride. Ethyl acetate was added to the reaction flask and the solution was filtered to remove the TEA salts and washed three times with 10 mM hydrochloric acid to remove any remaining salts and unreacted acrylic acid. Ethyl acetate was removed via rotovapping (40°C, 99 mbar) to leave a viscous liquid, which was redissolved in methylene chloride and stored at 4°C. The condensation product molecular weights were verified using GPC (Waters GPC System, Milford, MA) and the acrylation was assessed with 1H NMR spectroscopy (Bruker Advance 360 MHz, Bruker, Billerica, MA). Macromers were mixed with 0.5 wt% (with respect to the mass of macromer) of the photoinitiator 2,2-dimethoxy-2-acetophenone (DMPA, 10 wt% in methylene chloride).

6.2.2 Fabrication of Electrospun Acr-PGS/Gelatin Scaffolds

A solution containing 15 wt% of the macromer/photoinitiator and 4.5 wt% of gelatin B (from bovine skin) in 1,1,1,3,3,3 hexafluoro-2-propanol (HFIP) and a 10 wt% solution of poly(ethylene oxide) (PEO, 200 kDa, Polysciences, Inc. Warrington, PA) in 90% ethanol were prepared for electrospinning. First, a scaffold with non-aligned (NA) fibers was prepared by electrospinning Acr-PGS/gelatin alone at 1.5 mL hr⁻¹, +15 kV onto a flat collection plate located 10 cm from the spinnerette. The remaining scaffolds were prepared by electrospinning onto a rotating mandrel (Ø = 2", 12 m/s) using a custom setup as previously described by Baker et al. (17) (Figure 6.1). Briefly, a syringe containing the electrospinning solution was connected to a 5 cm piece of silicone tubing fitted with luer lock attachments and placed in a syringe pump programmed to operate at a flow rate of 1.5 mL hr⁻¹. A 6" blunt end 18-gauge needle was then attached to serve as the charged spinnerette. The spinnerette was inserted into a custom-built “fanner,” such that it translated along a 4 cm path across the mandrel. A power supply (Gamma High Voltage, Ormond Beach, FL) was used to apply a potential difference of +15 kV between the
spinnerette and the grounded collection apparatus located 10 cm away. The second scaffold was prepared by electrospinning Acr-PGS/gelatin alone (AL) onto the rotating mandrel, such as to generate a scaffold composed of aligned fibers. Next, stable Acr-PGS/gelatin fibers were electrospun concurrently with a second solution containing 10 wt% PEO (200 kDa, Polysciences, Inc. Warrington, PA) in order to introduce a sacrificial fiber population, thus generating a third aligned, composite scaffold (CO). This was accomplished using a second spinnerette and fanner located on the opposite side of the mandrel (Figure 6.1). A second power source was connected to this spinnerette and set up to operate using the same conditions described. However, this syringe pump was programmed to operate at 3.0 mL hr⁻¹, such that the resulting composite scaffold would contain 50% Acr-PGS/gelatin and 50% PEO by mass. All scaffolds were electrospun for 13 hours. A final scaffold consisting of PEO alone was also electrospun for comparison of mechanical properties. After electrospinning, scaffolds were stored under vacuum overnight. All scaffolds containing Acr-PGS were crosslinked with exposure to ~10 mW cm⁻² 365 nm ultraviolet light (Blak-Ray, Ultraviolet Products, Upland, CA) in a nitrogen atmosphere. All scaffolds were also gold sputter coated and viewed using scanning electron microscopy (SEM, Penn Regional Nanotech Facility, JEOL 7500 HR-SEM, Tokyo, Japan).

![Figure 6.1](image.png)

**Figure 6.1.** Schematic illustrating the set up for composite scaffold electrospinning.
6.2.3 Sacrificial Fiber Removal

Scaffolds were washed after fabrication to assess the amount of PEO sacrificial polymer. Specifically, samples from all three scaffold groups (n=3 per group) were weighed in the dry state and the PEO fibers were removed by incubation in DI water for three hours, with fresh water changes occurring at each hour, and incubation in phosphate buffered saline (PBS) overnight. The dry weight of the scaffolds was then collected following lyophilization (Freezone 4.5, Labconco, Kansas City, MO). SEM images were also taken prior to and following PEO removal.

6.2.4 Mechanical Testing

All scaffolds were subject to uniaxial tensile testing in both the parallel (PA) and perpendicular (PE) fiber directions after crosslinking as well as after PEO removal and lyophilization. Due to prior work (31) demonstrating an influence of hydration state on scaffold mechanics, samples were also tested at the conclusion of PEO removal, but before drying. All samples for mechanical testing (n=3-6 per group and 25 × 5 mm) were cut and uniaxial tensile testing was performed on an Instron 5848 mechanical tester (Canton, MA) at a strain rate of 0.1% s⁻¹ following a preload with 0.5 N at 0.5% strain for 60 seconds, and preconditioning with 10 sinusoidal cycles of 0.5% strain at 0.1 Hz prior to testing to failure. The tensile modulus of the construct was calculated from the linear region of the stress-strain curve (0-3%) and initial sample geometry.

6.2.5 Cell Adhesion

To investigate cellular interactions with the Acr-PGS/gelatin fibers, samples were prepared by electrospinning each macromer/gelatin solution onto glass coverslips (22 × 22 mm, Corning, Lowell, MA) for 20 minutes with the previously mentioned electrospinning parameters. To ensure that the fibers adhered to the glass, the coverslips were treated with poly(3-trimethoxysilyl)propyl methacrylate (TMSMA) as was previously described (32) prior to electrospinning. Briefly, coverslips were plasma treated for 3 minutes (700 mAmp, Plasma Prep II, SPI Supplies, West Chester, PA), coated with TMSMA, baked at 100°C for 30 minutes, baked at 110°C for 10 minutes, washed with DI water, and dried overnight. After electrospinning,
samples were maintained under vacuum overnight to ensure complete solvent removal, photocrosslinked as above with ultraviolet light (Blak-Ray) in a nitrogen atmosphere, and incubated in PBS overnight. Samples were placed in non-treated wells of a 6-well plate, sterilized upon exposure to germicidal ultraviolet light for 30 minutes, and seeded with freshly isolated neonatal rat cardiomyocytes (50,000 cells cm⁻¹), a kind gift from Dr. Kenneth Margulies’ lab (33). Media (DMEM:M199 4:1, 10% horse serum, 5% fetal bovine serum, 1% penicillin/streptomycin/glutamine, and 1% 1 M HEPES, all from Invitrogen, Carlsbad, CA) was replaced every other day and on day 5, cells were rinsed with PBS 3× and fixed in 4% formalin for 10 minutes. The cells were permeabilized with 0.25% Triton X-100 in PBS for 10 minutes, and nonspecific binding sites were blocked with 3% bovine serum albumin and 0.1% Tween-20 in PBS for 15 minutes. Actin stress fibers were stained using FITC-conjugated phalloidin (0.66 μg mL⁻¹ in blocking solution) for 40 minutes at 37°C. Cell nuclei were visualized following DAPI staining (2 μg mL⁻¹) for 5 minutes. Samples were rinsed three times with PBS at each step and images were taken on a fluorescent microscope (Olympus BX51, Center Valley, PA) with a digital camera (Olympus DP72). Images were post-processed using NIH ImageJ software to calculate fiber (n=128-135 per group) and cell (n=128-135 per group) alignment.

6.2.6 In Vivo Evaluation

Animals were cared for according to a protocol approved by the University of Pennsylvania Institute for Animal and Use Committee. Electrospun scaffolds were processed for PEO removal and sterilized using germicidal ultraviolet light as previously described. Samples (n=3-6 samples per group, 10 mm length × 5 mm width, NA 0.25-0.6 mm, AL 0.24-0.4 mm, CO 0.88-1.12 mm thick) were collected at 2, 3, and 4 weeks post-subcutaneous implantation into dorsal pockets of rats, fixed in 4% formalin for 24-36 hours, and processed using standard histological techniques. Following paraffin embedding, samples were sectioned into 7 μm thick sections in the scaffold cross-section, along the direction of the fibers for AL and CO samples. Samples were stained using Masson's trichrome (substituting hematoxylin Gill No. 2 for Weigret's hematoxylin). The amount of free (acellular) scaffold was calculated by determining
the thickness of the acellular region of the scaffold (n=10 per sample) using ImageJ, divided by the initial thickness of the sample, which was normalized to acellular scaffolds that were also processed and visualized using trichrome staining.

In order to evaluate matrix elaboration, samples were also stained using picrosirius red. Trichrome stained images were obtained on an upright microscope (Olympus BX51) with a digital camera (Olympus DP72) and post-processed using NIH ImageJ to determine the amount of free (lacking cellular infiltration) scaffold at each time point. Quantitative polarized light microscopy was used to visualize and quantify collagen alignment as described in Nerurkar et al. (24). Briefly, greyscale images were collected at 10× in 10° increments using a green band-pass filter (BP 546 nm) with a crossed analyzer and polarizer coordinately rotated through a 90° span on a Leica DM/LP microscope. The filter was replaced with a λ compensator and images were again collected through the same 90° span. A custom program was used to determine the collagen fiber orientation (n=25-30 collagen fibers for NA, 55-70 collagen fibers for AL and CO) located within the scaffold region per sample.

6.2.7 Statistical Analysis

All data is presented as mean ± standard deviation. Comparisons using a Student’s t-test assuming unequal variances was used for data in which the variances between groups was not equal, otherwise, single factor ANOVA with Tukey’s post hoc test was used to determine statistical significance among groups with p < 0.05.

6.3 Results and Discussion

Infarct restraints have been successful in reducing the maladaptive left ventricular remodeling response that occurs post-MI (7, 8). Specifically, their use has been shown to reduce the degradation of the existing extracellular matrix and influence the organization of matrix that is elaborated by the infiltrating fibroblasts (7, 8). Electrospinning is a simple, cost-effective technique to create fibrous scaffolds with a similar size scale and architecture to the native extracellular matrix, which has been shown to influence matrix elaboration and organization in
vitro (18, 24). Moreover, formation of composite electrospun scaffolds consisting of a stable fiber population and a sacrificial fiber population, which are subsequently dissolved following fabrication, have been shown to improve cellular infiltration, a common problem for electrospun scaffolds consisting of aligned fibers (17). The specific focus of this study was to evaluate the impact of fiber orientation and porosity on cellular infiltration and matrix organization both in vitro and in vivo as a subcutaneous implant. Our eventual goal is to utilize the results from this work to further develop infarct restraints to understand the influence of scaffold structure, material mechanics, and degradation on extracellular matrix composition, left ventricular geometry, and function post-MI.

6.3.1 Macromer Synthesis and Scaffold Fabrication

Acr-PGS was synthesized by a condensation reaction of glycerol and sebacic acid followed by reaction with acryloyl chloride in the presence of triethylamine. Previous work (Chapters 4 and 5) demonstrated the influence of molecular weight and % acrylation on the resulting mechanical properties and degradation kinetics of both Acr-PGS bulk polymers and fibrous scaffolds (30, 31). Based on this work, a macromer with a $M_w$ of 26 kDa and 12.7% acrylation was synthesized in order to more closely mimic the modulus of soft tissues (31). As expected, the bulk polymer had a modulus of 253.9 ± 18.6 kPa.

As described in Chapter 5, a carrier polymer, gelatin, is needed facilitate fiber formation with the relatively low molecular weight reactive Acr-PGS during electrospinning. In order to evaluate the impact of scaffold architecture and porosity on cellular interactions, three Acr-PGS scaffolds were fabricated that contained comparable masses of Acr-PGS and gelatin. One scaffold with non-aligned, randomly oriented fibers (NA) was fabricated by electrospinning an Acr-PGS/gelatin solution onto a flat collection plate. A second Acr-PGS/gelatin scaffold consisting of aligned fibers (AL) was fabricated by collecting electrospun fibers onto a rotating mandrel. Finally, a third scaffold consisting of a mixture of aligned distinct Acr-PGS/gelatin fibers and PEO fibers (CO) was fabricated using the device shown in Figure 6.1. Based on the study by Baker et al. (18), the composite scaffold was designed to be 50% Acr-PGS/gelatin and 50% PEO by mass, in an effort to increase cellular infiltration without sacrificing the mechanical integrity of the
scaffold. Representative SEM images of each scaffold are shown in Figure 6.2, with the arrow indicating the direction of fiber alignment for the aligned scaffolds. An increase in scaffold porosity was observed following PEO removal (Figure 6.2D).

![Figure 6.2.](image)

Figure 6.2. Representative SEM images of NA (A), AL (B), and CO (C) scaffolds following crosslinking and a CO scaffold following PEO removal and lyophilization (D). Scale bar = 20 μm.

In order to verify the mass composition of the composite scaffolds, all three scaffolds were subjected to the wash protocol by incubation in DI water for 3 hours and PBS overnight. NA and AL scaffolds lost 17.5 ± 2.2 and 12.3 ± 2.3% mass, respectively, due to loss of unreacted macromer and gelatin. However, CO scaffolds lost 63.0 ± 0.5% mass during processing for PEO removal. Based on the mass loss difference between the AL and CO scaffolds the composition of the CO scaffold was confirmed to be ~50% Acr-PGS/gelatin and ~50% PEO.
6.3.2 Mechanical Properties

Uniaxial tensile testing was conducted on all of the scaffolds both in the fiber direction (PA) and transverse to the fiber direction (PE). The anisotropy ratio for the AL and CO scaffolds was calculated as the ratio of the PA modulus to the PE modulus (Table 6.1). Representative PA stress versus strain curves of scaffolds tested following crosslinking, lyophilization after PEO removal in the dry state, and following PEO removal and incubation in PBS (the hydrated state) are shown in Figure 6.3. The anisotropy ratio for AL scaffolds decreased following washing and lyophilization in the dry state (Table 6.1). This is due to the increase in the AL PE modulus that was observed during mechanical testing. The anisotropy ratio of the CO scaffolds following PEO removal and hydration was increased due to water adsorption within the scaffold.

Table 6.1. Anisotropy ratio of AL and CO scaffolds.

<table>
<thead>
<tr>
<th>Scaffold</th>
<th>Post-Crosslinking</th>
<th>Post-Wash, Dry</th>
<th>Post-Wash, Hydrated</th>
</tr>
</thead>
<tbody>
<tr>
<td>AL</td>
<td>3.19 ± 0.24</td>
<td>1.65 ± 0.28</td>
<td>2.52 ± 0.26</td>
</tr>
<tr>
<td>CO</td>
<td>3.44 ± 0.21</td>
<td>3.11 ± 0.39</td>
<td>6.96 ± 0.37</td>
</tr>
</tbody>
</table>

PEO scaffolds were also tested for comparison to the CO scaffolds. Surprisingly, the modulus of the CO scaffolds (8.54 ± 1.68 MPa and 2.48 ± 0.21 MPa, PA and PE, respectively) tested after crosslinking was less than both the PEO (18.65 ± 2.43 MPa and 3.91 ± 0.73 MPa, PA and PE, respectively) and AL Acr-PGS scaffolds (39.89 ± 5.59 MPa and 12.49 ± 2.48 MPa, respectively) in both the PA and PE directions (Figure 6.4). This may be due to the reduced inter-fiber crosslinking that occurs as a result of PEO incorporation. As expected, the modulus of the CO scaffolds was drastically reduced after PEO removal (2.12 ± 0.45 MPa and 681.27 ± 219.72 kPa, PA and PE, respectively Figure 6.3, 6.4), whereas the modulus of the NA and AL scaffolds did not change significantly (Figure 6.3, 6.4).
Figure 6.3. Representative stress versus strain profiles for scaffolds following photocrosslinking up to 10% strain (A), lyophilization after PEO removal up to 10% strain (B), and hydration in PBS following PEO removal up to failure (C).
In general, the modulus of the AL scaffolds tested in the PA direction were greater than the NA scaffolds when tested in all conditions. Also, the modulus of all of the scaffolds was significantly reduced upon incubation in water (Figure 6.3, 6.4). This is potentially due to water adsorption of the gelatin component of the interpenetrating network that exists within the fibers. Furthermore, the % strain at break was increased to approximately 100% for all scaffolds when tested in the hydrated state, without significant differences between the groups (Figure 6.3). The elastic behavior of these scaffolds is attractive for many applications, including with cardiac tissue.

Figure 6.4. Moduli of PEO, NA, AL, and CO scaffolds tested in parallel (PA) and perpendicular (PE) fiber directions after crosslinking, lyophilization after PEO removal, and hydration in PBS following PEO removal. α denotes p<0.05 for post-wash dry scaffolds compared to after crosslinking and β denotes p<0.05 for hydrated scaffolds compared to post-wash dry scaffolds.

6.3.3 Neonatal Cardiomyocyte-Scaffold Interactions

As a model of a naturally aligned cell, neonatal cardiomyocytes were seeded onto thin (<100 μm) scaffolds, which were electrospun onto glass coverslips in order to confirm the capacity to influence cellular alignment with Acr-PGS/gelatin scaffolds. As expected, the cells maintained viability over the course of the 5-day study and appeared to interact with the fibers,
which is visualized with actin staining (Figure 6.4A). The alignment of the fibers and cells was determined by drawing a horizontal reference line across the image and determining the angle at which a given fiber or cell was with respect to the reference line. As expected, a Gaussian distribution of fiber angles was observed for the AL and CO scaffolds, with the greatest quantity of fibers being oriented perpendicular to the reference line (Figure 6.5B). However, this was not the case for the NA scaffolds, where as expected, the fibers displayed a random orientation (Figure 6.5B). Similar trends were observed upon calculation of the cellular alignment on the scaffolds, where the AL and CO scaffolds had highly aligned cells, whereas the NA scaffold did not (Figure 6.5C). Thus, Acr-PGS scaffolds have demonstrated the ability to influence cellular organization based on scaffold structure. This, along with the tunable mechanical properties of Acr-PGS scaffolds makes them attractive for use towards in vitro (e.g., improvement in cardiomyocyte electrical conduction) and in vivo (e.g., cardiac grafts) applications.
Figure 6.5. Neonatal cardiomyocyte interactions with TCPS, NA, AL, and CO scaffolds five days after seeding. Cells are stained with FITC-phalloidin for actin fiber visualization and DAPI, which also stains Acr-PGS/gelatin fibers (A). Histograms depicting fiber (B) and cellular (C) alignment. Scale bar = 100 μm.

6.3.4 In Vivo Cellular Infiltration

Electrospun scaffolds were implanted into dorsal subcutaneous pockets in rats. Samples were collected at 2, 3, and 4 weeks after implantation and processed using standard histological techniques. In general, there was no observed evidence of inflammation upon sample collection and samples appeared to be integrated with the host tissue. Complete cellular infiltration of composite scaffolds was observed as early as 2 weeks following implantation (Figure 6.6C). However, 13.4±7.4% and 16.3±10.8% of free (acellular) scaffold remained for NA and AL scaffolds at 2 weeks, respectively (Figure 6.6A, B). Similar observations were made when scaffolds were observed at three weeks post-implantation (Figure 6.5D-F), where 2.1±3.6% and 3.3±5.7% of free scaffold remained for NA and AL scaffolds, respectively (Figure 6.6D, E).
large deviation in cellular infiltration is due to complete cellular infiltration being observed for some, but not all of the samples at this time point. These differences may be due to slight variations in thicknesses of the samples prior to implantation. We have previously shown (Chapter 5) that scaffold thickness can impact in vivo degradation and cellular infiltration. Complete cellular infiltration was observed at 4 weeks for all groups. Toward this end, the increase in porosity associated with the CO scaffolds leads to an increase in cellular infiltration into the scaffolds at earlier time points (2 weeks), compared to NA and AL scaffolds. Also, there appears to be a trend, although not statistically significant, towards greater cell infiltration into the NA scaffolds at a given time point compared to the AL scaffolds.

Figure 6.6. Trichrome stained images depicting cellular infiltration into NA (A,D), AL (B,E), and CO (C,F) scaffolds at 2 (A, B, C) and 3 (D, E, F) weeks post-subcutaneous implantation. S denotes the scaffold region. Scale bar = 50 μm.
6.3.5 Matrix Elaboration and Organization

In order to evaluate the organization of matrix that developed within the scaffold following subcutaneous implantation, samples were collected 4 weeks post-implantation and sectioned along the fiber direction. Samples were collected at 4 weeks in order to obtain enough mature collagen for quantification. As a result of increased cellular infiltration at earlier time points, a greater quantity of collagen was evident in CO scaffolds (Figure 6.7C, F) compared to NA and AL scaffolds (Figure 6.7A, D and Figure 6.7B, E, respectively).

Figure 6.7. Picrosirius red stained images of NA (A, D), AL (B, E), and CO (C, F) scaffolds at 4 weeks post-implantation as viewed using brightfield (A-C) and polarized light microscopy (D-F). S indicates the scaffold region that was used for quantification. E indicates the edge of the scaffold. Scale bar = 50 µm.

The alignment of collagen fibers located within the scaffolds was quantified for the scaffolds (Figure 6.8). NA scaffolds had less collagen elaboration than AL and CO scaffolds (Figure 6.7D). However, the collagen that is present appears to be non-aligned (Figure 6.8). As expected, the collagen that was elaborated within the AL and CO scaffolds appears to be oriented in an aligned fashion (Figure 6.8).
The subcutaneous implant model was utilized in order to confirm the influence of scaffold architecture and porosity on cellular infiltration and matrix elaboration in vivo. Although it is well understood that the environment that exists subcutaneously is different than that of myocardium (35), we would expect to also observe alignment of the nascent collagen if these scaffolds were used as a cardiac graft in vivo. Previous work, described in Chapter 5, illustrated the capability of suturing these scaffolds onto the surface of the epicardium to be used as an infarct restraint (31). The tunable mechanical properties, degradation kinetics, and now scaffold architectures possible with Acr-PGS/gelatin scaffolds make them attractive candidates for cardiac graft fabrication in order to identify the optimal material properties for left ventricular restraints. Furthermore, use of Acr-PGS/gelatin scaffolds as left ventricular restraints could provide significant insight into the pathobiology that occurs post-MI and the mechanisms behind the reduction of left ventricular remodeling that occurs with use of left ventricular restraints.

Figure 6.8. Histogram representing the quantification of collagen fiber alignment within AL and CO scaffolds at 4 weeks post-implantation.
6.4 Conclusions

The objective of this work was to evaluate the impact of the fiber alignment and increased porosity of electrospun Acr-PGS/gelatin scaffolds on cellular infiltration and matrix elaboration. Three scaffolds were fabricated in order to accomplish this goal, with changes in fiber alignment and the introduction of a PEO sacrificial polymer population to introduce porosity. When the PEO component was removed, these scaffolds demonstrate an increase in porosity, yet retained their anisotropic nature. PEO component removal was verified visually with SEM, as well as through mass loss studies and evaluation of the mechanical properties of the scaffolds with uniaxial tensile testing after crosslinking and in the dry state after PEO removal and lyophilization. In an effort to mimic the hydrated nature experienced in vivo, mechanical properties were also evaluated following PEO removal and incubation in PBS. A significant decrease in the modulus of the scaffolds was observed due to water adsorption of the gelatin component of the interpenetrating network of Acr-PGS and gelatin that exists within the fibers. Furthermore, the moduli of the hydrated scaffolds tested in the PA and PE direction ranged between ~3-240 kPa and are thus in the range of properties desirable for soft tissue engineering. As expected, neonatal cardiomyocytes that were seeded onto the scaffolds maintained their viability up to 5 days and aligned along the surface of the AL and CO fibers. Scaffolds were also evaluated at 2 and 3 weeks following subcutaneous implantation. CO scaffolds were completely infiltrated at 2 weeks, whereas ~13% and ~16% of the NA and AL scaffolds, respectively remained free of cells. Similar trends were observed with only ~2% and ~3% of NA and AL scaffolds, respectively remained acellular at 3 weeks post-implantation. Scaffold samples were completely infiltrated at 4 weeks post-implantation and picrosirius red staining and polarized light microscopy was used to evaluate the collagen elaboration and orientation. An increase in the amount of collagen was observed for CO scaffolds compared to NA and AL scaffolds. Furthermore, as expected, alignment of the nascent collagen was observed for AL and CO scaffolds.
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Chapter 7

Synthesis and Characterization of Redox Initiated Hydrogels


7.1 Introduction

The goals of tissue engineering and regenerative medicine are to replace, repair, and/or regenerate tissues or organs (1). A variety of materials and scaffold processing techniques have been developed since the inception of these continually advancing fields in the late 1980s. Hydrogels, or three-dimensional crosslinked networks composed of water soluble monomers, have drawn particular attention due to their similarity in structure and composition to components of the native extracellular matrix (2, 3). The crosslinks in these networks can be formed through both physical (e.g., alginate (4)) and chemical (e.g., poly(vinyl alcohol) (5)) means. Chemical crosslinks are often formed through the use of free radical polymerization (2, 3).

In the tissue engineering community, photopolymerization, a type of free radical polymerization, is commonly used to prepare hydrogels for a variety of in vitro (5-7) and in vivo (8) applications. Although photopolymerization is advantageous due to its ability to spatially and temporally control gelation (i.e., insoluble network formation) (9), it is limited for in vivo applications to areas that can be exposed to light of the appropriate wavelength. In contrast, redox initiation can be used to inject monomers, such that gelation can occur in situ in areas where light penetration is not possible. For example, the bi-component redox initiators benzoyl peroxide and N,N-dimethyl-p-toluidine are clinically used with poly(methyl methacrylate) and
methyl methacrylate monomers for bone cement applications (10). Moreover, the water soluble bi-component initiation system of ammonium persulfate (APS) and N, N, N',N'-tetramethylethylenediamine (TEMED) has also been used to form hydrogels (11, 12), with the reaction kinetics of this system being dependent on the surrounding temperature and the concentration of the initiators (13).

Hyaluronic acid (HA) is an enzymatically degradable linear polysaccharide composed of D-glucuronic acid and N-acetyl-D-glucosamine. It is found in the native extracellular matrix and plays a role in several biological processes such as inflammation, wound repair, and cardiac morphogenesis (6, 14) and can be modified to introduce reactive methacrylate groups to form stable, crosslinked hydrogels through free radical polymerization mechanisms (6). The hydrogels can be modified to exhibit a range of mechanical properties (through macromer concentration) (6) or controlled degradation (7, 15). This work focuses on the characterization of methacrylated hyaluronic acid (MeHA) hydrogels, which are formed using the bi-component redox initiation system of APS and TEMED. Our goal is to optimize this system for **in situ** hydrogel formation.

### 7.2 Materials and Methodology

#### 7.2.1 Methacrylated Hyaluronic Acid Macromer Synthesis

All reagents were used as received from Sigma-Aldrich (St. Louis, MO) unless otherwise noted. Methacrylated hyaluronic acid (MeHA) was synthesized as previously described (6). Briefly, sodium hyaluronate (LifeCore, 74 kDa) was dissolved at 1 wt% in deionized water and reacted with varied amounts of methacrylic anhydride to influence the final macromer methacrylation at pH 8.0 on ice for 24 hours. The macromer was purified via dialysis (MW 6-8 kDa cutoff) against deionized water for 72 hours with the final product being obtained following lyophilization. $^1$H NMR (Bruker) was used to determine the % methacrylation.

#### 7.2.2 Bulk Hydrogel Network Mechanics and Swelling Characterization

In general, MeHA (2 or 4 wt%) was dissolved in phosphate buffered saline (PBS) with various concentrations of ammonium persulfate (APS, 5.0 or 12.5 mM, Sigma) and N,N,N',N'-tetramethylethylenediamine (TEMED) has also been used to form hydrogels (11, 12), with the reaction kinetics of this system being dependent on the surrounding temperature and the concentration of the initiators (13).
tetramethylethylenediamine (TEMED, 5.0 or 6.25 mM, Sigma). MeHA/APS and MeHA/TEMED solutions were loaded into different barrels of a dual barrel syringe and crosslinked by expulsion and mixing from the syringe. Compression testing was completed on hydrated samples (5 mm diameter, n=3) using a Dynamic Mechanical Analyzer (Q800 TA Instruments) at a strain rate of 10% min⁻¹. The modulus was determined as the slope between 10-20% strain (the linear region). Gelation onset (n=3) was quantified by monitoring the storage (G’) and loss (G”) moduli with time using an AR2000ex Rheometer (TA Instruments) at 37°C under 1% strain and 1 Hz in a cone and plate geometry (1°, 20 mm diameter).

The volumetric swelling ratio (Qᵥ) (n=3) was determined by swelling hydrogels in PBS for 48 hours to equilibrium and recording the wet weight and dry weight following lyophilization. Qᵥ can be determined using the following equations:

\[ Q_M = \text{wet weight/dry weight} \]  \hspace{1cm} 7.1

\[ Q_V = 1 + \left( \frac{\rho_P}{\rho_S} \right) \times (Q_M - 1) \]  \hspace{1cm} 7.2

where \( \rho_P \) is the density of the dry polymer (1.23 g cm⁻³) and \( \rho_S \) is the density of the solvent (1 g cm⁻³) (16). Additionally, Qᵥ can be related to the average molecular weight between crosslinks, \( M_c \), via a modified Flory-Rehner equation:

\[ Q_V = \left[ \left( \frac{\nu \times M_c}{V_1} \right) / V_1 \right] \times (0.5 - \chi) \]  \hspace{1cm} 7.3

where \( \nu \) is the specific volume of the dry polymer, \( V_1 \) is the molar volume of the solvent (18 mol cm⁻³), and \( \chi \) is the Flory polymer-solvent interaction parameter. In the literature, the value \( \chi \) has been estimated as 0.473 based on its similarity in structure to dextran, a well-studied polysaccharide (16). This can be further related to the effective crosslink density of the network, \( \nu_e \), using the equation:

\[ V_e = \frac{\rho_P}{M_c} \]  \hspace{1cm} 7.4
7.2.3 Hydrogel/Initiator Cytotoxicity

Cytotoxicity of the formed gels was evaluated by exposure to human mesenchymal stem cells (Lonza, 6,000 cells cm$^{-2}$) in a transwell format (n=3 per group). Hydrogel disks were sterilized upon exposure to germicidal ultraviolet light for 1 hour and placed into a transwell insert into a well 24 hours after seeding. Cell viability and proliferation was measured using the AlamarBlue™ (AB, Invitrogen) fluorescence assay. Growth media containing 10% (v/v) AB was added to each well. After a 4-hour incubation period, 100 μL aliquots (n=3/group) of the AB containing media was removed from each well for fluorescence measurement (530 nm excitation, 590 nm emission, Synergy HT, Biotek Winooski, VT). Blank control samples for each group (tissue culture plastic, MeHA High, and MeHA Low) in growth media without cells were also treated in order to remove any background fluorescence measurements. Samples were then fed with fresh growth media without AB. For continual assessment, the AB assay was completed every other day on the same population of cells up to day 3 after exposure to the hydrogels.

7.2.4 Hydrogel Degradation

The degradation (n=3 per time point) of the hydrogels in PBS at 37°C was monitored using an uronic acid assay (17) and 100 units of exogenous hyaluronidase per mL PBS was added at 20 weeks for complete degradation of the hydrogels. The PBS was changed at each time point. Additionally, the mechanics of the hydrogels under compression was assessed after incubation in PBS at 2, 4, and 8 weeks using the method described above.

7.2.5 Statistical Analysis

All data is presented as mean ± standard deviation. Comparisons using a Student’s t-test assuming unequal variances was used for data in which the variances between groups was not equal, otherwise, single factor ANOVA with Tukey’s post hoc test was used to determine statistical significance among groups with p < 0.05.
7.3 Results and Discussion

Free radical polymerization can be used to form hydrogels for a variety of tissue engineering applications. Although photopolymerization, a type of free radical polymerization, is frequently used due to its ability to spatially and temporally control gelation (9) as well as maintain cellular viability during in situ polymerization (5), it is limited for use with in vivo applications to areas that can be exposed to light. In these instances, redox initiation, another type of free radical polymerization, can be used to inject hydrogel precursor solutions to polymerize in situ. This polymerization rate is dependent on the surrounding temperature and initiator concentrations (11-13) and can be optimized for specific delivery approaches (e.g., delivery via a catheter).

7.3.1 Methacylated Hyaluronic Acid Synthesis and Network Formation

The methacrylated hyaluronic acid (MeHA) macromers were synthesized and purified following reaction with methacrylic anhydride at pH 8.0 for 24 hours, dialysis, and lyophilization. It is most likely that the free hydroxyl group on the C6 carbon is modified during this reaction (Figure 7.1A). $^1$H NMR confirmed the introduction of the methacrylate groups, as indicated by the peaks at 5.7 and 6.2 ppm (Figure 7.1C). The extent of methacrylate modification for the two macromers that were synthesized was found to be ~30% (MeHA Low) or ~60% (MeHA High) by calculating the area under the aforementioned peaks relative to the peak centered at 3.7 ppm, which corresponds to 10 protons on the HA backbone (Figure 7.1C). Therefore, the amount of methacrylic anhydride added during the synthesis directly impacted the amount of methacrylate group substitution on the HA backbone.

Hydrogels were formed from the MeHA macromer (2 or 4 wt%), APS (5 or 12.5 mM), and TEMED (5 or 6.25 mM). In this initiation scheme, TEMED behaves as an accelerator, increasing the polymerization rate compared to APS alone, which is also a thermal initiator (18). The radicals that are generated upon the combination of APS and TEMED subsequently attack the vinylic double bond of the methacrylate group to form the crosslinked network consisting of the HA backbone and poly(methyl methacrylate) kinetic chains (dashed lines, Figure 7.1B).
7.3.2 Hydrogel Mechanics, Swelling, and Reaction Kinetics

The formed hydrogel mechanical properties were first evaluated using compression testing to investigate the impact of macromer concentration, % methacrylation, and initiator concentration. The compressive modulus ranged from ~4 kPa to ~80 kPa for the eight groups that were tested. As expected, as the macromer concentration increased, the resulting hydrogels have an increase in modulus, for a constant initiator concentration (e.g., dark blue column compared to light blue column, Figure 7.2). Also, the compressive modulus significantly increases as the % methacrylation increases, for a constant initiator concentration (e.g., comparison of dark blue columns, Figure 7.2). This is due to the presence of a greater number of methacrylate functional groups available for crosslinking during propagation. In general, there is
an increase in the compressive modulus of the hydrogels as the initiator concentration is increased for a constant macromer concentration and % methacrylation (blue column compared to green column, Figure 7.2). This is especially true for the 4 wt% gels for both MeHA macromers. Based on this work, the 4 wt% MeHA macromer concentration was selected for any remaining studies due to its large difference in moduli for both MeHA macromers at both of the initiator concentrations that were tested.

**Figure 7.2.** Compressive modulus of MeHA gels formed using various macromer and initiator concentrations or % methacrylate substitution. A denotes APS, T denotes TEMED, # denotes p<0.05, whereas * denotes p<0.01 and ** denotes p<0.01 compared to MeHA Low (with same initiator concentration).

The volumetric swelling ratio for hydrogels formed from the two MeHA macromers (4 wt%) and the two different initiator combinations were calculated using Equation 7.2. In general, $Q_v$ decreased significantly as the % methacrylation increased when the initiator concentration remained constant (Figure 7.3). For example, $Q_v$ is ~37 for hydrogels formed from MeHA Low with 5 mM APS and 5 mM TEMED, where as it is only ~25 for hydrogels formed from MeHA High using the same initiator system. Moreover, there was a trend, although not-statistically significant, towards decreasing $Q_v$ as the initiator concentration is increased when the type of MeHA macromer is held constant.
The modified Flory-Rhener equations described in Equation 7.3 and Equation 7.4 above, describe an inverse relationship between $Q_V$ and $v_e$. This appears to correlate well with our observations for this redox initiated hydrogel system and the difference in compressive mechanics shown in Figure 7.2. As the initiator concentration is increased, there is an increase in the number of radicals that are generated during the initiation phase, resulting in a greater number of propagating species for a given time period. The outcome of this is a greater number of shorter crosslinks (i.e., smaller $M_c$) due to the associated diffusion limitations and reactive group availability. This is manifested by a reduced $Q_V$ value and higher modulus, such as observed for networks formed from the MeHA High macromer at the two different initiator concentrations.

![Graph showing volumetric swelling ratio for 4 wt% MeHA hydrogels formed using different initiator concentrations or % methacrylate substitutions. A denotes APS, T denotes TEMED, * denotes $p < 0.05$ compared to MeHA Low (with same initiator concentration).](image)

**Figure 7.3.** Volumetric swelling ratio for 4 wt% MeHA hydrogels formed using different initiator concentrations or % methacrylate substitutions. A denotes APS, T denotes TEMED, * denotes $p < 0.05$ compared to MeHA Low (with same initiator concentration).

The reaction kinetics for networks formed from the different MeHA macromers and initiator combinations was monitored using rheology. A representative example of the time sweep curve of the storage ($G'$) and loss ($G''$) moduli are shown in Figure 7.4A. The gelation onset time was determined as the point in time at which $G'>G''$ and is depicted by the arrow (Figure 7.4A). As expected from the compression testing results, a significant difference between...
G' (defined as the fourth consecutive point with <1% change) of the two hydrogels formed with identical initiator concentrations, as well as a significant difference between hydrogels formed from the same macromer but different initiator concentrations, were observed (Figure 7.4B).
Figure 7.4. Hydrogel mechanical properties and gelation time behavior. Representative MeHA High (5.0 mM APS and 5.0 mM TEMED) time sweep depicting gelation onset, as indicated by the arrow (A). Storage modulus (A) and gelation onset time (B) for various macromer and initiator combinations. A denotes APS, T denotes TEMED, * denotes p<0.01.
As expected, the gelation onset time decreased with increasing initiator concentration and ranged from ~2.5 to 4 minutes. Importantly, the gelation onset time was only dependent on the initiator concentration and not dependent on the MeHA macromer (i.e., MeHA High versus Low) used for hydrogel formation. Moreover, the hydrogel gelation was completed by 25 minutes for all of the groups.

7.3.3 Hydrogel Cytoxicity

Since our goal is to optimize our system for in situ gelation, we chose to assess cellular viability using a transwell apparatus as opposed to cellular encapsulation within our formed hydrogels. Human mesenchymal stem cells (hMSCs) were cultured in the presence of hydrogels prepared from the two different MeHA macromers using the different initiator concentrations and viability was assessed using the AlamarBlue™ assay. The AlamarBlue™ reagent interacts with the by-products of cellular metabolism and therefore the fluorescence measurement is related to cellular viability/proliferation. The viability of the hMSCs cultured in the presence of all of the formed hydrogels remained similar to the unexposed controls at Days 1 and 3 post-exposure (Figure 7.5).

**Figure 7.5.** hMSC metabolic activity/proliferation, as measured by fluorescence over 3 days using the AlamarBlue™ assay. hMSCs were cultured in the presence of hydrogels formed from the different MeHA macromers using different initiator concentrations.
7.3.4 Hydrogel Network Degradation

In order to evaluate the degradation behavior of these networks, hydrogels formed from both MeHA macromers using 5.0 mM APS and 5.0 mM TEMED initiator system were studied. The degradation of the hydrogels was monitored over 20 weeks prior to the addition of exogenous hyaluronidase, at which point the gels degraded completely within one week (Figure 7.6A). The %HA release was quantified using an assay to detect uronic acid, a by-product of HA degradation. In general, the MeHA Low hydrogels have a slight, although not significant, increase in the % HA released (Figure 7.6A). This is potentially due to the increased water content, as evidence by the swelling data that was previously described. However, hydrogels formed using both the MeHA High and Low macromers only demonstrated ~20% HA release over this 20-week period, without statistical differences between the groups (Figure 7.6A). As expected, upon introduction of hyaluronidase, hydrogels formed from both of the MeHA High and Low macromers degraded within 1 week.

The change in mechanical properties with time of the MeHA hydrogel networks was investigated by performing compression testing on gels which had been incubated in PBS at 37°C for 2, 4, and 8 weeks. In general, the MeHA Low hydrogel modulus only decreased by ~15% at the 8 week time point. In contrast, MeHA High hydrogels maintained their initial modulus of ~40 kPa up to 4 weeks of in vitro degradation. However, the modulus of the hydrogels dropped by almost 50% at its 8-week time point (Figure 7.6B). This is initially somewhat surprising due to the small amount (~20%) of mass loss, as was previously described (Figure 7.6A). However, since these hydrogels undergo bulk degradation throughout their scaffold it is possible that between the 4 and 8-week time points a large enough amount of the ester bonds were subject to degradation and were broken such that the mechanics of the network were affected. However, since a single HA polymer is bound at multiple locations throughout the network, all of these bonds would have to be broken in order for the HA to be released from the network and be detected with the uronic acid assay.
Figure 7.6. MeHA hydrogel degradation behavior. %HA release of MeHA hydrogels as assessed using the uronic acid (HA degradation product) assay. The dashed line represents the addition of 100 U/mL exogenous hyaluronidase enzyme (A). The compressive modulus of MeHA hydrogels incubated in PBS at 37°C with time (B).

7.4 Conclusions

In this work, two MeHA macromers of varying amounts of methacrylate substitution were synthesized and their network formation using different concentrations of the bi-component redox-initiation system of APS and TEMED were investigated. Compression testing was used to
confirm the impact of macromer concentration (2 or 4 wt%), % methacrylate group modification (~30 or ~60% by $^1$H NMR), and initiator concentration (5.0 or 12.5 mM APS and 5.0 or 6.25 mM TEMED) on the resulting network mechanical properties. In general, the compressive modulus of the hydrogels increased as the macromer concentration, methacrylate group substitution, or initiator concentration increased. As expected, $Q_v$ was increased as the macromer modification or initiator concentration was decreased. Furthermore, rheology confirmed an increase in $G'$ with increasing macromer methacrylate group modification as well as decreased gelation onset time with increasing initiator concentration. Moreover, hMSCs cultured in the presence of hydrogels prepared from the two different macromers using the two different initiator combinations remained viable and were indistinguishable from controls. Although there was some decrease in the mechanics of the hydrogels at 8 weeks of *in vitro* degradation, the mass loss profiles were not statistically different between hydrogels formed using the different macromers and 5.0 mM APS and 5.0 mM TEMED. Overall, we were successful in creating a tunable, redox-initiated system that can be used for *in situ* hydrogel formation.
References:


Chapter 8

Injectable Hydrogel Properties Influence Infarct Expansion and Extent of Post-Infarction Left Ventricular Remodeling in an Ovine Model


8.1 Introduction

Left ventricular (LV) remodeling caused by a myocardial infarction (MI) is responsible for almost 70% of the 5 million cases of heart failure that have occurred in the United States in recent years (1). Early infarct expansion or stretching has been associated with poor long-term prognosis (2-4) and has been identified as the mechanical phenomenon that initiates and sustains the process of adverse post-MI LV remodeling that leads to heart failure (5-10). Infarct expansion causes abnormal stress distribution in myocardial regions outside the infarction, especially in the adjacent borderzone (BZ) region, putting this region at a mechanical disadvantage. With time, increased regional stress is the impetus for several maladaptive biologic processes, such as myocyte apoptosis and matrix metalloproteinase activation that inherently alter the contractile properties of normally perfused myocardium (11, 12). Once initiated, these maladaptive processes lead to a heart failure phenotype that is difficult to reverse by medical or surgical means.

We have demonstrated that ventricular restraint early after MI reduces infarct expansion and limits long-term global LV remodeling in large animal infarction models (10, 13-16). In order to circumvent the surgical placement of restraining devices early post-MI, our group and others
have begun to explore the use of injectable materials to limit infarct expansion and normalize the regional stress distribution (17-26). Such an approach offers the potential for a non-invasive, catheter-based treatment that could be administered early post-MI to attenuate the remodeling process and prevent the development of heart failure. Despite the range of material types and properties that have been injected, there have been no studies that systematically investigate the importance of material properties (e.g., mechanics) on structural and functional outcomes. Such information will be extremely important in developing the optimal material for prevention of infarct expansion and the associated remodeling.

One molecule, hyaluronic acid (HA) is a linear polysaccharide that plays a role in several biological processes such as inflammation, wound repair, and cardiac morphogenesis (21, 22) and can be modified with methacrylate groups to form stable, crosslinked hydrogels through free radical polymerization mechanisms (21). Redox initiators (e.g., ammonium persulfate (APS) and N,N,N’,N’-tetramethylethylenediamine (TEMED)) can be used to facilitate injectable HA hydrogels via a dual-barrel syringe that mixes the solution at the injection site. These HA hydrogels can be modified to exhibit a range of mechanical properties (e.g., via extent of methacrylation) or for controlled degradation (23, 24).

This study focuses on assessment of injectable HA hydrogels to stiffen/thicken the infarct area to limit the associated borderzone expansion, limit LV dilation, and improve global function. Specifically, injectable formulations of HA hydrogels were designed that have similar degradation and gelation behavior to eliminate mass loss and tissue distribution as variables, yet have varied mechanical properties, both as hydrogels and upon injection. An established, clinically relevant in vivo ovine model of infarction was used to evaluate the associated salutary effect of hydrogel mechanical properties in the attenuation of post-LV remodeling.
8.2 Materials and Methodology

8.2.1 Methacrylated Hyaluronic Acid Macromer Synthesis

Methacrylated hyaluronic acid (MeHA) was synthesized as previously described (21). Based on previous work described in Chapter 7, two modified versions of MeHA were synthesized upon addition of different amounts of methacrylic anhydride. ¹H NMR was used to confirm the ~30% (MeHA Low) and ~60% (MeHA High) methacrylate modification. MeHA hydrogels were formed using the bi-component initiation system of ammonium persulfate (APS) and N,N,N',N'-tetramethylethlenediamine (TEMED). In general, MeHA (4 wt%) was dissolved in saline with various concentrations of APS (5.0, 12.5 mM, Sigma) and TEMED (5.0, 6.25 mM, Sigma) in PBS. MeHA/APS and MeHA/TEMED solutions were loaded into different barrels of a dual barrel syringe and crosslinked by expulsion and mixing from the syringe.

8.2.2 Characterization of In Situ Hydrogel Formation

The delivery and penetration of the injected hydrogel, formed using the different initiator concentrations, into normal (not infarcted) myocardial tissue was investigated using explanted ovine myocardial tissue from the LV apex (i.e., the intended infarct region). The dye methacryloxethyl thiocarbamoyl rhodamine B (125 μM) was added for visualization purposes. This dye is both macroscopically visible and crosslinks into the hydrogel. To confirm gelation within the tissue, 0.3 mL of the macromer/initiator solution with dye was injected into the LV apex. After 30 minutes post-injection, biopsy punches were used to remove 5 mm diameter disks of tissue or tissue containing hydrogel composite for compression testing (n=5 per group from multiple samples from two hearts). Compression testing was completed on hydrated samples (5 mm diameter, n=3) using a Dynamic Mechanical Analyzer (Q800 TA Instruments) at a strain rate of 10% min⁻¹. The modulus was determined as the slope between 10-20% strain (the linear region).

The tensile properties of cardiac tissue and cardiac tissue/hydrogel composites were also assessed. Samples (20 × 5 × 2 mm, n=4-8 per group from 4 different hearts) were removed from the mid-wall of the left ventricle of explanted (not infarcted) tissue in the circumferential and
longitudinal directions. Uniaxial testing was completed using an Instron 5848 Microtester with a 50 N load cell and equipped with custom grips and a phosphate buffered saline reservoir. A 0.05 N preload was applied for 60 seconds. Samples were preconditioned with 15 cycles of 0.005% of gauge length at 0.1% sec$^{-1}$ followed by a ramp to failure at 0.1% strain sec$^{-1}$. The modulus was determined as the slope between 10-15% strain (the linear region) of the resulting stress versus strain curve using a custom Matlab program.

8.2.3 In Vivo Infarction Model and Assessment

The animals used in this work received care in compliance with the protocols approved by the Institutional Animal Care and Use Committee at the University of Pennsylvania in accordance with the guidelines for humane care (National Institutes of Health Publication No. 85-23, revised 1996).

A clinically relevant ovine model of infarction and LV remodeling was used to assess the impact of the injected hydrogels (17). Twenty-one adult male Dorset sheep (35-40 kg) were anesthetized. The arterial, ventricular, and pulmonary artery pressures and electrocardiogram were monitored continuously throughout the procedure. A left thoracotomy was performed to expose the heart. Baseline echocardiographic and hemodynamic data were obtained. Infarction was induced via ligation of the left anterior descending and the second diagonal coronary artery in such a manner as to create an infarct the basal extent of which was 40% of the distance from the apex to the base of the heart. This procedure previously demonstrated the creation of a reproducible, moderately sized infarct involving ~20% of the LV mass at the anteroapex (25).

Animals were split into the following three cohorts: Infarct Control (n=9), MeHA High (n=7), and MeHA Low (n=5). Historical data of Normal (non-infarct) tissue thickness was used for comparisons. Cohorts receiving MeHA treatment received 20 injections of 0.3 mL macromer/initiator solution at 3 minutes post-mixing immediately following the echocardiograph at 30 minutes following infarction. The injection sites were uniformly distributed within the ischemic territory and located at a depth of approximately 2 mm into the mid-wall of the myocardium. An example is shown in Figure 8.1.
Echocardiographic data were collected and analyzed as previously described (17). Briefly, transapical epicardial real-time three-dimensional echocardiography was performed through the left thoracotomy using a Philips IE 33 platform with a 7 MHz ultrasound probe (Philips Medical Systems, Bothell, WA). Full volume 3D datasets were acquired. These were exported to a dedicated workstation for image manipulation and analysis using QLAB 3D Advanced Quantification software (Philips Medical Systems, Bothell, WA). The three dimensional image acquired was manipulated to display two orthogonally related long axis views, bisecting each other on the central long axis of the left ventricle. Ventricular volumes were obtained according to the software manufacturer's recommended method: in both end diastole (defined as the frame prior to closure of the mitral valve) and in end systole (defined as the frame prior to closure of the aortic valve), the basal and apical limits of the left ventricle are defined by manually placing reference points on the image in the two orthogonally related long axis views. The software then defines the interface between the endocardium and left ventricular cavity and thus the left ventricular envelope by inserting splines to connect the manually inserted reference points for each of these frames. The 3D image for each of these two time points may then be rotated about its long axis and thus the line defining the endocardial envelope of the left ventricle may be manually fine tuned to correct for interpolation error. Once these two frames are traced in this manner the remaining frames are traced in sequence by means of automated contour detection. The resulting 4-dimensional LV model is then automatically divided into the 17-segment model of...
the American Society of Echocardiography with the global and segmental volume-time curves being exported to Microsoft Excel. At each time point, global end-diastolic and end-systolic volumes were defined as the maximum and minimum LV cavity volumes, respectively. Global ejection fraction was defined as $\frac{(EDV-ESV)}{EDV}$. The length of the anterior apical wall motion abnormality (i.e. infarct length) was measured in the 2 dimensional (2D) apical 2 chamber view. A pulmonary artery catheter was used to measure cardiac output via the thermodilution method.

Echocardiographic and hemodynamic data were collected again at two weeks post-infarct, as well as at 8 weeks. Animals also underwent dobutamine (2.5 and 5.0 mg kg$^{-1}$ min$^{-1}$) stress echocardiographic testing at 8 weeks. Following these evaluations, animals were sacrificed, the hearts were harvested, and the infarct thickness was measured with a digital micrometer. Samples were also collected and fixed for histological analysis using hematoxylin and eosin staining, as well as Mason’s trichrome staining. One MeHA High treatment subject was sacrificed at 24 hours post-injection and processed for histology in order to evaluate gel distribution in vivo.

8.2.4 Statistical Analysis

Data is presented as mean ± standard deviation or mean ± S.E.M. as indicated in the respective figure caption. For hydrogel or tissue/hydrogel composite characterization experiments, differences between groups were assessed using the Student’s t-test. Changes in tissue dimensions as well as echocardiograph and LV function readings, were assessed using a one-way ANOVA with Tukey’s post-hoc evaluation. Echocardiographic and LV function readings were compared using a paired t-test for comparisons to baseline values for a respective group. For all comparisons, $p<0.05$ was considered to be statistically significant.

8.3 Results and Discussion

The LV remodeling that occurs post-MI is a complex process and increased understanding of this process, as well as the impact of various treatment paradigms is needed to develop valuable therapies to impact patient outcomes and welfare. LV remodeling evolves with
time post-MI to involve myocardium more remote from the infarct in a process known as borderzone expansion (1-4). The reduced wall thickness in the infarct region and a global change to a more spherical geometry correspond to increases in wall stress (1, 3, 4). These alterations increase the mechanical burden on the injured heart and initiate maladaptive biological processes that act together to produce heart failure (1, 3). Although recent data has demonstrated the potential of injectable materials to affect the post-MI LV remodeling (8, 10-14, 17-20), only a few groups (16, 17, 19) have evaluated the impact of acellular intramyocardial injections in large animal models of MI (e.g., swine or ovine). Furthermore, the influence of specific material properties on the associated LV remodeling response has not been explored experimentally and is not well understood. This is partially due to the difficulty in developing materials that can systematically investigate the influence of one property (e.g., mechanics) without altering other potentially confounding parameters (e.g., mass loss).

8.3.1 In Situ Hydrogel Distribution Characterization

In this study, we investigated HA hydrogels, where mechanical properties are modified through a simple alteration in the number of reactive methacrylate groups (i.e., MeHA Low versus MeHA High) on the MeHA macromer, which is easily changed during synthesis. The work in Chapter 7 described the impact of % methacrylate modification (i.e., MeHA Low vs. MeHA High) on the resulting hydrogel network properties. Briefly, the compressive modulus of the MeHA Low modulus ranged between ~7-25 kPa, while the compressive modulus of MeHA High ranged between ~40-70 kPa, depending on the initiator concentration.

The delivery and penetration of the hydrogel (with dye for visualization) was evaluated through injection into the apical region of explanted ovine left ventricle tissue. A greater distribution of the hydrogel in the tissue was observed with a lower initiator concentration and slower gelation time when compared to hydrogels formed with a higher initiator concentration and faster gelation (Figure 8.2A and 8.2B). However, gel distribution was not dependent on the MeHA formulation, as long as the initiator concentration was constant. Furthermore, due to the amount of time required to prepare the syringe, delivery with the higher initiator concentration (12.5 mM APS/6.25 mM TEMED) often led to an increase in viscosity and premature gelation within the
syringe such that complete injection of the entire content was not possible. Based on these results, the 5.0 mM APS and 5.0 mM TEMED initiator combination was selected for all remaining studies. As described in Chapter 8, this initiator combination led to gelation times of $4.49 \pm 0.4$ and $3.83 \pm 0.2$ min and gelation completion times of $23.2 \pm 0.6$ min for MeHA Low and $25.3 \pm 0.4$ for MeHA High. When the hydrogel was injected in vivo and assessed 24 hours post-MI (Figure 8.2C), histological staining demonstrates a clear integration of the gel within the tissue and distribution throughout cell layers (Figure 8.2C).

**Figure 8.2.** Explanted cardiac tissue with 4 wt% MeHA High (outlined with dashed line) gelled with either 5.0 mM APS/5.0 mM TEMED (A) or 12.5 mM APS/6.25 mM TEMED (B), showing differences in gel distribution based on initiator concentrations, scale bar = 10 mm. Representative hematoxylin and eosin stained image of the cardiac tissue at the apex of a MeHA High gel (labeled with G) treated infarct 24 hours post-injection, demonstrating integration with the tissue (C), scale bar = 100 μm.

### 8.3.2 Hydrogel Injection Impacts Mechanics of Cardiac Tissue

A finite element study simulation of the theoretical impact of injection of a material into the myocardium after MI illustrates the suspected stress reduction potential of intramyocardial stiffening. Furthermore, stiffer materials were shown to bear more of the load in the remote and borderzone regions, resulting in a decrease in stresses within these regions (28). Moreover, using a closed-loop lumped-parameter model of the ovine cardiovascular system, we have also
demonstrated that a reduction in compliance in the infarct area (i.e., infarct stiffening) should reduce dilation of the LV and improve EF (30). For this reason, specific formulations were investigated to form hydrogel/tissue composites that have similar (MeHA Low) and greater (MeHA High) moduli than native cardiac tissue.

Investigation of the mechanics of cardiac tissue prior to and after injection of the hydrogels revealed an increased modulus with hydrogel injection, dependent on the MeHA modification. A constant initiator system of 5.0 mM APS/5.0 mM TEMED initiator combination was selected to maximize material distribution at each injection site. The injection process leads to a series of hydrogel pockets that distribute from the injection point, which made it difficult to obtain uniform samples for composite material testing. Thus, compression was used to minimize non-uniformity of the samples and it was possible to core the samples that appeared uniform.

Under compression, the modulus of untreated cardiac tissue was 5.8 ± 1.5 kPa, whereas the bulk hydrogels had moduli of 7.7 ± 1.0 kPa and 43.0 ± 12.3 kPa for MeHA Low and MeHA High, respectively (Figure 8.3). The moduli of the tissue/hydrogel composites were greater than explanted cardiac tissue alone for the MeHA High group, but not for the MeHA Low group (Figure 8.3B). Specifically, there was no statistical difference in the modulus between the cardiac tissue and the composite with the MeHA Low gel; however, there was a statistically significant increase in modulus with the MeHA High composite when compared to normal cardiac tissue.
Cardiac tissue and tissue/hydrogel constructs also underwent uniaxial tensile testing in the longitudinal and circumferential directions since tensile testing is indicative of the type of loading that will be experienced *in vivo*. Similar trends were observed when the tissue/hydrogel composites underwent uniaxial tensile testing in both directions (Figure 8.4). However, due to the thickness of the wall, samples were isolated from the mid-wall, leading to variability in fiber angle,
which further contributed to the observed variations. Moreover, a lack of sample uniformity (i.e., amount of hydrogel within tissue composite) may also contribute to the observed variability between the groups that were tested. However, based on these and the aforementioned compressive testing results, it appears that the modulus of the tissue can be impacted by the injection of our hydrogel system, specifically MeHA High.

Figure 8.4. The tensile modulus of cardiac tissue and tissue/hydrogel composites in the circumferential and longitudinal directions. Data is presented as mean ± standard deviation.

8.3.3 Hydrogel Injection Maintains Normal Tissue Thickness and Reduces Infarct Size Depending on Modification.

Hydrogels were injected 30 minutes post-infarct into 20 injection sites in the apex and borderzone region and outcomes were assessed after 2 and 8-weeks, as well as with Dobutamine stress evaluation immediately prior to sacrifice. To limit animal mortality, we chose to intervene at 30 minutes post-infarction; this timing is likely faster than could be achieved clinically since the average time from onset of symptoms to hospital presentations averages from 2-6 hours (31, 32). However, recent reports have also demonstrated attenuation of LV remodeling with polymer injection several weeks after infarct (33, 34). Future work will evaluate the impact of MeHA treatment time and infarct reperfusion status on LV remodeling. This MI model involves a permanent occlusion, which is generally associated with a more profound remodeling stimulus

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(35, 36) compared to reperfused infarct models that have been used to evaluate the impact of other injectable materials with reperfusion (17, 29, 37).

Unfortunately, little is known regarding changes in infarct material properties with time and reperfusion status, factors that may affect the optimal properties of the injectable material. It was our intent to utilize our unique MeHA system to isolate one variable (e.g., injected hydrogel mechanics) in order to systematically evaluate the resulting impact on infarct expansion and remodeling. This work not only contributes to the development of injectable materials for therapy, but also provides further insight into the pathobiology that occurs post-MI.

Injection of the MeHA hydrogels 30 minutes after infarction (see Figure 8.1 for representative figure) led to maintenance of tissue thickness compared to control infarct samples, as evident upon sacrifice at 8 weeks (Figure 8.5A, 5B, and 5C). The regional thickness from the apex to the base was quantified and demonstrated significant differences in tissue thickness in the apex (7.02 and 6.54 mm) and basilar infarct (7.15 and 6.96 mm) regions for MeHA High and MeHA Low hydrogel injections, respectively, compared to control infarct (2.13 and 4.89 mm) apex and basilar infarct regions, respectively (Figure 8.5D).
Figure 8.5. Hydrogel treatment influences myocardial wall thickness. Representative samples of control infarct (A), MeHA High treatment (B), and MeHA Low treatment (C) at 8 weeks post-MI and hydrogel injection. Quantified regional thicknesses at 8 weeks (D). Data is presented as mean ± standard error of the mean and * denotes p<0.05 compared to all other groups in the same region.

This prevention of tissue thinning is also evident upon examination of the representative histological images in Figure 8.6. Magnified images of samples with MeHA High (Figure 8.6D) and MeHA Low (Figure 8.6E) treatment depict that the gels are present and maintain their integration with the tissue at 8 weeks. Thus, the stability of the materials indicates that the primary difference between the two groups was the mechanical properties of the hydrogel. Furthermore, the cellular response to MeHA is very limited, with no evidence of increased macrophage or myofibroblast infiltration. The change in infarct expansion and functional
properties appear to relate to the properties of the injectate rather than a biologic response to the material.

**Figure 8.6.** Histological images at 8 weeks post-MI and injection. Representative hematoxylin and eosin stained samples of control infarct (A), MeHA High (B,D), and MeHA Low (C,E) treatment where the gel (labeled with G) stains purple. Scale bar = 1 mm (A-C) or 100 μm (D,E).

The length of the anteroapical wall motion abnormality immediately after coronary occlusion and before injection was similar in all groups, indicating that the stimulus for remodeling (i.e., initial infarct size) was comparable between the three groups (Figure 8.7A). However, the infarct area at the time of sacrifice was reduced with MeHA treatment (23.9% MeHA High, p<0.05 and 26.4% MeHA Low) compared to the control infarct (28.6%) indicating that the treatment groups experienced less infarct expansion during the 8 week follow-up period (Figure 8.7A). The non-statistically significant trend toward smaller lengths of the anteroapical wall motion abnormality in the MeHA High group lend support to this conclusion (Table 8.1). Therefore, although infarct thicknesses increased in both treatment groups relative to control, there was significantly less infarct expansion and reduced LV remodeling only with the MeHA High
formulation. This result suggests that the properties of the injected material (i.e., increased stiffness) may be of greater importance in stress reduction than infarct stiffening alone.
Figure 8.7. Quantified infarct dimensions, *in vivo* volume, and functional metrics for control infarct, MeHA High treatment, and MeHA Low treatment for initial infarct length and infarct area at 8 weeks (A), for normalized end diastolic volume and normalized end systolic volume (B), and for cardiac output and ejection fraction (C). Data is presented as mean ± standard error of the mean and ** denotes *p*<0.01 versus infarct control and # denotes *p*<0.05 versus respective baseline value.
8.3.4 Hydrogel Injection Attenuates LV Dilation and Improves Cardiac Function Depending on Hydrogel Mechanics

Real-time 3D echocardiography was used to assess the LV dimensions and cardiac function for each animal prior to and immediately after (i.e., baseline) infarction. The normalized end diastolic volume (NEDV) and normalized end systolic volume (NESV) increased for all groups after 2 weeks, with the MeHA High treatment group tending to have the smallest changes in volume (Table 8.1). A similar trend was observed after 8 weeks, with the MeHA High treatment group again demonstrating an improvement in NEDV (1.7, Figure 8.7B) and NESV (1.9, Figure 8.7B) compared to the infarct control (2.1 and 2.4, respectively) and the MeHA Low treatment group (2.1 and 2.5, respectively). This improvement was even more dramatic upon stress testing with Dobutamine (2.5 and 5.0 mg/kg/min), which was conducted prior to sacrifice (Table 8.1). These observations are important since ESV is indicative of adverse effects post-MI (38).

LV function was assessed through monitoring CO and EF throughout the study. The cardiac output (CO) was reduced for all groups at 2 and 8 weeks following MI, although this difference was only statistically different from the baseline for the control infarct group (Figure 8.7C and Table 8.1). As expected, a reduction in the ejection fraction (EF) at 2 and 8 weeks was also observed for all groups relative to their respective baseline values (Figure 8.7C and Table 8.1). Stress testing prior to sacrifice demonstrated a greater improvement in EF of the MeHA High treatment group (Table 8.1).

The goal of this study was to investigate any salutary effects associated with differences in the mechanics of the injectable hydrogels developed to limit infarct expansion and suppress the LV remodeling response that occurs post-MI. Our findings clearly indicate that the higher modulus (MeHA High) hydrogel treatment group demonstrated less infarct expansion and reduced LV dilation, as well as improved function compared to our lower modulus hydrogel and infarct control groups. In agreement with the simulations described by Wall and colleagues (28), we believe that the higher modulus MeHA High hydrogel is better able to stabilize the myocardium and reduce wall stresses compared to the lower MeHA Low treatment group. Since,
the modulus of the tissue/MeHA Low composite group is similar to that of the excised cardiac tissue, it is likely that these values are closer to the passive material properties of the myocardium during diastole, whereas the modulus of tissue/MeHA High composite is likely closer to passive myocardial material properties at end systole. Since the increase in wall stress during systole is likely driving the maladaptive remodeling process, the tissue treated with MeHA High may be capable of normalizing myocardial stress distribution than tissue treated with MeHA Low.

To our knowledge, this study is the first to evaluate the impact in vivo of the effect of injectable material properties on the post-MI LV remodeling response. This provides fundamental information towards the development of hydrogels for treatment of LV remodeling. However, further work to determine the impact of hydrogel degradation (i.e., changes in material properties with time) is needed. Moreover, the timing of therapy (e.g., acute versus chronic), mode of delivery (e.g., via a catheter), and possible encapsulation of cells or drugs should be investigated in order to develop an optimal system for clinical use in the treatment of MI.
Table 8.1. *In vivo* effects of MeHA treatment on infarct size, geometry, and function. Data is plotted as the mean ± standard error of the mean. *a* denotes *p*<0.01 compared to control infarct, *b* denotes *p*<0.05 compared to respective baseline value, DoB denotes dobutamine (mg/kg).

<table>
<thead>
<tr>
<th>Metric</th>
<th>Control Infarct</th>
<th>MeHA High</th>
<th>MeHA Low</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><em>n</em></td>
<td>9</td>
<td>6</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>40.6 ± 0.7</td>
<td>40.8 ± 0.7</td>
<td>39.4 ± 1.3</td>
</tr>
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<td>Infarct Area</td>
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<td>23.87 ± 0.93<em>a</em></td>
<td>26.42 ± 1.56</td>
</tr>
<tr>
<td>Infarct Length</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Post-MI</td>
<td>7.41 ± 0.23</td>
<td>7.37 ± 0.16</td>
<td>7.34 ± 0.10</td>
</tr>
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<td>2 week</td>
<td>8.28 ± 0.39</td>
<td>8.04 ± 0.12</td>
<td>8.26 ± 0.14</td>
</tr>
<tr>
<td>8 week</td>
<td>8.91 ± 0.55</td>
<td>8.47 ± 0.16</td>
<td>8.98 ± 0.15</td>
</tr>
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<td>NEDV</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Post-MI</td>
<td>1.30 ± 0.08</td>
<td>1.32 ± 0.08</td>
<td>1.50 ± 0.13</td>
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<tr>
<td>2 week</td>
<td>1.76 ± 0.22</td>
<td>1.63 ± 0.12</td>
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<td>2.06 ± 0.20</td>
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<td>2.08 ± 0.25</td>
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<td>1.41 ± 0.15</td>
<td>1.22 ± 0.07</td>
<td>1.58 ± 0.29</td>
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<tr>
<td>DoB 5.0</td>
<td>1.19 ± 0.13</td>
<td>0.90 ± 0.07</td>
<td>1.20 ± 0.28</td>
</tr>
<tr>
<td>NESV</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Post-MI</td>
<td>1.38 ± 0.08</td>
<td>1.45 ± 0.08</td>
<td>1.66 ± 0.18</td>
</tr>
<tr>
<td>2 week</td>
<td>2.10 ± 0.30</td>
<td>1.90 ± 0.12</td>
<td>2.22 ± 0.40</td>
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<td>8 week</td>
<td>2.43 ± 0.29</td>
<td>2.00 ± 0.16</td>
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<td>1.32 ± 0.10</td>
<td>1.88 ± 0.44</td>
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<td>1.38 ± 0.18</td>
<td>0.95 ± 0.10</td>
<td>1.50 ± 0.43</td>
</tr>
<tr>
<td>EDV</td>
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<td>51.64 ± 2.27</td>
<td>56.97 ± 2.92</td>
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<tr>
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<td>71.73 ± 3.81</td>
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<td>2 week</td>
<td>88.08 ± 7.99<em>b</em></td>
<td>91.23 ± 5.22<em>b</em></td>
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<td>8 week</td>
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<td>95.78 ± 6.50<em>b</em></td>
<td>106.08 ± 8.84<em>b</em></td>
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<tr>
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<td>71.53 ± 7.63</td>
<td>69.75 ± 5.64</td>
<td>79.5 ± 11.68</td>
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<td>DoB 5.0</td>
<td>60.46 ± 6.29</td>
<td>51.48 ± 3.73</td>
<td>60.26 ± 10.97</td>
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<tr>
<td>ESV</td>
<td></td>
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<td></td>
</tr>
<tr>
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<td>34.17 ± 1.58</td>
<td>30.20 ± 1.36</td>
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<tr>
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<td>47.42 ± 2.64</td>
<td>46.50 ± 2.65</td>
</tr>
<tr>
<td>2 week</td>
<td>63.38 ± 6.67<em>b</em></td>
<td>64.22 ± 3.77<em>b</em></td>
<td>65.94 ± 9.98<em>b</em></td>
</tr>
<tr>
<td>8 week</td>
<td>74.03 ± 6.38<em>b</em></td>
<td>67.18 ± 4.64<em>b</em></td>
<td>75.32 ± 8.72<em>b</em></td>
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<tr>
<td>DoB 2.5</td>
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<td>44.98 ± 3.80</td>
<td>55.82 ± 11.00</td>
</tr>
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<td>33.13 ± 3.17</td>
<td>43.64 ± 11.24</td>
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<td>Cardiac Output</td>
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<tr>
<td>Baseline</td>
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<td>4.32 ± 0.26</td>
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<td>3.18 ± 0.26</td>
<td>3.30 ± 0.33</td>
</tr>
<tr>
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<td>4.18 ± 0.75</td>
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<td>4.38 ± 0.35</td>
<td>5.02 ± 1.08</td>
</tr>
<tr>
<td>DoB 5.0</td>
<td>4.92 ± 0.61</td>
<td>5.15 ± 0.42</td>
<td>5.14 ± 0.62</td>
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<td>Ejection Fraction</td>
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<td>41.22 ± 0.94</td>
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<td>33.80 ± 1.69</td>
<td>35.76 ± 1.67</td>
</tr>
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<td>2 week</td>
<td>28.39 ± 1.41<em>b</em></td>
<td>29.40 ± 0.95<em>b</em></td>
<td>29.18 ± 3.37<em>b</em></td>
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<td>8 week</td>
<td>28.65 ± 0.99<em>b</em></td>
<td>29.67 ± 1.21<em>b</em></td>
<td>29.60 ± 2.67<em>b</em></td>
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<td>35.33 ± 1.97</td>
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<td>DoB 5.0</td>
<td>31.23 ± 1.29</td>
<td>35.97 ± 2.00</td>
<td>30.26 ± 4.31</td>
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</table>
8.4 Conclusions

This work focuses on the investigation of injectable hyaluronic acid (MeHA) hydrogels that have tunable mechanics and gelation behavior. Specifically, two MeHA formulations that exhibit similar degradation and tissue distribution upon injection, yet have differential moduli (~8 versus ~43 kPa) were injected into a clinically relevant ovine MI model to evaluate the associated salutary effect of intramyocardial hydrogel injection on the remodeling response based on hydrogel mechanics. Treatment with both hydrogels significantly increased the wall thickness in the apex and basilar infarct regions compared to the control infarct. However, only the higher modulus (MeHA High) treatment group had a statistically smaller infarct area compared to the control infarct group. Moreover, reductions in normalized end diastolic and end systolic volumes were observed for the MeHA High group. This group also tended to have better functional outcomes (cardiac output and ejection fraction) compared to the low modulus (MeHA Low) and control infarct groups. This study provides fundamental information towards the rational design of therapeutic materials for treatment of MI.
References:


Chapter 9

Summary, Limitations, and Future Directions

9.1 Summary

The left ventricular (LV) remodeling response that occurs following myocardial infarction (MI) is a maladaptive process that often contributes to the onset of congestive heart failure. The limited success and availability of current clinical care has inspired the development of alternative treatments. Chapter 1 outlined ongoing strategies in cardiac tissue engineering that are aimed at the development of novel therapies to address this unmet need. Although the use of cells and growth factors has demonstrated some beneficial effects in vivo, there are several concerning issues associated with their use, such as a lack of a functional cell source and poor cellular engraftment. Therefore, acellular approaches, such as the use of left ventricular restraints and intramyocardial hydrogel injections, are attractive treatment options to reduce the remodeling response that occurs post-MI. However, the mechanisms behind their benefits still remain poorly understood. With that in mind, it was the goal of this thesis to develop biomaterials, such as fibrous scaffolds and injectable hydrogels with tunable properties (e.g., mechanics), for each of these approaches, in hopes that their use in vivo could not only attenuate this remodeling process, but also lead to some insight as to the optimal biomaterial properties and the mechanism behind their benefit. The general material class investigated is radically polymerized and crosslinked biomaterials, an overview of which is covered in Chapter 3.

In order to accomplish this goal, we first developed a collection of biodegradable elastomers with mechanical properties applicable for a wide range of soft tissues described in Chapter 4. Specifically, we modified the synthetic scheme of the biodegradable elastomer poly(glycerol sebacate) (PGS) to add the reactive acrylate group (Acr-PGS) in order to utilize free radical polymerization for crosslinking, as opposed to the high temperature and long time under vacuum traditionally required to cure PGS. The structure-property relationships of the macromers and resulting networks were elucidated through changing the molecular weight of the...
condensation product and % acrylation during synthesis. In general, the Young’s modulus increased with % acrylation for a constant molecular weight, and and the % strain at break increased with increasing molecular weight. One macromer, with a Young’s modulus within the range of that of soft tissues (~150 kPa) was further characterized for in vitro and in vivo degradation, as well as biocompatibility. Samples lost ~33% and 37% for in vitro and in vivo mass loss, respectively at 8 weeks. Furthermore, there was no evidence of necrosis or inflammation of the adjacent tissue for bulk samples that were crosslinked using photopolymerization or redox initiation for in situ crosslinking when implanted subcutaneously.

In Chapter 5, electrospinning was utilized to fabricate non-aligned scaffolds from three candidate macromers with varying bulk properties (i.e., mechanics and degradation) from the aforementioned collection of biodegradable elastomers. Gelatin was selected as the optimal carrier polymer to facilitate fiber formation of the relatively low molecular weight Acr-PGS macromers, due to improved cell attachment compared to poly(ethylene oxide) (PEO). Similar trends in mechanical properties were observed for the bulk polymers and scaffolds after hydration, in terms of the direct relationship between % acrylation and modulus. The % acrylation also dictated the rate of degradation for the samples, with the higher % acrylation leading to slower degradation. Cell adhesion and proliferation on the scaffolds was evaluated using human mesenchymal stem cells (hMSCs) and was indistinguishable from control samples. Finally, the scaffolds were sutured onto the epicardial surface in a rat model in order to evaluate the biocompatibility. The scaffolds could readily be sutured to the surface of the heart and demonstrated a dependence of sample thickness on degradation. Thin samples (~150 μm) degraded entirely within 2 weeks and an increase in the inflammatory response was observed with increasing amounts of macromer acrylation. Thicker (~ 300 μm) samples of the high % acrylation macromer were collected at 4 weeks post-implantation and limited cellular infiltration into the scaffold was observed. This motivated further work to control cellular interactions such as alignment and infiltration into the scaffolds.

In order to better mimic the anisotropic nature of the myocardium and to evaluate the impact of scaffold structure on nascent collagen formation in vivo, scaffolds consisting of
randomly oriented fibers (NA) and aligned fibers (AL) were fabricated in Chapter 6. Moreover, in an effort to improve the cellular infiltration throughout the depth of the scaffold, Chapter 6 also focused on increasing the porosity of the scaffolds. To accomplish this, a composite scaffold (CO) consisting of stable Acr-PGS/gelatin fibers and sacrificial PEO fibers were simultaneously electrospun from two polymer jets onto one mandrel. Porosity of the CO scaffolds increased after photocrosslinking and subsequent submersion into aqueous medium to dissolve the PEO component from within the scaffold. PEO removal was verified visually using SEM as well as through uniaxial tensile testing of samples following crosslinking and incubation in aqueous medium for 18 hours and lyophilization. The mechanical properties of scaffolds upon hydration ranged from ~3-240 kPa (for both parallel and transverse to the fiber directions) and are thus in the range suitable for the engineering of soft tissues, such as myocardium. As an example of a fiber aligned cell, neonatal cardiomyocytes were used to verify the capacity of the AL and CO scaffolds to induce cellular alignment. Cellular infiltration into the scaffolds at 2, 3, and 4 weeks was evaluated following subcutaneous implantation into the dorsal pocket of a rat model. CO scaffolds were completely infiltrated at 2 weeks, whereas ~13% and 16% of NA and AL scaffolds, respectively, remained free of cells. A similar trend was observed at 3 weeks post-implantation with ~2% and 3% of NA and AL scaffolds, respectively remaining cell-free. All scaffolds were fully infiltrated within 4 weeks and picrosirius red staining and polarized light microscopy was used to evaluate collagen formation and orientation. The greatest amount of collagen elaboration was observed in CO scaffolds. Furthermore, the collagen within the AL and CO scaffolds was aligned, whereas the collagen found within the NA scaffolds was not.

In a different biomaterial strategy, Chapter 7 focused on the synthesis and characterization of redox initiated hydrogels based on hyaluronic acid for injectable tissue engineering applications. The natural, enzymatically degradable polymer hyaluronic acid (HA) was reacted with varying amounts of methacrylic anhydride to form reactive macromers (MeHA) capable of crosslinking using free radical polymerization. Specifically, two macromers with ~30% (MeHA Low) or ~60% methacrylation and their network formation using the bi-component redox initiation scheme of ammonium persulfate (APS) and N, N, N', N'-tetramethylethylenediamine
(TEMED) were characterized. Compression testing of the hydrogels demonstrated an increase in the modulus with increasing macromer concentration (2 or 4 wt%), % methacrylation (30% or 60%) and initiator concentration (5.0 or 12.5 mM APS and 5.0 or 6.25 mM TEMED). Moreover, rheology was used to confirm the increase in storage modulus and decrease in gelation time observed with increasing initiator concentration. hMSCs cultured in the presence of the hydrogels remained viable and were indistinguishable from controls, indicating a lack of toxicity to the gels. Although there was decrease in the modulus of the hydrogels with degradation at 8 weeks, the mass loss profiles were not statistically different between hydrogels polymerized using MeHA High or MeHA Low and 5.0 mM APS and 5.0 mM TEMED.

In Chapter 8, the influence of the MeHA hydrogel material properties, specifically the mechanics on infarct expansion and LV remodeling was evaluated in vivo in an ovine model of infarction. Based on the distribution profiles of the hydrogels upon injection into explanted myocardium, the 5.0 mM APS and 5.0 mM TEMED initiator concentrations were used for all remaining studies. The ability of the hydrogels to impact the mechanical properties of explanted myocardial tissue was evaluated under compression and uniaxial tensile testing in the circumferential and longitudinal directions. As expected, hydrogel/tissue composites formed using the MeHA Low macromer (~8 kPa) were not statistically different in modulus than either cardiac tissue alone (~6 kPa) or the MeHA Low bulk hydrogel (~8 kPa). However, hydrogel/tissue composites formed using the MeHA High macromer (~38 kPa) were statistically different than cardiac tissue alone (~6 kPa), but not the MeHA High bulk hydrogel (~43 kPa). Treatment with both hydrogels significantly increased the wall thickness in the apex and basilar infarct regions of the heart compared to the control infarct. However, only treatment with MeHA High significantly decreased the infarct area compared to the control infarct. Moreover, reductions in normalized end diastolic volume and end systolic volume and improvement in cardiac function were observed with MeHA High treatment compared to MeHA Low treatment or the control infarct group.
9.2 Limitations and Future Directions

9.2.1 Specific Aim 1: To synthesize and characterize a tunable collection of photopolymerizable and biodegradable elastomers.

9.2.1.1 Limitations

The polycondensation reaction is very difficult to control and leads to very disperse condensation products, thus making it problematic to obtain condensation products of the same molecular weight and polydispersity between batches. In order to avoid as much variability as possible, all macromers were prepared using the same batch of prepolymer for a given set of experiments. Furthermore, due to the complexity of the $^1$H NMR spectra, it is difficult to quantify the exact quantity of free hydroxy groups that remain at the conclusion of the condensation reaction for acrylate modification. Therefore, for all remaining studies, the assumption that approximately one third of the initial hydroxy groups remain after the condensation reaction was used to determine the quantity of acryloyl chloride to add during the acrylation reaction. Although these limitations do not influence the work presented, they should be considered in the future when synthesizing these materials. Also, only one injectable formulation was investigated and this needs to be further characterized if the elastomers are to be used for injectable material applications in the future.

9.2.1.2 Future Work

In order to fully evaluate the structure-property relationships that exist within this collection of materials, the effect of condensation product branching on the resulting network material properties (i.e., mechanics and degradation) needs to be evaluated. The amount of branching on the condensation product likely increases with reaction time and molecular weight due to the secondary hydroxyl group on the glycerol monomer. This could be evaluated through incorporation of 1,3 propanediol, which has the same chemical structure as glycerol, except for the secondary hydroxyl group, during the condensation reaction. Macromers synthesized with the same molecular weight and % acrylation could be synthesized with variable ratios of 1,3 propanediol to glycerol could lead to a wider range of network properties. Also, the properties
obtained with these biodegradable polymers could be expanded further to apply to other applications than those presented here.

9.2.2 Specific Aim 2: To process the biodegradable elastomers into fibrous scaffolds using photopolymerization and to investigate in vitro and in vivo cellular interactions based on material and structure.

9.2.2.1 Limitations

During uniaxial mechanical testing, several of the scaffolds broke at the clamp, thus indicating a premature failure, and most likely contributing to the variability in the % strain at break observed in Chapter 5. Use of dog bone-shaped scaffolds may reduce this variability and demonstrate a trend similar to that obtained for bulk samples. Moreover, the reaction conversion during photopolymerization of the scaffolds is not possible to determine using the same method described for the bulk samples in Chapter 4 due to the overlap of the acrylate peak with the chemical groups in the gelatin component. However, reaction characterization carried out with a similar reactive macromer that was electrospun with PEO as the carrier polymer exhibited a high level of conversion (1). Therefore, we suspect to observe similar results with our scaffolds. This is also supported by the maintenance of the fibrous architecture of the scaffold upon submersion into aqueous medium for in vitro degradation (as shown in Chapter 5).

In Chapter 5, a possible influence of scaffold mechanics on the host response was observed when samples were implanted onto the epicardial surface, yet this observation was not investigated further. However, Chapter 6 focused on evaluating cellular infiltration, as well as matrix elaboration and organization into three scaffolds (NA, AL, and CO) that were formed using the same macromer. The mechanics of the CO scaffolds were drastically decreased upon removal of the sacrificial PEO fibers. In order to fully elucidate the role of mechanics, additional macromers would have to have been synthesized, such that the CO modulus is more similar to that of the AL scaffold. Moreover, the scaffolds ranged in thickness (NA 0.2-0.6 mm, AL 0.2-0.4 mm, and CO 0.88-1.15 mm), which may have contributed to some of the variability in cell infiltration observed in vivo. Fabrication of scaffolds with the same thicknesses would have been
difficult to predict since there is some loss of material to the surroundings during fabrication. The same initial mass of macromer was used in each scaffold with the hope that this mass loss would be similar for all scaffolds. Furthermore, there was some variation in thickness of the NA scaffold due to the build-up of the scaffold occurring regionally during collection on a flat plate as opposed to a rotating mandrel. In order to avoid this, electrospinning onto the mandrel rotating at a slow speed was investigated as an alternative collection apparatus. However, some degree of alignment was observed (compared to the flat plate), even at the lowest operational speed of rotation possible. Also, the anisotropy ratio for CO scaffolds in the hydrated state (~6.9) was much greater than that reported for non-infarcted myocardium (~2.4 for rat LV as reported in (2)). For future studies, the alignment of the fibers could be tuned to better mimic this anisotropic ratio through adjustment of the rotational speed of the mandrel.

Some limitations also exist with determination of the matrix orientation following subcutaneous implantation. First, although there was an increase in the amount of collagen observed within the CO scaffolds, relative low amounts of collagen for quantification existed compared to other studies in the literature (3, 4). However, isolation of the scaffolds at time points later than 4 weeks, when a greater amount of collagen would have been elaborated, would have been quite difficult, since the scaffold would have been completely infiltrated and difficult to identify. Use of a slower degrading macromer for these studies may have been more useful, such that longer time points could be collected. Also, studies in the literature (3, 4) that have used polarized light microscopy to quantify collagen fiber alignment following in vitro culture have sectioned their samples in the same plane as the fibers. Unfortunately, this was not possible for samples collected in vivo due to the fact that it would be difficult to ensure that a given image was generated from a section within the depth of the scaffold as opposed to the fibrous capsule. Thus, samples were sectioned in the cross-section of the sample, along the length of the fibers.

Finally, although these scaffolds were designed for use as an LV restraint post-MI, they were not ultimately tested for this application after the re-design to improve tissue organization and infiltration in Chapter 6. This is in part due to the difficulty associated with selecting an appropriate animal model for in vivo application post-infarct. The Wagner group has evaluated
the use of a similar but macroporous biodegradable poly(ester urethane urea) elastomer as an LV restraint in vivo in a rat model and saw a beneficial response (5). However, using sonomicrometry analysis and biaxial mechanical testing, the Holmes group (6) has recently demonstrated a difference in the mechanical properties in the rat model compared to studies in the literature on models of infarction in large animals, such as swine (7, 8). Specifically, infarcts in a rat model become isotropic with time (6), whereas the degree and direction of the anisotropy ratio has been shown to change with time in an ovine model of infarction (7). Although the rat model is beneficial due to the small size of scaffold necessary for epicardial placement, a high sample number (~100) would be necessary due to the high mortality rate (~35% (6)) and in order to have enough samples to complete a full characterization, which is described in the future work below. Although large animal models, such as swine or ovine, may better mimic human infarcts, they require the use of larger sizes of scaffolds, which may introduce further variability, since it is unlikely that one electrospun scaffold would be sufficient for a complete evaluation. Furthermore, mechanical testing on swine and ovine myocardium is difficult, due to the thickness of the wall. Although less is known about the mechanical properties of rabbit hearts post-infarct, the future work described below will be described using this model with hopes that the number of samples will be reduced due to the possibility of mechanical testing and biochemistry samples being completed using the same specimen, instead of two specimens, as would be necessary if a rat model was used.

9.2.2.2 Future Work

For future studies, an initial assessment of scaffold structure (i.e., isotropic NA versus anisotropic AL) and porosity (anisotropic AL versus anisotropic CO) would first be completed in a model of myocardial infarction. Scaffolds (n=3-6 per group) consisting of the same thickness (300-500 μm) and with mechanics on the same order of magnitude (~150 kPa) would be sutured onto the surface of the epicardium one-week post-infarction. AL and CO scaffolds would also be evaluated by placement of the scaffolds with the direction of the fibers in both the circumferential and longitudinal directions of the heart. LV function (i.e., cardiac output, max dp/dt, min dp/dt) and echocardiography (i.e., LV end diastolic volume, end systolic volume) would be recorded at
baseline, following infarct, prior to and following scaffold placement, as well as 2, 4, and 6 weeks post-implantation, at which point specimens would be sacrificed and samples LV regional wall thickness from the apex to the base would be recorded. Samples would also be collected for mechanical testing, histological examination, and biochemistry evaluation.

In order to evaluate the mechanical properties of the tissue during remodeling, a sample of the LV will be removed to perform bi-axial mechanical testing. A second sample would be removed for a hydroxyproline assay in order to quantify the collagen content within the specimen. The collagen crosslink concentration would be quantified using a hydroxylysyl pyridinoline assay, as described by Fomovsky and Holmes (6). Samples removed for histological analysis would be stained using hematoxylin and eosin, trichrome staining, as well as picrosirius red for analysis of collagen fiber orientation using quantitative polarized light microscopy. Furthermore, samples would be stained using α-smooth muscle actin for visualization of vascularity and myofibroblasts concentration, troponin I (a contractile protein), and connexin 43 for gap junction formation. Based on the results from this study, scaffolds containing the same structure and porosity as determined from the first study would be fabricated with a higher modulus (e.g., 500 kPa) and a lower modulus (e.g., 50 kPa) to further investigate the influence of scaffold mechanics on outcomes. Additional experiments would be necessary in order to identify the optimal scaffold size (i.e., scaffold covers infarct area exclusively or infarct region and borderzone) and time of therapy (i.e., one week or four weeks following infarct). Ultimately, the hope would be to determine the optimal material properties for left ventricular restraints and possibly elucidate some of the mechanisms behind their benefit.

9.2.3 Specific Aim 3: To develop crosslinkable hyaluronic acid based hydrogels for injection in vivo.

9.2.3.1 Limitations

The goal of this work was to synthesize and characterize two enzymatically degradable hydrogels with similar mass loss and without significant degradation over an 8-week period. The modulus of the MeHA High hydrogels was reduced more than expected at the 8-week time point,
whereas that of the MeHA Low hydrogels was not. This is most likely due to the additional hydrolytically degradable methacrylate groups introduced during synthesis. Unfortunately, this experiment could not be repeated due to a lack of remaining macromer following the conclusion of other studies.

9.2.3.2 Future Work

Although cells that were cultured in the presence of the hydrogels formed using the bi-component redox initiation system of APS and TEMED remained viable, reports in the literature (9) have demonstrated reduced viability of cells encapsulated within gels polymerized using APS and TEMED. Therefore, additional mechanisms for in situ gelation could be evaluated. For example, alternative redox initiators such as sodium persulfate and ascorbic acid or through enzymatic means could be characterized.

9.2.4 Specific Aim 4: To assess the functional and geometric sequelae of intramyocardial infarct stiffening using injectable hydrogels.

9.2.4.1 Limitations

In order to evaluate the impact of hydrogel material properties on infarct size and remodeling, hydrogels were injected 30 minutes post-infarction, representing the best-case scenario for observing an impact on remodeling. However, this time frame is not necessarily clinically relevant and therefore the impact of treatment time should be evaluated. Furthermore, in order to reduce batch-to-batch material variations, all samples were evaluated using the same batch of MeHA. Unfortunately, there was only enough material in a given batch to test n=6 samples per MeHA group. Ideally, a greater number of samples (i.e., n=7-10) would be evaluated in order to observe statistical differences in NEDV and NESV. Additionally, there was no assessment performed on the tissue properties at the final time point, which may have provided some insight into the remodeling process.
9.2.4.2 Future Work

In order to elucidate the influence of injectable hydrogel material properties on infarct remodeling, the influence of material degradation must also be evaluated. Hydrolytically degradable HA based hydrogels with tunable properties, such as those described by Chung and colleagues (10), could be used to accomplish this goal. Hydrogels with initial mechanics similar to MeHA High and MeHA Low, however that have significantly reduced mechanics at 1, 2, 4, and 8 weeks could be synthesized and evaluated. Furthermore, sonomicrometry crystals and bi-axial mechanical testing could be utilized to directly evaluate the impact of hydrogel mechanics and degradation on LV remodeling with time. Furthermore, delivery of the hydrogel via a catheter could be optimized prior to translation to the clinic to present a more minimally invasive approach to hydrogel delivery.

9.3 Conclusion

Although, a substantial amount of work remains to develop alternative treatment strategies to attenuate LV remodeling post-MI, this thesis contributes significant advances towards the development of biomaterials with tunable properties in order to identify the optimal material properties for LV restraints and intramyocardial hydrogel injection. In order to accomplish this task, a collection of biodegradable elastomers with tunable properties and that are capable of crosslinking through free radical polymerization mechanisms was developed. Next, these materials were fabricated into fibrous scaffolds with tunable properties (i.e., mechanics and degradation) that were biocompatible and able to be sutured onto the epicardial surface for use as an LV restraint. Finally, additional tunability with regards to scaffold structure (e.g., isotropic versus anisotropic) and porosity were introduced through fiber alignment and composite scaffold formation with the inclusion of a sacrificial fiber population. Increases in cellular infiltration and matrix elaboration were observed with implantation of composite scaffolds in vivo. In an effort to utilize injectable approaches to limit LV remodeling, injectable hydrogels based on hyaluronic acid were developed using redox initiation. The infarct area was significantly reduced from control infarct samples with treatment by hydrogels with a greater modulus (~40
kPa) than hydrogels with a modulus similar to cardiac tissue (~6-8 kPa). The work described in this thesis represents an advancement in the development of biomaterials for novel treatment strategies to prevent the onset of congestive heart failure in patients afflicted with post-MI remodeling.
References:


