RICE UNIVERSITY

Development and Characterization of a Poly(ethylene glycol) Hydrogel Scaffold System for Adipose Tissue Engineering

by

Parul Natvar Patel

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Doctor of Philosophy

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ABSTRACT

Development and Characterization of a Poly(ethylene glycol) Hydrogel

Scaffold System for Adipose Tissue Engineering

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As the number of soft tissue correctional procedures increases yearly and the current methods being inadequate, alternative methods of rectifying defects has become a focus for tissue engineers. Our long-term goal is to engineer adipose tissue to correct soft tissue defects resulting from aging, trauma, congenital abnormalities, and tumor resection (including lumpectomies and mastectomies). The immediate objective is to develop and characterize a photopolymerizable synthetic hydrogel system to act as a bioactive scaffold that promotes preadipocyte (precursor cell to adipocyte) adhesion and proliferation for adipose tissue engineering. We have shown that poly(ethylene glycol) diacrylate derivatized with enzyme-sensitive degradation sites and cell-adhesion ligands promote preadipocyte adhesion, viability, proliferation and differentiation, and demonstrates mechanical properties able to withstand physiological strains and frequencies. The following specific aims address the scope of this thesis:

1. Determine the required polymer chemistry and fabricate a series of hydrogels with and without degradation peptides and cellular binding sites.
2. Quantitatively compare the physical properties (e.g., viscosity, elastic modulus, viscous modulus, complex modulus, and % recovery) of human adipose tissue with hydrogels fabricated.

3. Demonstrate that hydrogels containing adhesion ligands and degradation sites optimally promote preadipocyte adhesion, viability, and proliferation compared to other hydrogel configurations.

4. Demonstrate that preadipocyte-loaded hydrogels promote adipogenesis within a low-shear bioreactor system.
Acknowledgments

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Chapter I. Introduction

This chapter addresses the types of soft tissue defects prevalent in the United States today, the standards of care for correcting these defects and their disadvantages, and the research efforts currently being conducted to find alternative means to repair a defect.
A. Soft Tissue Defects

The correction of soft tissue defects caused by trauma, tumor resection, congenital abnormalities, and aging presents a challenge in reconstructive surgery. Soft tissue defects run the gamut in terms of volume, from restoring the fullness of the face by removing wrinkles (small scale defects) to restoring the breast mound after mastectomy (large scale defects). This section addresses the anatomy and clinical occurrences of both scales of defects (small and large) and the problems associated with the current standards of care for treating these defects.

i. Small Scale Defects

Skin Anatomy and Clinical Incidences

Over a million correctional procedures are performed every year to repair soft tissue defects (Table 1). Resections of various tumors of the head and neck, soft tissue, and bone, as well as trauma, congenital abnormalities, changes associated with aging and a myriad of other medical conditions commonly result in contour deformities due to defects in the dermis and underlying subcutaneous adipose tissue (Figure 1). These can impair the aesthetic appearance, function, and psychological well-being of patients. The large number of reconstructive and cosmetic surgeries and underlying clinical limitations warrants focus on adipose tissue strategies for tissue engineering. Soft tissue restoration as a means of correcting these defects poses particular challenges for both the tissue engineer and the plastic surgeon.
Table 1. Possible applications for a tissue engineered adipose strategy. (1)

<table>
<thead>
<tr>
<th>Category</th>
<th>Application</th>
<th>Specifics</th>
<th>Incidence or Number of Procedures/Year</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reconstructive</td>
<td>Oncologic resection</td>
<td>Mastectomies</td>
<td>81,729 Breast reconstructions</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Parotidectomies</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Complex trauma</td>
<td>Soft tissue deficits</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Congenital abnormalities</td>
<td>Hemifacial microsomia</td>
<td>1 in 4,000 to 1 in 5,600</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Poland's syndrome</td>
<td>1 in 20,000 to 1 in 32,000</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Romberg's syndrome</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Other</td>
<td></td>
<td>44,147 Reconstructions</td>
</tr>
<tr>
<td>Cosmetic</td>
<td>Augmentation</td>
<td>Breast</td>
<td>219,883 Breast augmentations (mammaplasty)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>55,176 Breast lifts (mastoplexy)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Cheek, chin, jaw</td>
<td>8,494 Cheek implants (malar augmentation)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>28,736 Chin augmentations (mentoplasty)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Lips</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Buttocks</td>
<td></td>
<td>1,339 Buttock lifts</td>
</tr>
<tr>
<td>Rejuvenation</td>
<td>Wrinkles</td>
<td></td>
<td>2,495 Fibril injections</td>
</tr>
<tr>
<td></td>
<td></td>
<td>796,526 Collagen injections</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>855,846 Botox injections</td>
<td></td>
</tr>
<tr>
<td>Nonspecific revision/resculpting</td>
<td>Various locations</td>
<td></td>
<td>72,632 Fat injections</td>
</tr>
<tr>
<td>Correctional</td>
<td>Implant removal</td>
<td>Breast</td>
<td>62,861 Removals</td>
</tr>
<tr>
<td></td>
<td>Bulking agent</td>
<td>Stress urinary incontinence</td>
<td>1,500,000</td>
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<td></td>
<td></td>
<td>Vesicoureteral reflex</td>
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<tr>
<td></td>
<td></td>
<td>Intrinsic sphincter defect</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Vocal cord insufficiency</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Orthotic-related</td>
<td>Atrophied &quot;cushion&quot; in</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>ball/heel of aged foot</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Augmentation</td>
<td>Soft tissue deficits</td>
<td></td>
</tr>
</tbody>
</table>
Figure 1. Anatomy of normal skin and skin defects  
(a) Anatomical layers of skin and subcutaneous tissues. 
(b) Soft tissue defects result from tissue deficits in the subcutaneous fat layer. 
(c) Wrinkles result from tissue deficits in the dermis.  
*Illustrations courtesy of Charles W. Patrick, Jr., Department of Plastic Surgery, M.D. Anderson Cancer Center.*
Limitations of Current Standards of Care

The clinical strategy used to date to repair soft tissue defects has been to restore the missing volume with a natural, synthetic, or hybrid material. The first substances used as filler materials, including candlewax, beeswax, paraffin, and various oils, proved to be not only ineffective for soft tissue repair but dangerous to the patient due to allergic reactions, local chronic edema, lymphadenopathy, scarring, ulcerations, and migration with associated reactive processes (i.e., oil granulomas). (2) Although many materials are currently used clinically and new materials are being investigated, none have proven to be the “perfect” material for soft tissue repair. Many materials possess severe limitations including, but not limited to, unpredictable outcome, fibrous capsular contraction, allergic reaction, suboptimal mechanical properties, distortion, migration, and long-term resorption. (3) The ideal material for soft tissue restoration would be nonallergenic, nonpyrogenic, produce no disease states, permit ambient temperature storage, look and feel natural, be economical relative to standard of care, be stable after implantation or injection, be fully integrated or replaced by host tissue over time, and be easy to use in the operating room and outpatient setting. For relatively small defects, the material should be versatile enough to be injected through small gauge needles yet capable of being molded into a solid implant after injection. (4)

Numerous substances, including reconstituted bovine collagen, silicone fluid, autologous fat and tissues, gelatin powder mixes, polymethylmethacrylate microspheres, crosslinked polydimethylsiloxane, Teflon paste, and autologous collagen, have been used in the last two decades to repair small soft tissue defects. (3, 5) Reconstituted bovine collagen
(Zyderm or Zyplast) has been widely used but it is far from the optimal material. Bovine collagen consists of sterile, purified bovine Types I and III collagen and is injected intradermally. Over time, this material is recognized as foreign and is degraded by collagenases and inflammatory cells. *In vivo* experiments demonstrate loss of collagen within 3 months of injection. (6, 7) Therefore, collagen injections must be repeated frequently to maintain clinical results. Although allergic reactions occur rarely (3%-5% incidence), hypersensitivity reactions are observed.(8-10) In addition, erythema and induration in the presence of circulating antibodies, granulomatous responses, and serum sickness-like illnesses have all been documented. (11, 12) Other synthetic materials, including Fibril and silicone, are associated with technical and regulatory difficulties and can cause hypersensitivity and inflammatory reactions.

Autologous tissue preparations are theoretically ideal materials for repairing tissue defects because of their biocompatibility and lack of allergic reactions and tissue rejection. Allografts or homologous tissue grafts are less ideal because of the potential for viral transmission and potential immunogenic or allergic reactions. Autologous materials have been used as filler materials such as dermal cellular and extracellular matrix constituents and injectable fat. One disadvantage of such procedures is that they require harvesting and processing the patient’s own tissues. Also, resorption rates are notably high, up to 20% and 60% for autologous dermal and fat grafts, respectively, requiring the plastic surgeon to overcorrect during reconstruction and make subsequent revisions. (13, 14)
Among the commercially available products for soft tissue repair is Isolagen which consists of autologous dermal fibroblast injections based on the theory being that collagen-producing cells should result in longer duration of correction compared with bovine collagen alone. Although the prospect of an alternative to bovine collagen is exciting, there are several limitations to Isolagen. The clinical effects are often subtle and not as impressive as other forms of restoration. Logistical factors involving transport, preservation, and processing of tissues may limit the usefulness of this method. Finally, additional costs incurred by the repeated labor-intensive harvesting process, as well as by material storage and transport, reduce the feasibility of this process on a large scale. (15)

Another commercial product, Autologen, is an injectable collagen harvested from the dermis of the patient during surgical procedures in which excessive skin provides a source for extraction of collagen fibers. Autologen, like Isolagen, has little potential for causing allergic reactions since it is an autologous material. Due to the maintenance of the physical and chemical structure of the collagen fibers, Autologen provides the added advantage of longer duration compared to bovine collagen. The great disadvantage of Autologen is its limited supply and the initial requirement of a large amount of tissue for harvesting and processing. (16-18)

Early on, autologous fat (i.e., mature adipose tissue) was investigated as a virtually limitless source of material for soft tissue repair. It is easily harvested, readily available, and most patients possess excessive amounts that can be harvested without producing significant contour defects. Despite the theoretical advantages, however, autologous fat
transplantation has demonstrated poor results, with a 40%-60% reduction in graft volume due to resorption. (19-21) The adipose tissue resorption is postulated to be related to insufficient revascularization and mechanical damage. Some limited success has been obtained by using small diameter “pearls” of fat tissue where mass transport occurs readily via diffusion. (22, 23) However, this strategy is not viable for the majority of clinically-sized defects. The advent of liposuction led investigators to attempt using single-cell suspensions of mature adipocytes. However, since adipocytes possess a cytoplasm composed of 80%-90% lipid, they tend to be traumatized by the mechanical forces of aspiration, resulting in about 90% damaged cells. The remaining 10% tend to form cysts or localized necrosis after injection. Moreover, mature adipocytes cannot be expanded \textit{ex vivo} because they are terminally differentiated. (24)

\textbf{ii. Large Scale Soft Tissue Defects}

\textbf{Breast Anatomy and Clinical Incidences}

The breast is a specialized organ composed of glandular, ductal, connective and adipose tissue. (25) Each breast contains 15-25 lobes of compound glands that are embedded in fibrous and adipose tissue (Figure 2). (26) These lobes, each containing an excretory duct that drains into the lactiferous sinus, radiate from a central nipple-areolar complex. The breasts are typically the most prominent superficial structures of the anterior thoracic wall, overlying the pectoral muscles and extending from the clavicle superiorly to the sixth rib inferiorly, and from the midsternal line medially to the axilla laterally, forming the axillary tail of Spence. The size and shape of a breast is determined by the bounding skin envelope and the adipose tissue surrounding the glandular structures. The presence
of adipose tissue lobules interspersed between fibrous and glandular components of the breast help determine the bulk, softness, and contour of the breast mound. The deep aspect of the breast is separated from the underlying pectoralis major muscle by the deep fascia. Between the breast and this deep fascia is a thin layer of loose connective tissue (within the retromammary space) devoid of adipose tissue that allows the breast to move freely over the deep fascia. The breast is firmly attached to the skin and underlying structures by fibrous bands referred to as suspensory ligaments (Cooper’s ligaments), which provide additional support and also contribute to determining the shape and contour of the breast. (27)

Figure 2. Anatomy of the female breast. [Adapted from breastcancer.org]
Breast cancer is the second leading cause of cancer in women (after skin cancer), affecting one in every eight women in America and one in every three women with cancer. (28) Due to the large number of clinical occurrences, breast reconstruction following lumpectomy (partial removal of breast tissue) or radical mastectomy (total removal of the breast) has become the sixth most common reconstructive procedure performed in the United States. (1) Lumpectomy defects less than 25% of the total breast volume can be corrected by rearranging the local breast tissue. (29) Larger lumpectomies and mastectomies require more drastic reconstruction modalities. Studies show that many women who have had a mastectomy tend to suffer from a syndrome “marked by anxiety, insomnia, depressive attitudes, occasional ideas of suicide, and feelings of shame and worthlessness.” (30) Reconstruction of the breast mound following a mastectomy has proven to alleviate the sense of mutilation and suffering that women experience post-surgery. (31) As a result, breast reconstruction is offered as an option to any woman undergoing surgery for breast cancer. Breast reconstruction was performed on 74,090 patients in 2002 with 50% of them having the procedure at the same time as mastectomy. Furthermore, 236,888 breast augmentation procedures were performed in 2002, being the third most common surgical cosmetic procedure. (1)

Current Standards of Care

When planning breast augmentation or reconstruction, one of three options is currently considered. First, either a fixed-volume or expandable prosthesis is placed alone for breast augmentation. This option is most popular with women desiring an increase in
breast size. (32) Second, autologous tissue is used to reconstruct the breast. This option is most commonly performed for patients following a mastectomy. Finally, autologous tissue in combination with a prosthetic device is used. If the patient's own tissue does not contribute an adequate donor volume for reconstruction, then a prosthetic device can be additionally implanted to fill the defect. (29, 33) Each of these clinical strategies is briefly discussed below.

**Implants and Expanders**

There are two basic types of prosthetic devices available: fixed-volume breast implants and tissue expanders. The advantage of using an implant or expander is that these devices can be manufactured in a broad range of sizes, contours, profiles, and textures. Fixed-volume implants have an outer layer or envelope of vulcanized silicone and are usually filled with a saline solution. (29, 33) Implants filled with silicone gel are also available, but on a limited basis due to the controls placed by the FDA in 1992. (32, 34) Tissue expanders are implants in which the volume can be altered after implantation. Breast reconstruction using tissue expanders involves a temporary expander being inserted and inflated to stretch the skin to a slightly larger than necessary state, at which point the device is removed and replaced with a permanent implant of desired size. Expanders possess an outer envelope of silicone elastomer and serial injections of saline are made over time, usually once per week, to inflate the implant. (29, 33) Both implants and expanders are available with a smooth or textured silicone surface. Textured surfaces result in a lower incidence of capsular contracture after implantation than smooth surface implants. Textured surfaces also serve to stabilize the implant and facilitate tissue
expansion. One drawback to having a textured envelope is that these implants have a slightly thicker vulcanized silicone shell that may be more visible in some patients by exhibiting a rippled appearance through the skin. The thicker shell may also contribute to the implant folding and potentially deflating following insertion. (29, 33)

The most common complication resulting from implants and expanders is capsular contracture (Figure 3). (29, 33) Inserting an implant into the body inevitably leads to the formation of a capsule of firm, fibrous scar tissue around the implant due to a foreign body reaction. (35-38) The implant capsule constricts over time, making the augmented breast feel harder and firmer than desired. (29, 33, 34, 39) Capsular contracture often results in a spherical breast appearance, (29, 33, 34) chronic chest wall discomfort, and restricted shoulder or arm movement. (29, 33) Although capsule formation is normal after breast augmentation, not all capsules result in severe contraction. (40) Capsular contracture is graded on the Baker scale of I-IV, where I denote a breast that looks and feels soft and IV denotes a breast that feels firm, is beginning to become distorted in shape, and causes pain to the patient. The potential for calcium deposits accumulating in the capsule can also interfere with tumor detection. (29, 33)
Figure 3. Examples of capsular contracture around breast implants. (A) Patient possesses capsular contracture of both breasts, resulting in breast hardness, an unnatural spherical profile, and breast asymmetry. (B) Similarly, this patient possesses capsular contracture of her right breast. *Photographs are courtesy of Elisabeth Beahm, M.D., Department of Plastic Surgery, The University of Texas M.D. Anderson Cancer Center.*

Implants are also subject to other local complications such as rupture, possible silicone or saline leakage, (29, 33) displacement, deformation, (29, 33, 41) chronic seroma, implant exposure, (29, 33) hematoma, and loss of nipple sensation. (38) Breast augmentation using tissue expanders also results in more complications than using fixed-volume implants alone. Infection is most common with expanders due to the bacteria that can be introduced from the serial saline injections. Fill-valve problems and skin loss are also problems associated with using tissue expanders. (33)
Autologous Tissue

Breast reconstruction considers the aesthetic and anatomic properties of the breast mound in order to achieve optimal clinical results. Autologous adipose tissue was investigated as a source of material for lumpectomy repair. Problems associated with autologous adipose tissue injections include microdeposits of serum calcium from fat resorption that result in obscure mammographies and interfere with tumor detection, (42, 43) along with high resorption rates.

Breast reconstruction using the patient’s own tissues, rather than implantable devices, tends to produce better results (29) with fewer complications. (33) The use of autologous tissues tends to produce a breast mound that better recreates the shape, contour, softness, and fullness of the natural breast than the use of implants. (29) There are a number of sites for donor tissue for breast reconstruction. The primary areas include the abdomen (transverse rectus abdominis musculocutaneous [TRAM] flap) (29, 33) and the back (latissimus dorsi musculocutaneous flap). The buttocks (inferior gluteus maximus musculocutaneous flap) and the hips (Rubens Flap) are seldom used to reconstruct the breast mound. Tissue availability must be considered for each donor site based on the patient. (33)

Abdominal flap reconstruction is the most common, yet technically demanding, procedure performed for breast reconstruction using autologous tissues. (29) The TRAM flap conveniently provides abdominal wall skin and adipose tissue, along with the blood vessels that supply these tissues to reconstruct the breast. Breasts reconstructed using the
TRAM flap have shown to uphold symmetry over time. Patients also view the accompanying abdominoplasty, or “tummy tuck,” as an added benefit to having reconstruction using the TRAM flap. A disadvantage to having this surgery performed is that additional procedures to reconstruct the nipple-areola and to refine the breast mound are required. Additionally, due to the complexity of the TRAM flap procedure, complications such as prolonged pain, abdominal weakness, an extended period of recovery, and occasional areas of necrosis accompany the surgery. (33) Finally, contour abnormalities of the abdomen may result after TRAM flap reconstruction, including upper and lower abdominal bulge, upper fullness, and hernia. (44-46) The latissimus dorsi muscle is a large triangular muscle that extends over the lower half of the thorax and lumbar region. Although the procedure involving the latissimus dorsi flap is less involved than the TRAM flap procedure, the long-term results of the latissimus dorsi flap procedure are inferior. The muscle tends to atrophy over time and there is typically a volume discrepancy in the back from the donor site. (33)

Breast augmentations and reconstructions are adversely affected by radiation therapy. A high percentage of breast cancer patients need radiation therapy, which causes an increase in the risk of capsular contracture around the implant and eventual loss of the implant. Radiation therapy also increases the incidence of fibrosis, and hence capsular contraction around breasts reconstructed with implants or autologous tissues. (29)
B. Literature Review: Contemporary Adipose Tissue Engineering Strategies

Due to the complexity of soft tissue augmentation and the complications associated with various reconstruction measures, various tissue engineering strategies are being investigated as a means to repair and restore defects. Tissue engineering strategies in general involve seeding cells and introducing appropriate tissue induction and differentiation growth factors in a three-dimensional natural, synthetic, or hybrid scaffold in order to develop “biological substitutes that restore, maintain, or improve tissue function.” (3, 5, 47) Numerous scaffold materials, ranging from nonwoven fiber and hydrogel extracellular matrix structures to biodegradable polymers in the form of foams, nonwoven fibers, and hydrogels, are being investigated (Table 2). Scaffold materials must be able to mechanically support and guide tissue formation, such as adipogenesis. Materials must also be biocompatible, biodegradable, easily processed, (48, 49) resistant to mechanical strain, and easily shaped to the surgeon’s specifications. (31) Also, materials must permit variability in shape and volume in order to personalize the scaffold meet the patient’s specific contour and size needs. Ideally, the scaffold should recapitulate the endogenous extracellular matrix as much as possible. Acellular tissue engineering constructs are implanted in patients and rely on recruitment of surrounding cells or are tailored to remain acellular, whereas cellular tissue engineering constructs either are grown ex vivo in sophisticated bioreactors and then implanted in the patient or are placed directly in vivo with the patient serving as a bioreactor. Preadipocytes, precursor cells that differentiate into mature adipocytes, can be seeded onto a scaffold and allowed to proliferate and differentiate to promote the formation of adipose tissue.
The materials that are under investigation as potential scaffolds for adipose tissue growth are discussed in the next sections (Figure 4).

Table 2. Natural and synthetic materials employed in adipose tissue engineering strategies (references).

<table>
<thead>
<tr>
<th>Natural Materials</th>
<th>Synthetic Materials</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alginate</td>
<td>Poly(glycolic) acid (60-62)</td>
</tr>
<tr>
<td>Collagen</td>
<td>Poly(L-lactic-co-glycolic) acid (50, 51, 71, 72)</td>
</tr>
<tr>
<td>Fibrin</td>
<td>Polytetrafluoroethylene (52)</td>
</tr>
<tr>
<td>Hyaluronic acid</td>
<td></td>
</tr>
<tr>
<td>Matrigel</td>
<td></td>
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</table>

i. Natural and Synthetic Polymer Foams and Meshes

Rigid poly(L-lactic-co-glycolic-acid) (PLGA) polymer foams seeded with rat preadipocytes have been implanted subcutaneously into male Lewis rats in an effort to generate *de novo* adipose tissue (Figure 4 a,b). Results from short-term (2-5 weeks) and long-term (1-12 months) studies demonstrated the successful formation of adipose tissue for up to 2 months. (50, 51) The volume of generated adipose tissue then began to decrease and was completely resorbed by 5 months. The resorption of adipose tissue after 2 months may be due to factors such as a lack of adequate vascularization, lack of support structure after the PLGA degraded, the anatomical site of implantation not being conducive to long-term maintenance of adipose tissue, or a limitation related to the small
animal model used. The long-term maintenance or persistence of generated tissue is a challenge for all tissue engineering applications.

In addition to foams or sponges, nonwoven fiber meshes have been studied as potential scaffolds for adipose tissue engineering (Figure 4 c,d). Fluortex monofilament-expanded polytetrafluoroethylene (52 micron pore size) is one such material. Since preadipocytes do not attach to polytetrafluoroethylene, the material was coated with fibronectin to optimize cell seeding efficiency. Fibronectin coating resulted in a significantly higher number of attached human preadipocytes than collagen or albumin coatings. Human preadipocytes were able to proliferate and differentiate into adipocytes on the fibronectin-coated expanded polytetrafluoroethylene in vitro over a period of 120 hours. (52) In vivo studies have not been conducted.

Von Heimburg and colleagues have studied porous collagen scaffolds (53-55) and two types of hyaluronan based materials (55, 56) for adipose tissue engineering. The collagen scaffolds are constructed based on a directional solidification method followed by freeze-drying to obtain a uniformly porous structure. (57, 58) The hyaluronic acid-based devices were manufactured into sponges or scaffolds composed of nonwoven fibers. The sponges demonstrated open, interconnecting pores ranging from 50 - 340 microns while the nonwoven mesh had an interfiber distance of 100- 300 microns. In vivo studies conducted in mice showed that the hyaluronan sponge proved to be a better scaffold than the freeze-dried collagen construct or the hyaluronan nonwoven carrier due to the larger, interconnected pores. After 8 months, a greater number of adipocytes were found in the
sponge compared to the nonwoven mesh. The large pore size is important for the preadipocytes to incorporate lipids and enlarge during differentiation. Pore size was also found to be a factor in adipogenesis in several proprietary Johnson & Johnson biodegradable polymer sponges and nonwoven fibers. (59)

Beahm and colleagues have demonstrated recruitment of resident preadipocytes and formation of de novo adipose tissue in silicone molds of various shapes (sheet, hemisphere, sphere). (60-62) Packed with poly(glycolic acid) (PGA) fibers, Matrigel, and bFGF, the molds were sutured to the superficial inferior epigastric vessel of nude rats to create a vascular pedicle model. Qualitative assessment over 4-20 weeks demonstrated de novo adipogenesis. This approach is currently being translated to a large animal model (Yucatan micropig) to assess adipose tissue engineering strategies.

ii. Hydrogels

Numerous natural and synthetic hydrogels are being investigated for primarily small breast defects, soft tissue repair, and cosmetic applications. As a natural wound healing matrix, fibrin is often studied as a potential injectable, cell-seeded scaffold. Investigators in Germany have seeded human preadipocytes within defined fibrin gels and demonstrated cell proliferation and differentiation (Figure 4 e). (63) Similarly, reconstituted basement membrane of a mouse tumor, or Matrigel, has been shown by several investigators to induce migration, proliferation, and differentiation of preadipocytes when supplemented with basic fibroblast growth factor (bFGF). (64-66) It is speculated that when Matrigel plus bFGF is injected into a subcutaneous space,
preadipocytes residing in the adjacent connective tissue migrate into the extracellular matrix hydrogel. Results demonstrate that endothelial cells are also recruited. (66) It remains unclear whether the observed angiogenesis drives the adipogenesis or vice versa. When gelatin microspheres loaded with bFGF were coimplanted with Matrigel, similar results were obtained. (67)

Porous alginate material (a naturally derived hydrogel) has also been investigated as a construct for soft tissue engineering. Halberstadt et al. modified the alginate material with the peptide sequence arginine, glycine, and aspartic acid (RGD) to allow cells to adhere to the construct. (56) In vitro studies demonstrated that the porous alginate-RGD material supported cell attachment, adhesion, and proliferation. (68) Small animal studies performed over 6 months showed that the implanted material was also conducive to tissue ingrowth and did not elicit major inflammatory responses. (69) In a large animal model (sheep), the material was seeded with preadipocytes and injected into the nape of the neck. Well-defined adipose tissue was identified within the hydrogel at 1 and 3 months. Unfortunately, it cannot be determined whether the adipose tissue growth resulted from the previously seeded preadipocytes in the material or from resident preadipocytes. (56)

Collagen hydrogels have also been investigated as a three-dimensional biological matrix upon which preadipocytes are co-cultured with human mammary epithelial cells in order to form tissue that closely resembles the normal human breast. Histologic analysis of the collagen gels from in vitro studies indicates a pattern of ductal structures of human
mammary epithelial cells within clusters of adipocytes similar to the architecture of breast tissue. These findings indicate that there is a potential for breast tissue to be regenerated \textit{in vitro} on a three-dimensional scaffold and later implanted for breast reconstruction purposes. (70)

iii. \textbf{Materials as Delivery Vehicles for Adipogenic Factors}

Sophisticated biomaterials can be modified to present factors that promote adipogenesis. Modifications include encapsulating a factor or tethering a factor to polymer chains. PLGA/polyethylene glycol (PLGA/PEG) microspheres have been investigated as growth factor delivery vehicles in the absence of Matrigel (Figure 4 f). (71) Insulin, insulin-like growth factor-1 (IGF-1), and bFGF have been administered to the muscular fascia of the rat abdominal wall via these PLGA/PEG microspheres. (72) At the 4 weeks harvest, adipose tissue was grossly observed at the site of implantation. Histologic and image analysis showed that the microspheres treated with both insulin and IGF-1 demonstrated statistically greater increases in adipose tissue (composed of fibroblasts and adipocytes) than did empty microspheres or microspheres treated with insulin, IGF-1, or bFGF alone or with all three growth factors. This \textit{de novo} generation of adipose tissue may result from one of three mechanisms. Stem cells may be present in the fascia and stimulated to differentiate into preadipocytes and subsequently adipocytes. Another possible explanation is that preadipocytes residing in the fascia are stimulated to differentiate into adipose tissue. The third explanation, and the most speculative, is that the fibroblasts in the fascia dedifferentiate and then differentiate into preadipocytes and subsequently adipocytes. Regardless of the means, results indicate that adipose tissue can be generated
in fascia within a short period (4 weeks) via delivery of microspheres treated with insulin and IGF-1. (72)

Figure 4. Examples of materials employed in adipose tissue engineering. (A) PLGA foam (bar denotes 600 microns) (B) PLGA foam with preadipocytes within the pores (bar denotes 300 microns) (C) Nonwoven polymer fiber (bar denotes 100 microns) (D) Nonwoven polymer fiber with preadipocytes spanning the fibers (bar denotes 100
microns) (E) Preadipocytes differentiating within a fibrin hydrogel (300X magnification) (F) PLGA/PEG microsphere used to deliver bioactive factors (bar denotes 30 microns).

Photographs courtesy of Charles W. Patrick, Jr., Ph.D., Department of Plastic Surgery, M.D. Anderson Cancer Center.
Chapter II. Synthesis of Poly(ethylene glycol) Derivatives and Photoinitiating Systems

Synthetic polymer hydrogels are actively being studied as potential tissue engineering scaffolds because of the ability to derivatize the polymers with bioactive functional groups and, thereby, precisely control molecular and cell function on spatial and temporal scales. Hydrogels are viscoelastic polymeric structures that contain a significant volume fraction of water (usually >90%). Currently, hydrogels are being employed in biomedicine for controlled drug release, soft tissue augmentation (Section I.B.ii), cell separations, and biosensors. (73, 74) In the tissue engineering arena, hydrogels can be used as nascent materials, or they can be modified with bioactive peptides that aid them in mimicking cell adhesion properties of the extracellular matrix. Specifically, poly(ethylene glycol) or its derivatives have been investigated as potential scaffold materials for bone tissue engineering (75) and cartilage tissue engineering, (76-78) as well as used in wound healing (49) and for the treatment of enzyme deficiencies. (79)
A. Poly(ethylene glycol) Diacrylate

Hydrogels formed with poly(ethylene glycol) diacrylate are not degradable by hydrolysis or enzymatic degradation. Poly(ethylene glycol) in its basic form is also resistant to cellular adhesion and essentially acts as a “blank slate” to which desired biofunctionality can be added via defined chemistries. (48)

Structure

\[
\text{CH}_2=\text{CH} - \text{C} - \text{O} - (-\text{CH}_2\text{CH}_2\text{O})_n - \text{C} + \text{CH}=\text{CH}_2
\]

Figure 5. Poly(ethylene glycol) diacrylate. Examples of n values for poly(ethylene glycol) polymers: (a) polymers with molecular weight 400 Da, \(n = 5\), (b) polymers with molecular weight 6000 Da, \(n = 132\), and (c) for polymers with molecular weight 10000 Da, \(n = 223\).

Synthesis of Poly(ethylene glycol) Diacrylate

Nondegradable hydrogels are prepared with poly(ethylene glycol) diacrylate of molecular weight 6000 Da. Poly(ethylene glycol) is acrylated by dissolving 0.1 mmol/mL dry poly(ethylene glycol) (Fluka, Buchs, Switzerland), 0.4 mmol/mL acryloyl chloride (Sigma), and 0.2 mmol/mL triethylamine (Sigma) in anhydrous dichloromethane and stirring under argon overnight. Potassium carbonate (2 M, 0.8 mmol/mL, Sigma) is added to the resulting poly(ethylene glycol) in a separatory funnel. Carbon dioxide is
vented and the remaining emulsion is allowed to separate by gravity. The dense organic layer containing the poly(ethylene glycol) is separated from the aqueous phase and dried with anhydrous magnesium sulfate (Sigma). The solution is filtered to remove the magnesium sulfate and precipitated with diethyl ether. The solution is again filtered to separate poly(ethylene glycol) from the solution. poly(ethylene glycol) is dried under a vacuum and stored frozen under argon.
B. Derivatized Poly(ethylene glycol)

Poly(ethylene glycol) can be modified with bioactive peptides that permit enzymatic degradation or aid in cell-adhesion. Two types of modified poly(ethylene glycol) were fabricated for our studies. For a polymer degradable by collagenase (which is secreted by preadipocytes), the peptide sequence LGPA was used. For preadipocyte adhesion, the peptide sequence YIGSR was incorporated onto the polymer.

i. Degradable Poly(ethylene glycol)

Proteolytically degradable peptides can be incorporated into the backbone of the polymer to form hydrogels that are degraded by cell-secreted enzymes. (48) For instance, polyethylene glycol can be modified with the peptide sequence leucine-glycine-proline-alanine (LGPA) to form a polymer degradable by collagenase.

**Structure/Synthesis**

![Chemical structure](image)

Figure 6. Synthesis of poly(ethylene glycol) degradable by collagenase.
Degradable poly(ethylene glycol) is synthesized by modifying acryloyl-poly(ethylene glycol)-N-hydroxysuccinimide (acrl-PEG-NHS, 3400 Da, Shearwater Polymers, Huntsville, AL) with the collagenase-sensitive peptide sequence glycine-glycine-leucine-glycine-proline-alanine-glycine-glycine-lysine (GGLGPAAGK) in a 2:1 molar ratio (Figure 6). The polymer-peptide mixture is then dissolved in 50 mM sodium bicarbonate (pH 8.5) and allowed to react for 2 hours. The solution is filter sterilized, lyophilized, and stored frozen under argon. The resulting poly(ethylene glycol) derivative is a block copolymer, ABA, where A is poly(ethylene glycol) and B is the collagenase-sensitive peptide. The poly(ethylene glycol) derivative is terminated with acrylated groups for photocrosslinking. An in vitro degradation assay is used to validate the specific degradation of this polymer.

ii. Poly(ethylene glycol) Coupled with Cell-Adhesion Ligands

Cell adhesion sites can also be coupled to polyethylene glycol using the peptide tyrosine, isoleucine, glycine, serine, and arginine (YIGSR) (Figure 7). YIGSR is one of many cell binding peptides found on laminin. Wu and Patrick have shown that preadipocytes bind preferentially to laminin-1 and that cell adhesion to and migration on laminin-1 is mediated, in part, by the $\alpha_1\beta_1$ integrin. (80)
Structure/Synthesis

\[
\begin{align*}
\text{(OCH}_2\text{CH}_2\text{)}_{75}^\text{N-\text{YIGSR-COOH}} + \text{Acrl-PEG-NHS} & \rightarrow \\
\text{(OCH}_2\text{CH}_2\text{)}_{75}^\text{YIGSR}
\end{align*}
\]

Figure 7. Synthesis of poly(ethylene glycol) coupled with cell-adhesion ligands.

Acrl-PEG-NHS is combined with YIGSR in a 1:1 molar ratio and then dissolved in 50 mM sodium bicarbonate (pH 8.5) and allowed to react for 2 hours. The solution is filter sterilized, lyophilized, and stored frozen under argon.
C. Polymerization Via Photoinitiating Systems

The three-dimensional polymeric structures of the hydrogel are held together primarily by cross-linking. Photopolymerization is a common method of cross-linking for tissue engineering applications. (81) The clinical benefit of having a polymerizable hydrogel such as polyethylene glycol is that the polymer solution can easily be injected into the defect to be corrected and then photopolymerized in situ into a hydrogel. The need for complex surgical intervention would thus be eliminated. (77) Photopolymerization of poly(ethylene glycol) hydrogels involves exposing the polymer solution and photoinitiating system to ultraviolet light and allowing networks to form via free radical reactions of the acrylated end groups. Hence, it is important to note the effects of photoinitiating systems on cell viability.

i. Photoinitiating Systems

Various photoinitiators can be used to polymerize the poly(ethylene glycol) hydrogels. The ideal photoinitiator for the system however, is one that is cytocompatible with the cell type used. Three photoinitiating systems were studied for preadipocyte cytocompatibility. 2,2-dimethoxy-2-phenylacetophenone (Irgacure 651) has a cross-linking time of less than 60 seconds and has been shown to be compatible with endothelial cells. (48) 2-hydroxy-1-[4-(hydroxyethoxy)phenyl]-2-methyl-1-propanone (Darocur 2959) has a relatively long cross-linking time (>20 minutes) and has been shown to be compatible with chondrocytes, while 1-hydroxycyclohexyl phenyl ketone (Irgacure 184) has a cross-linking time less than 5 minutes and has been shown to be compatible with fibroblasts. (76) The structures of the photoinitiating systems are shown
in Figure 8. The next two sections describe the methods and results for a preliminary study conducted to determine preadipocyte viability in the presence of these three photoinitiators.

(a)

(b)
Figure 8. Chemical structures for the photoinitiators examined: (a) Irgacure 651, (b) Darocur 2959, and (c) Irgacure 184.

ii. Materials/Methods

Preadipocyte Isolation

Preadipocytes are isolated from epididymal fat pads of young male Lewis rats (250g, Harlan) via enzymatic digestion. Briefly, rats are euthanized with CO₂ asphyxiation and the shaved harvest site is sterilized with alcohol. Epididymal adipose tissue is aseptically harvested postmortem and placed in 4°C PBS supplemented with 500 U/mL penicillin and 500 μg/mL streptomycin (Gibco). Harvested adipose tissue is finely minced and enzymatically digested in Ca\(^{2+}\)/Mg\(^{2+}\)-free PBS supplemented with 2% (w/v) type I collagenase (Sigma Chemical Co.) and 5% (w/v) bovine serum albumin (BSA) for 20 minutes at 37°C on a shaker. The digested tissue is filtered through a 250 μm mesh followed by a 40 μm nylon mesh to separate undigested debris and capillary fragments from preadipocytes. The filtered cell suspension is centrifuged for 5 minutes at 4°C, 200
g. The resulting pellet of preadipocytes is then plated onto plastic culture flasks. Preadipocytes are cultured in Dulbecco’s Modified Eagle’s Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U/ mL penicillin, and 100 μg/mL streptomycin.

**Cytocompatibility of Photoinitiators with Preadipocytes**

Primary preadipocytes are seeded at 40,000 cells per well of 12-well plates in DMEM with serum. Three concentrations of each photoinitiator, varying from 0.025% (w/v) to 0.1% (w/v), are added to each plate. Control wells contained no photoinitiating system. Number of viable cells is recorded after two days using a Coulter cell counter (n=3/test condition). Viability of counted cells is verified with trypan blue exclusion.

**iii. Results**

**Cytocompatibility of Irgacure 651 with Preadipocytes**

Figure 9 illustrates that even minimal amounts of Irgacure 651 caused significant preadipocyte death over the control group. The cytoincompatibility of Irgacure 651 with preadipocytes makes this photoinitiator a poor choice for use in crosslinking our polymer for preadipocyte seeding.
Figure 9. Preadipocyte response to varying concentrations of Irgacure 651. Data are average cell count (n=3) ± standard error of the mean.

**Cytocompatibility of Darocur 2959 with Preadipocytes**

Figure 10 shows that Darocur 2959 is cytocompatible with preadipocytes at all concentrations tested. While the photoinitiator does not affect cell viability, the relatively lengthy crosslinking time makes this photoinitiator an unsuitable choice for use in the clinical setting.
Figure 10. Preadipocyte response to varying concentrations of Darocur 2959. Data are average cell count (n=3) ± standard error of the mean.

**Cytocompatibility of Irgacure 184 with Preadipocytes**

Figure 11 shows that Irgacure 184 is cytocompatible with preadipocytes for all concentrations tested. The crosslinking time is also reasonably short, which makes this photoinitiator an adequate photoinitiating system for preadipocyte seeding and polymer crosslinking.
Figure 11. Preadipocyte response to varying concentrations of Irgacure 184. Data are average cell count (n=3) ± standard error of the mean.

Based on the results of these cytocompatibility studies, Irgacure 184 was used in subsequent studies.
Chapter III. Physical Properties of Poly(ethylene glycol) Hydrogels and Adipose Tissue

In order to better understand the behavior of poly(ethylene glycol) hydrogels in varying physiological conditions, we must characterize the physical properties of the polymer and verify that they are qualitatively similar or superior to those of adipose tissue. Hydrogel swelling was measured since uptake properties are important to note as to accurately fill defects with the appropriate amount of polymer. The viscosity, elastic modulus, viscous modulus, and complex modulus of the hydrogel and adipose tissue were also measured as a function of varying shear rates, frequencies, and strains that the human body could experience on a daily basis. Additionally, the % recovery of the hydrogels and adipose tissue after deformation was measured as a function of strain. These properties can then determine whether the polymer is an appropriate material for initial implantation into the soft tissue defect.
A. Hydrogel Swelling

In order to determine the appropriate amount of poly(ethylene glycol) solution to inject into a defect site without overfilling, the amount of hydrogel swelling is necessary to calculate.

i. Materials/Methods

Poly(ethylene glycol) diacrylate is dissolved in Hepes buffered saline (pH 7.4) to obtain a solution that is 0.1 g/mL. Irgacure 184 (60 mg/mL in 100% ethanol) is added such that the photoinitiator concentration is 27 μL per mL of polymer solution. Varying amounts of this polymer solution is placed in a 48- or 96-well plate and exposed to ultraviolet light (10 mW/cm² at 365 nm) for 5 minutes, until polymerization is complete. Each hydrogel sample is weighed after polymerization and the weight is recorded as initial weight. The samples are then allowed to swell in buffered saline for 4 hours and the weight of each gel is again measured and recorded. The volume uptake of each sample is then determined using density calculations and the swelling properties can be determined as a function of surface area of hydrogel.
## ii. Results

Table 3. Results of hydrogel swelling experiment

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<th>Final Volume (µL)</th>
<th>% Volume Increase</th>
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**Average Swelling** 38.1%  
**SEM** 0.9%

Based on the results from the swelling experiments, we found that the amount of hydrogel swelling is independent of volume or initial surface area of the samples. There is a 38.1% ± 0.9% increase in the volume of each hydrogel after swelling is complete.
B. **Rheological Properties**

In order to successfully correct for soft tissue defects, one must ideally use scaffolds or implantable materials that possess physical properties similar to or superior to those of adipose tissue. The hydrogel must also be able to withstand forces experienced during normal daily activity. Hence, the rheological behavior of poly(ethylene glycol) hydrogels was investigated using an Advanced Rheometric Expansion System (ARES) and compared to the behavior of adipose tissue. These results are then used to determine whether the poly(ethylene glycol) hydrogels are adequate for initial implantation to correct for soft tissue defects. Steady and dynamic tests were run to determine the viscosity, elastic modulus, and viscous modulus at physiological conditions.

i. **Materials/Methods**

**Hydrogel Formation**

Hydrogels were formed as described in Section III.A. Briefly, poly(ethylene glycol) diacrylate is dissolved in Hepes buffered saline to obtain a solution that is 0.1 g/mL. Irgacure 184 (60 mg/mL in 100% ethanol) is added such that the photoinitiator concentration is 27 µL per mL of polymer solution. The polymer solution is injected into a 25 mm diameter mold (Figure 12) and exposed to ultraviolet light for 5 minutes, until polymerization is complete. The hydrogels are stored and allowed to swell in buffered saline until use.
Figure 12. Schematic of mold system used in formation of 25 mm diameter hydrogels.

**Adipose Tissue Acquisition**

Human adipose tissue from the abdomen of the female body was used for all tests. Tissue samples are incidental tissue remaining from reconstructive procedures. Tissue accrual adheres to Institutional and National Institutes of Health human subjects guidelines. Donor patients were 45-65 years in age and tissue samples were sectioned from approximately 2-4 mm below the dermis.

**Rheometric Testing**

Rheological measurements employ a strain-controlled ARES 100FRT (Advanced Rheometric Expansion System - TA Instruments, New Castle, DE) rheometer using the 25 mm parallel plate geometry (Figure 13). The viscosity (η), elastic modulus (G'), and viscous modulus (G'') are measured (n=6/test). Viscosity is described as “resistance to shear or flow” (82) and was measured with the motor in steady mode. The elastic modulus can be described at the “solid-like” component of the material and the viscous modulus can be described as the “liquid-like” component. (82) The complex modulus G*, which denotes firmness of the material, can also be determined for the samples. The
elastic and viscous moduli make up the real and imaginary parts of the complex modulus as shown below:

\[ G^* = G' + iG'' \quad (82) \]

Dynamic oscillatory modes are used to measure the elastic, viscous, and complex moduli. All tests are performed at 37.1 °C over a range of strains (up to 25-40% pending location) and frequencies (up to 50 Hz) that the human body physiologically experiences on a daily basis.

Figure 13. Illustration of parallel plate geometry used to determine rheometric properties of hydrogel.

ii. Results

Steady Rate Sweep Tests

One of the first properties determined during new material characterization is the relationship between viscosity and shear rate. A plot of viscosity (\( \eta \)) of poly(ethylene glycol) hydrogels and adipose tissue vs. shear rate (\( \gamma \)) (Figure 14) indicates that viscosity
decreases for both materials as the shear rate increases. This behavior is known as shear thinning and is common to many polymeric materials.

Figure 14. Viscosity vs. shear rate for poly(ethylene glycol) hydrogels and adipose tissue. Data shown are average viscosity ($n = 10$) ± standard error of the mean.

**Dynamic Frequency Sweep Tests**

Figure 15 plots the elastic modulus ($G'$) and viscous modulus ($G''$) of poly(ethylene glycol) hydrogels vs. frequency for strains ranging from 1% to 20%. The elastic modulus remains relatively constant over the strains and frequencies tested. In contrast, the viscous modulus increases as the strain increases over the range of frequencies tested. This indicates that the material is becoming firmer as the strain increases for the range of
frequencies tested. The viscous modulus at 1% strain is not shown on the plot due to limitations in the resolution of the measured torque of the rheometer.

![Graph showing elastic and viscous moduli](image)

Figure 15. Elastic and viscous moduli of poly(ethylene glycol) hydrogel vs. frequency for strains set at 1%, 5%, 10%, and 20%. Data are average moduli (n=6) ± standard error of the mean.

Figure 16 shows plots of elastic (G') and viscous moduli (G'') of human adipose tissue vs. frequency for strains ranging from 0.1 to 20%. The viscous modulus is relatively constant over the strains and frequencies tested. As the strain increases however, the elastic modulus decreases, indicating that the material is decreasing in firmness.
Figure 16. Elastic and viscous moduli of adipose tissue vs. frequency for strains set at 0.1%, 5%, 10%, and 20%. Data are average moduli (n=6) ± standard error of the mean.

**Dynamic Strain Sweep Tests**

The complex modulus ($G^*$) of poly(ethylene glycol) hydrogels vs. strain for frequencies set at $\pi$, $5\pi$, 10$\pi$, and 15$\pi$ Hz is illustrated in Figure 17. The complex modulus stays relatively constant below 1% strain for all frequencies tested. This is known as the Hookean region and indicates that the material is linearly viscoelastic under these conditions. Above 1% strain, the complex modulus becomes non-linear and the curves at constant strain begin to separate more as frequency increases. This trend also verifies that the material is becoming firmer as the strain increases.
Figure 17. Complex modulus of poly(ethylene glycol) hydrogels vs. strain for frequencies set at $\pi$, $5\pi$, $10\pi$, $15\pi$. Data are average moduli ($n=6$) ± standard error of the mean.

Figure 18 shows the complex modulus ($G^*$) of adipose tissue vs. strain for frequencies set at $\pi$, $5\pi$, $10\pi$, and $15\pi$ Hz. The complex modulus is not significantly different for each of frequencies tested and decreases with increasing strain. For the adipose tissue, the complex modulus shows no linear viscoelastic region as was seen for the hydrogels. These results indicate that the sample is becoming less firm (i.e., breaking apart) as strain and frequency increase.
Figure 18. Complex modulus of adipose tissue vs. strain for frequencies set at $\pi$, $5\pi$, $10\pi$, $15\pi$. Data are average moduli ($n=6$) ± standard error of the mean.
C. Recovery Properties

The ideal material for the correcting soft tissue defects should be able to recover to its original state after being subjected to deformation experienced on a daily basis. A creep indentation apparatus was used to measure the % recovery of poly(ethylene glycol) hydrogels. The % recovery of adipose tissue and skin was also measured for comparison.

i. Materials/Methods

Hydrogel Formation

Hydrogels disks were formed as described in Section III.B. Briefly, poly(ethylene glycol) diacrylate is dissolved in Hepes buffered saline to obtain a solution that is 0.1 g/mL. Irgacure 184 (60 mg/mL in 100% ethanol) is added such that the photoinitiator concentration is 27 μL per mL of polymer solution. The polymer solution is injected into a 25 mm diameter mold (Figure 12) and exposed to ultraviolet light for 5 minutes, until polymerization is complete. The hydrogels are allowed to swell in buffered saline and stored at room temperature until use.

Tissue Acquisition

Human skin and adipose tissue from the abdomen of the female body was used for all tests. Tissue samples are incidental tissue remaining from reconstructive procedures. Tissue accrual adheres to Institutional and National Institutes of Health human subjects guidelines. Donor patients were 45-65 years in age. Skin samples consisted of the epidermis and dermis only. Adipose tissue samples were sectioned from approximately 2-4 mm below the dermis.
Recovery Testing

Recovery properties are measured at varying strains using a Creep Indentation Apparatus (K. Athanasiou, Rice University, Houston, TX). Samples are exposed to a constant stress and the resulting strain is monitored (creep testing) to equilibrium. The deforming force is then removed and the recovery of the sample height at equilibrium is determined. The percent recovery is calculated based on the final deformation of the hydrogel as follows.

\[
\frac{\text{Initial deformation} - \text{Final deformation}}{\text{Initial deformation}} \times 100\% = \% \text{ Recovery}
\]

ii. Results

The percent recovery of hydrogels was measured when exposed to varying strains using a Creep Indentation Apparatus (Figure 19). As the hydrogel is exposed to greater deformations, the percent recovery to its original state is lessened (78% recovery after 15% deformation).
Figure 19. Percent recovery of poly(ethylene glycol) hydrogels from initial deformation as a function of strain.

Testing of skin and adipose tissue on the creep indentation apparatus resulted in no recovery of the tissue when exposed to even minimal deformations. Although hydrogels were not able to recover fully to their original state even after low deformation, these recovery properties are still superior to those of adipose tissue since the tissue was not able to recover over the range of deformations tested.
D. Discussion

poly(ethylene glycol) hydrogels were found to exhibit rheological properties that are qualitatively superior to human adipose tissue, thus making it a suitable material for initial correction of soft tissue defects. Results of the tests to determine the relationship between viscosity of poly(ethylene glycol) hydrogels and shear rate (Figure 14) indicate that the samples exhibit shear thinning (viscosity decreases as shear rate increases). There are two possible reasons for this phenomenon. First, as the shear rate increases, some of the forces holding the gel structure together could weaken and the viscosity of the hydrogel decreases as a result. Alternatively, as the shear rate increases, the polymer chains could become partially elongated and line up with the shearing streamlines, which would cause a decrease in viscosity. To determine which of these events was causing the shear thinning of the material, dynamic tests were conducted to determine the viscoelastic behavior of the material.

Dynamic testing indicates that at low strains, the hydrogel is predominantly elastic over the range of frequencies tested. As the strain is increased, the viscous modulus increases while the elastic modulus remains constant, indicating that viscous elements are beginning to take effect. Plotting the complex modulus $G^*$ vs. strain (Figure 17) shows that at strains above 1%, the complex modulus increases as the frequency increases. These trends indicate that the hydrogel is becoming firmer as strain and frequency are increased. This can be attributed to the large number of entanglements within the system. As the hydrogel deforms, the polymer chains may begin to elongate but overlapping chain entanglement causes increased firmness of the hydrogel. The greater loss in
elasticity at lower frequencies can also be attributed to the polymer chains having more
time to slip.

It is important to also note the recovery behavior of the hydrogel in order to determine if
the hydrogel has appropriate material properties for soft tissue defect correction. In other
words, the material must be able to withstand the daily forces exposed to the site of
correction. Exposing the poly(ethylene glycol) hydrogels to creep indentation followed
by recovery testing showed that as the hydrogel is deformed, the ability to recover to its
original state is lessened (Figure 19). Water loss resulting from creep indentation could
cause a decrease in the sample height. Greater deformations of the hydrogel could also
cause the polymer chains to break, which would result in the hydrogel being less able to
recover to its original state. However, the hydrogel is still able to recover to 78% of its
original state even after exposure to high strains, which makes it an acceptable material
for initial implantation into the site defect.

After subjecting adipose tissue to oscillatory testing, it was found to be viscoelastic,
much like many other biological tissues. The samples of adipose tissue tested exhibit
shear thinning which is similar to the behavior of the hydrogels. As with the hydrogels,
dynamic tests were also conducted to determine the reason for the decrease in viscosity
with increasing shear rates. From the dynamic oscillatory testing (Figures 16 and 18), we
found that the adipose tissue becomes less firm with increasing strains and frequencies.
These results indicate that the adipose tissue is being broken apart with increasing forces
on the material. Lipid lobules in adipose tissue are known to be susceptible to rupturing
with minimal forces. This is also confirmed with the creep indentation/recovery testing. When placed on the creep indentation apparatus, the adipose tissue samples were not able to recover after even the smallest deformations were imposed.

Due to the inability of adipose tissue to recover after being deformed, the recovery properties of skin were tested for comparison. The skin samples tested were also not able to recover after deformation. The age of our patient base was between 45 and 65 and hence, the skin samples received were not capable of recovering to its original state. This is attributed to the loss of collagen commonly occurring in an elder person’s skin.

A number of variables can be manipulated to alter the material properties of the hydrogels. For example, the material properties of the poly(ethylene glycol) hydrogels can be altered using varying concentrations of polymer solutions. The firmness of the hydrogel would increase with increasing polymer concentrations. This trait is attributed to the increase in the number of polymer chains in the system, which causes an increase in the number of entanglements in the system. Varying the polymer chain length at a given concentration will also affect the physical properties of the hydrogel. At a given concentration, increasing the polymer chain length results in fewer polymer chains in the system, which in turn causes a decrease the firmness of the hydrogel. Incorporation of degradation sites and adhesion ligands into the polymer (common in tissue engineering applications) influences chain length as well, and can affect the material properties.
In order to better understand the behavior of poly(ethylene glycol) hydrogels in varying physiological conditions, we characterized the physical properties of the polymer and verified that they are qualitatively superior to those of adipose tissue. The viscosity and elastic, viscous, and complex modulus of the hydrogel and adipose tissue were measured as a function of varying shear rates, frequencies, and strains that the human body could experience on a daily basis. Additionally, the percent recovery of the hydrogels and adipose tissue after deformation was measured as a function of strain. Results indicate that the hydrogels are able to withstand the range of frequencies and strains that it could potentially face on a daily basis when incorporated into the human body. Adipose tissue alone, however, breaks apart when exposed to these same forces. Based on the results from these tests, we then determined that the polymer is an appropriate material for initial implantation into the soft tissue defect.
Chapter IV. Poly(ethylene glycol)-based Hydrogels as a Scaffold for Preadipocytes

Preadipocyte adhesion, proliferation, and viability was tested in varying polymeric conditions in order to determine the optimal conditions for adipose tissue engineering. Four hydrogel configurations were seeded and studied: (1) nondegradable scaffold system, (2) nondegradable scaffold system containing cell-adhesive ligands, (3) degradable scaffold system, and (4) degradable scaffold system containing cell-adhesive ligands.
A. Preadipocyte Adhesion to YIGSR Ligands

i. Materials/Methods

Preadipocyte Isolation

Preadipocytes are isolated from epididymal fat pads of young male Lewis rats (250g, Harlan) via enzymatic digestion. Briefly, rats are euthanized with CO₂ asphyxiation and the shaved harvest site is sterilized with alcohol. Epididymal adipose tissue is aseptically harvested postmortem and placed in 4°C PBS supplemented with 500 U/mL penicillin and 500 μg/mL streptomycin (Gibco). Harvested adipose tissue is finely minced and enzymatically digested in Ca²⁺/Mg²⁺-free PBS supplemented with 2% (w/v) type I collagenase (Sigma Chemical Co.) and 5% (w/v) bovine serum albumin (BSA) for 20 minutes at 37°C on a shaker. The digested tissue is filtered through a 250 μm mesh followed by a 40 μm nylon mesh to separate undigested debris and capillary fragments from preadipocytes. The filtered cell suspension is centrifuged for 5 minutes at 4°C, 200 g. The resulting pellet of preadipocytes is then plated onto plastic culture flasks.

Preadipocytes are cultured in Dulbecco’s Modified Eagle’s Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 μg/mL streptomycin.

Hydrogel Formation

To verify preadipocyte adhesion to the YIGSR ligands in the scaffold, poly(ethylene glycol) diacrylate hydrogels are prepared with and without the cell-adhesion peptide YIGSR. Poly(ethylene glycol) diacrylate is dissolved in Hepes buffered saline (pH 7.4) to achieve a 10% w/v solution. For the system containing adhesion sites, poly(ethylene
glycol) coupled with YIGSR ligands is also added to achieve a 2.8 μmol per mL of polymer solution. Irgacure 184 (60 mg/mL in ethanol) is added to each system such that the photoinitiator concentration is 27 μL per mL of polymer solution. Results from the cytocompatibility study described above showed that Irgacure 184 is non-toxic to preadipocytes with the concentration employed. The polymer solution is placed in a 48-well plate at 200 μL/ well and exposed to ultraviolet light (10 mW/cm² at 365 nm) for 5 minutes. Hydrogels are incubated in DMEM with serum and allowed to swell at 37°C, 5% CO₂.

Adhesion Study

For the following study, preadipocytes are isolated as described above and used prior to confluence since contact inhibition initiates adipocytes differentiation and ceases preadipocyte proliferation. (83, 84). A solution of preadipocytes in DMEM with serum (1*10⁶ cells/mL) is placed on the top surface of each system of hydrogels formed for this study. The hydrogel-cell system is then incubated for 3 hours at 37°C, 5% CO₂. The hydrogels are removed from incubation and each sample is gently washed twice with sterile buffered saline to remove any free cells. The hydrogels are then viewed for preadipocyte attachment using a Nikon Eclipse E600 upright microscope and 20x objective. Eight images are taken per system using a Hamamatsu EB-CCD camera (C7190, Nikon) and MetaMorph software (Universal Imaging, Downingtown, PA). The number of cells attached per square area of each hydrogel system is then determined to assess the adhesive functionality of the YIGSR ligands used. The means of data measurements are presented. Error bars are calculated as the standard error of the mean.
ii. Results

Results indicate that the YIGSR binding peptide used in these studies are functional for preadipocyte adhesion. Preadipocytes did not adhere to the surface of the hydrogels without the adhesion ligands. Cells were however able to attach to the surface of the hydrogels containing YIGSR, with the surface concentration calculated to be 107 cells/mm² (Figure 20). Thus, the adhesive functionality of the YIGSR is proven for preadipocyte attachment to this peptide sequence.

![Figure 20](image)

Figure 20. Average concentration of preadipocytes (± standard error of the mean) on surface of hydrogels with and without the YIGSR adhesion ligand.
B. Preadipocyte Proliferation in Hydrogels

i. Materials/Methods

Preadipocyte Isolation

Preadipocytes are isolated from epididymal fat pads of young male Lewis rats (250g, Harlan) via enzymatic digestion. (50) Briefly, rats are euthanized with CO₂ asphyxiation and the shaved harvest site is sterilized with alcohol. Epididymal adipose tissue is aseptically harvested postmortem and placed in 4°C PBS supplemented with 500 U/mL penicillin and 500 μg/mL streptomycin (Gibco). Harvested adipose tissue is finely minced and enzymatically digested in Ca⁺²/Mg⁺²-free PBS supplemented with 2% (w/v) type I collagenase (Sigma Chemical Co.) and 5% (w/v) bovine serum albumin (BSA) for 20 minutes at 37°C on a shaker. The digested tissue is filtered through a 250 μm mesh followed by a 40 μm nylon mesh to separate undigested debris and capillary fragments from preadipocytes. The filtered cell suspension is centrifuged for 5 minutes at 4°C, 200 g. The resulting pellet of preadipocytes is then plated onto plastic culture flasks. Preadipocytes are cultured in Dulbecco’s Modified Eagle’s Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U/ mL penicillin, and 100 μg/mL streptomycin.

Hydrogel Formation

For the following studies, preadipocytes are isolated as described above and passaged prior to confluency since contact inhibition initiates adipocytes differentiation and ceases preadipocyte proliferation. (83, 84). Four configurations of scaffold systems are studied:
(1) nondegradable hydrogel, (2) nondegradable hydrogel incorporated with cell-adhesion peptides, (3) degradable hydrogel, and (4) degradable hydrogel incorporated with cell-adhesion peptides. For the first two configurations studied, nondegradable poly(ethylene glycol) diacrylate is dissolved in HEPES buffered saline (pH 7.4) at 0.2 g/mL. For the third and fourth configurations, poly(ethylene glycol) containing degradation sites is dissolved in HEPES buffered saline at 0.2 g/mL. A volume of solution of phenyl red-free DMEM and preadipocytes (1 X 10^6 cells/mL) is added equivalent to that of HEPES buffered saline added such that the final polymer concentration is 10% w/v. In hydrogel configurations (2) and (4), poly(ethylene glycol) coupled with YIGSR ligands is also added to achieve a 2.8 μmol per mL of cell-polymer solution. The photoinitiator, Irgacure 184 (60 mg/mL in 100% ethanol), is added to each system such that the photoinitiator concentration is 27 μL per mL of cell-polymer solution. Irgacure 184 was previously (Section II.C.) shown to be non-toxic to preadipocytes with the concentration employed. The cell-polymer solution is placed in a 96-well plate at 100 μL/well and exposed to ultraviolet light (10 mW/cm² at 365 nm) for 5 minutes, until polymerization is complete. Hydrogels are incubated in supplemented DMEM at 37°C, 5% CO₂.

**Pico Green Double-Stranded DNA Assay**

Hydrogel samples are analyzed in triplicate for DNA content at 0, 2, 4, and 6 days to assess cell proliferation. Samples from the *in vitro* experiments described above are digested at each time point in 250 μL of 0.5 N sodium hydroxide and neutralized, and the DNA content in the gels is then determined using a pico green double-stranded DNA assay (P-7581, Molecular Probes). This assay measures the amount of DNA in each
sample at the time of digestion and is related to the cell proliferation within each hydrogel. poly(ethylene glycol) hydrogels containing no cells are used as the negative control. Hydrogels containing known amounts of calf thymus DNA (D-4522, Sigma) are used as standards to determine the DNA content of the sample gels. Samples are analyzed according to the manufacturer’s instructions using a Bio-Rad fluorometer (excitation ~ 480 nm, emission ~ 520 nm).

**Statistical Analysis**

The means of data measurements are presented. Error bars are calculated as the standard error of the mean. Statistical significance is defined as $p \leq 0.05$ using a two-tailed, two-sample Student’s $t$-Test assuming unequal variance.

**ii. Results**

Hydrogel polymerizations for each of the configurations studied were successful and reproducible. DNA content in the nondegradable hydrogels does not increase over time as shown in Figure 21. Similarly, DNA content in nondegradable hydrogels containing YIGSR remains constant over the experimental time course (Figure 22). These results suggest that preadipocyte proliferation does not occur in these two polymer configurations. In contrast, the DNA content in the degradable hydrogels (without YIGSR) increases initially and then decreases to initial levels (Figure 23). Only DNA content at day 4 is statistically different when compared to day 0. Unlike the previous three polymer configurations, the DNA content in degradable gels with cell adhesion
sites increases four- to five-fold over the experimental time course (Figure 24). DNA content at days 4 and 6 are statistically different when compared to day 0.

Figure 21. DNA content in nondegradable gels vs. time. Data are average DNA content (n=3) ± standard error of the mean.
Figure 22. DNA content in nondegradable gels containing adhesion ligands vs. time.

Data are average DNA content (n=3) ± standard error of the mean.
Figure 23. DNA content in degradable gels vs. time. Data are average DNA content (n=3) ± standard error of the mean. (*) denotes statistical significance when compared to day 0.
Figure 24. DNA content in degradable gels containing adhesion ligands vs. time. Data are average DNA content (n=3) ± standard error of the mean. (*) denotes statistical significance when compared to day 0.

Cellular migration within a scaffold is also an important feature of development, tissue organization, and biological function. (85) Hence, the migratory process of preadipocytes within the degradable, adhesive hydrogel was observed to verify that the cells possess the necessary capabilities for tissue formation in this particular scaffold configuration. Cell processes are degrading the polymer network and extending through the hydrogel a distance equivalent to a cell diameter (Figure 25).
Figure 25. Image of preadipocytes migrating through a degradable poly(ethylene glycol) hydrogel containing YIGSR ligands (day 6).
C. Preadipocyte Viability in Hydrogels

i. Materials/Methods

Preadipocyte Isolation

Preadipocytes are isolated from epididymal fat pads of young male Lewis rats (250g, Harlan) via enzymatic digestion. Briefly, rats are euthanized with CO₂ asphyxiation and the shaved harvest site is sterilized with alcohol. Epididymal adipose tissue is aseptically harvested postmortem and placed in 4°C PBS supplemented with 500 U/mL penicillin and 500 μg/mL streptomycin (Gibco). Harvested adipose tissue is finely minced and enzymatically digested in Ca⁺²/Mg⁺²-free PBS supplemented with 2% (w/v) type I collagenase (Sigma Chemical Co.) and 5% (w/v) bovine serum albumin (BSA) for 20 minutes at 37°C on a shaker. The digested tissue is filtered through a 250 μm mesh followed by a 40 μm nylon mesh to separate undigested debris and capillary fragments from preadipocytes. The filtered cell suspension is centrifuged for 5 minutes at 4°C, 200 g. The resulting pellet of preadipocytes is then plated onto plastic culture flasks. Preadipocytes are cultured in Dulbecco’s Modified Eagle’s Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 μg/mL streptomycin.

Hydrogel Formation

For the following studies, preadipocytes are isolated as described above and passaged prior to confluence since contact inhibition initiates adipocytes differentiation and ceases preadipocyte proliferation. (83, 84). Four configurations of scaffold systems are studied: (1) nondegradable hydrogel, (2) nondegradable hydrogel incorporated with cell-adhesion
peptides, (3) degradable hydrogel, and (4) degradable hydrogel incorporated with cell-adhesion peptides. For the first two configurations studied, poly(ethylene glycol) diacrylate is dissolved in Hepes buffered saline (pH 7.4) at 0.2 g/mL. For the third and fourth configurations, poly(ethylene glycol) containing degradation sites is dissolved in Hepes buffered saline at 0.2 g/mL. A volume of solution of phenyl red-free DMEM and preadipocytes (1 X 10^6 cells/mL) is added equivalent to that of buffered saline added, such that the final polymer concentration is 10% w/v. In hydrogel configurations (2) and (4), poly(ethylene glycol) coupled with YIGSR ligands is also added to achieve a 2.8 \( \mu \text{mol per mL} \) of cell-polymer solution. The photoinitiator, Irgacure 184 (60 mg/mL in ethanol), is added to each system such that the photoinitiator concentration is 27 \( \mu \text{L per mL} \) of cell-polymer solution. The cell-polymer solution is placed in a 96-well plate at 100 \( \mu \text{L/well} \) and exposed to ultraviolet light (10 mW/cm^2 at 365 nm) for 5 minutes. Hydrogels are incubated in DMEM with serum at 37°C, 5% CO_2.

**Live/Dead Viability Stain**

A two-color fluorescence viability assay (L-3224, Molecular Probes) is used to demonstrate the viability of preadipocytes in all four described hydrogel configurations six days subsequent to polymerization. Hydrogels with cells are incubated for 45 minutes in a combined 2mM calcein AM and 2 mM ethidium homodimer solution in sterile phosphate buffered saline. The calcein AM permeates live cells and produces a bright green fluorescence (ex ~495 nm/em ~515 nm), while the ethidium homodimer penetrates cells with injured membranes and produces a red fluorescence (ex ~495 nm/em ~635 nm).¹⁹-²⁰ Samples are viewed using a Retiga EXi FAST Cooled Mono 12-bit camera.
(QImaging, Burnaby, B.C. Canada) and IPLab software (Scanalytics, Inc., Fairfax, VA) for image analysis. The number of live and dead/dying cells is recorded for five planes in each of three samples for each hydrogel configuration and the percent live cells is then calculated.

ii. Results

Results indicate that as many as 90% of the cells in the hydrogels containing cell-adhesion ligands, configurations (2) and (4), remained viable for the duration of the experiment. Viability within the hydrogel configurations without YIGSR ligands was approximately 60% for the degradable scaffold and less than 10% in the nondegradable scaffold (Figure 26).

![Graph of % Live Cells over Time](image)

Figure 26. Average percent of viable cells (± standard error of the mean) in each hydrogel configuration after six days.
D. Discussion

This chapter concludes that a photopolymerizable poly(ethylene glycol) hydrogel system is able to act as a three-dimensional scaffold system to aid in the support and proliferation of preadipocytes. This polymer meets our initial specifications for an ideal scaffold material because it can be modified to incorporate the necessary degradation sites and adhesion ligands to accommodate cellular adhesion and proliferation which is crucial to cell function. The preadipocytes can be mixed into a poly(ethylene glycol) solution and then photopolymerized into a hydrogel containing cells in suspension. Three derivatives of poly(ethylene glycol) were fabricated: poly(ethylene glycol) diacrylate which is nondegradable, poly(ethylene glycol) incorporated with a collagenase-sensitive degradable sequence, and poly(ethylene glycol) coupled with cell-adhesion ligands. Four variations of scaffold configurations were created using these three polymer derivatives: nondegradable scaffold, nondegradable scaffold containing cell-adhesion ligands, degradable scaffold, and degradable scaffold containing cell-adhesion ligands. Adhesion, proliferation, and viability assays were completed to determine which scaffold configuration is best suitable for preadipocyte growth.

Next, the proliferative capabilities of preadipocytes within the hydrogels were assessed. DNA assays demonstrated that preadipocytes are anchorage dependent cells. In other words, cell viability and proliferation are dependent on the adhesion to the underlying substratum. One of the first studies performed was to assess the adhesivity of the incorporated ligand. It was observed that preadipocytes were able to adhere to hydrogels containing YIGSR within three hours.
Preadipocytes did not proliferate while in the nondegradable poly(ethylene glycol) scaffold due to the lack of degradation sites. In order for cells to undergo morphological changes during proliferation, there must be adequate room. Nondegradable poly(ethylene glycol) hydrogels do not provide the additional space required for continued cell proliferation. In addition, due to the absence of adhesion sites, cells cannot adhere to the polymer to provide traction for cell division. Moreover, DNA content in nondegradable hydrogels containing YIGSR remained constant during the course of the study. Cell-adhesion sites allowed the preadipocytes to bind to the hydrogel, but due to the nondegradable nature of the network, the cells are contact inhibited and no longer proliferate.

In contrast, the DNA content in the degradable scaffold increases initially, but subsequently decreases to initial levels. Serum protein from the DMEM used to form the hydrogels permits temporary preadipocyte binding and initial degradation of the network by cellular secretion of collagenase results in initial proliferation. As degradation continues to occur, the serum protein is no longer entrapped, thus cell proliferation decreases. As a result, there is initial cell proliferation followed by cell death, as the hydrogel cannot support continued cell adhesion. Only when poly(ethylene glycol) scaffolds containing both cell-adhesion ligands and degradation sequences does the DNA content increase steadily over the course of the study. Cells are able to attach to the supporting network, proliferate, and secrete collagenase to degrade the polymer. Hence a four-to-five-fold increase in cell growth is observed over the course of the week.
Results of *in vitro* experiments indicate that degradable networks allow for cellular growth while nondegradable networks do not. In addition, incorporating cell-adhesion sites are necessary for supporting cell growth. Results also demonstrate that preadipocyte adhesion and the sustenance of cellular growth depend upon both the degradation and adhesion sites in the system. Combining the degradable poly(ethylene glycol) with the polymer coupled with cell-adhesion sites produces a synthetic hydrogel scaffold that has been shown to promote preadipocyte proliferation and viability.

Poly(ethylene glycol) modified with LGPA and YIGSR peptide sequences proves to be a suitable biocompatible material for this application. Poly(ethylene glycol) is also relatively cost effective and may ideally provide longer term results than current methods of repairing defects. It is easily injectable and can be polymerized and molded to fill the defect volume *in vivo*, which makes it an ideal material to be used in the clinical setting.
Chapter V. Bioreactor Study

preadipocytes must also be able to differentiate in the hydrogel scaffold in order to eventually form adipose tissue. Preadipocytes begin to lipid load once the differentiation process has begun. The increase in lipid content causes the cells to become more buoyant and hence ‘float’ out of the scaffold in static culture. A bioreactor can however be used to contain the cell-polymer sample in a single entity so that differentiated cells can be monitored. Bioreactors are already being extensively used to culture various cell types in suspension. This chapter uses a Rotating Cell Culture System (RCCS) to verify that preadipocytes are able to differentiate within a poly(ethylene glycol) scaffold modified with adipogenic factors such as adhesion ligands and degradation sites for adipose tissue engineering.
A. Rotating Cell Culture System

The Rotating Cell Culture System (RCCS, Synthecon, Houston, TX) has been used to date to culture hepatocytes, (86-88) skeletal muscle satellite cells, (89) chondrocytes, mesenchymal progenitor cells, and cardiac myocytes. (90, 91) Studies have also been conducted to grow bone tissue on poly(lactic-co-glycolic) acid scaffolds. (92) This chapter investigates the use of the RCCS for monitoring preadipocyte differentiation in a poly(ethylene glycol) hydrogel scaffold for adipose tissue engineering.

The benefit of using the RCCS for tissue engineering is that cells in the scaffold can be grown in free suspension with low shear forces and higher mass transfer of nutrients. The change in boundary layer conditions from a two-dimensional to a three-dimensional system increases mass transport and reduces shear stress on the sample. (93) The RCCS is used to establish “suspension modality” and hence, if the cells in the scaffold can be grown without the influence of gravity, they may assemble and form tissue that more closely resembles tissue in the body. (94) The hydrogels are essentially in a freefall state. (93) The low gravity environment provides minimal shear forces (0.2 dyn/cm²) (95) on the cells as the media and gel rotate as a single solid body. (89) Tissue growth however, is limited in size by mass transfer in the bioreactor.

In this chapter, preadipocyte differentiation within a modified poly(ethylene glycol) hydrogel is measured in a three-dimensional rotating tissue culture vessel in order to contain the cell-polymer sample as a single entity and mimic the in vivo effects on the scaffold. In previous static studies, preadipocyte proliferation was found to be optimal in
poly(ethylene glycol) incorporated with degradation sites and cellular adhesion ligands. For these experiments, this optimal scaffold configuration is studied for differentiation in the RCCS. The control set for these studies is nondegradable poly(ethylene glycol) containing cellular adhesion ligands, which has been found to promote preadipocyte adhesion to the scaffold but not cellular proliferation. The hydrogels seeded with preadipocytes and adipogenic factors are suspended in media by constant turning of the bioreactor vessel.

Figure 27. Schematic of Synthecon Rotary Cell Culture System.

i. **Materials/Methods**

Preadipocyte differentiation within the hydrogel will be measured in a three-dimensional rotating tissue culture vessel. Two configurations were studied: (1) nondegradable hydrogels containing cell adhesion ligands and (2) degradable hydrogels containing cell adhesion ligands. The hydrogels seeded with preadipocytes and adipogenic factors in dynamic culture are suspended in media by constant turning of the bioreactor vessel.
Preadipocyte Isolation

Preadipocytes are isolated from epididymal fat pads of young male Lewis rats (250g, Harlan) via enzymatic digestion. (50) Briefly, rats are euthanized with CO₂ asphyxiation and the shaved harvest site is sterilized with alcohol. Epididymal adipose tissue is aseptically harvested postmortem and placed in 4°C PBS supplemented with 500 U/mL penicillin and 500 μg/mL streptomycin (Gibco). Harvested adipose tissue is finely minced and enzymatically digested in Ca⁺²/Mg⁺²-free PBS supplemented with 2% (w/v) type I collagenase (Sigma Chemical Co.) and 5% (w/v) bovine serum albumin (BSA) for 20 minutes at 37°C on a shaker. The digested tissue is filtered through a 250 μm mesh followed by a 40 μm nylon mesh to separate undigested debris and capillary fragments from preadipocytes. The filtered cell suspension is centrifuged for 5 minutes at 4°C, 200 g. The resulting pellet of preadipocytes is then plated onto plastic culture flasks.

Preadipocytes are cultured in Dulbecco’s Modified Eagle’s Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U/ mL penicillin, and 100 μg/mL streptomycin. A cell concentration of 1 X 10⁶ primary preadipocytes/mL is used in the differentiation within the nondegradable hydrogel study. A cell concentration of 1 X 10⁷ primary preadipocytes/mL is used in the differentiation within the degradable hydrogel study. A higher concentration is used in this latter study in order to determine whether cells will undergo adipogenesis once in contact with other cells. The lower concentration of cells used in the nondegradable scaffold in order to show that these cells differentiate independently of contact with other cells in the hydrogel.
Hydrogel Formation

Preadipocytes are isolated as described above and passaged prior to confluency since contact inhibition initiates adipocytes differentiation and ceases preadipocyte proliferation. (83, 84). Two configurations of scaffold systems are studied: (1) nondegradable hydrogel incorporated with cell-adhesion peptides, and (2) degradable hydrogel incorporated with cell-adhesion peptides. For the first configuration studied, nondegradable poly(ethylene glycol) is dissolved in HEPES buffered saline (pH 7.4) at 0.2 g/mL. For the second configuration, poly(ethylene glycol) containing degradation sites is dissolved in HEPES buffered saline at 0.2 g/mL. A volume of solution of phenyl red-free DMEM and preadipocytes is added equivalent to that of HEPES buffered saline added such that the final polymer concentration is 10% w/v. In both hydrogel configurations, poly(ethylene glycol) coupled with YIGSR ligands is also added to achieve a 2.8 μmol per mL of cell-polymer solution. The photoinitiator, Irgacure 184 (60 mg/mL in 100% ethanol), is added to each system such that the photoinitiator concentration is 27 μL per mL of cell-polymer solution. Irgacure 184 was previously shown to be non-toxic to preadipocytes with the concentration employed. The cell-polymer solution is placed in a half-area 96-well plate at 50 μL/well and exposed to ultraviolet light (10 mW/cm² at 365 nm) for 5 minutes, until polymerization is complete. Hydrogels are incubated in supplemented DMEM at 37°C, 5% CO₂.

Bioreactor Operating Parameters

A 50-mL vessel with two syringe ports (for feeding and sampling) and an oxygenator membrane (for oxygen diffusion into the media) is used to incubate the hydrogels seeded
with preadipocytes in supplemented DMEM. Fifteen samples are placed in each bioreactor. The bioreactors are kept in a standard incubator with 95% air and 5% CO₂, and constant atmosphere is maintained at a temperature of 37°C. Feeding and sampling are performed every other day under sterile conditions in a standard biological hood. Hydrogels are maintained in suspension by equalizing the sedimentation gravity with the vessel rotational velocity (20-30 rpm). (86)

**Differentiation Assay**

Cell differentiation is monitored using the lipid stain Oil Red O. The hydrogels are placed in formalin at each time point to fix the cells. The samples are removed from the formalin, washed twice in distilled water, and once in 70% ethanol. The samples are then covered with Oil Red O and allowed to sit for 10 minutes. Finally, samples are rinsed once with ethanol and once with distilled water. Stained samples are viewed at 40x using bright field microscopy, IPLab software, and a RT SPOT camera.

**ii. Results**

Static *in vitro* studies showed that poly(ethylene glycol) alone does not permit preadipocyte adhesion or proliferation. The incorporation of adhesion ligands into the scaffold allows for preadipocyte adhesion and the addition of degradation sites further allows for cellular proliferation. In this chapter, differentiation of preadipocytes in modified poly(ethylene glycol) scaffolds was monitored in a three-dimensional rotating bioreactor system.
Figure 28 quantitatively compares the lipid loading within the preadipocytes, which indicates differentiation into mature adipocytes. If the cell concentration within the degradable hydrogel is such that the cells are not in contact with other cells, the preadipocytes do not differentiate. For this reason a higher concentration of cells is used and the data set ‘degradable + YIGSR’ is the percent of cells that are only in contact with other cells that contain lipid. A lower concentration of cells is used in the ‘nondegradable + YIGSR’ set to show that the cells in this hydrogel differentiate independently of contact with other cells. In Figure 28, the ‘nondegradable + YIGSR’ set is the percent of the total cells in a plane of view that contain lipid. None of the cells in this set were in contact with other cells.

![Bar graph showing lipid loading over time](image)

**Figure 28.** Average percent (± standard error of the mean) of cells containing lipid. Degradable+YIGSR set is % of cells in contact in degradable scaffold containing lipid. Nondegradable+YIGSR set is % of total cells in nondegradable scaffold containing lipid.
Figure 29. Differentiated preadipocytes (day 2) within a nondegradable poly(ethylene glycol) hydrogel containing YIGSR adhesion ligands. Cells differentiate due to contact inhibition from the nondegradable network.

Figure 30. Differentiated preadipocytes (day 6) within a degradable poly(ethylene glycol) hydrogel containing YIGSR adhesion ligands. Only cells contact inhibited by other surrounding cells have begun to differentiate.
B. Discussion

Preadipocytes were previously found to proliferate in the hydrogel configuration containing degradation sites and adhesion ligands over the nondegradable hydrogel configuration. Preadipocyte proliferation was not seen in the nondegradable, cell-adhesive scaffold, however, cells were able to remain viable in the hydrogel. Cell-adhesion sites allow for the preadipocytes to bind to the hydrogel, but due to the nondegradable nature of the network, the cells are barred by the polymer and do not proliferate. Hence, the cell number was found to remain constant over the course of the week (Section IV.A.ii.).

Proliferation is not the only cellular function necessary in the tissue engineering of fat. The preadipocytes must also be able to differentiate in the hydrogel system in order to eventually form adipose tissue. This cellular function was tested using a bioreactor. Previous work (Section IV.A.ii.) has shown that the cells do not proliferate, but remain viable and differentiate in nondegradable hydrogels containing adhesion ligands. Previous work also indicated that the cells do not differentiate in degradable hydrogels with adhesion ligands if the cellular concentration was such that the cells were not in contact with each other. In order to determine if cells are able to differentiate in the degradable hydrogels containing adhesion ligands, a high concentration of preadipocytes was used. Results indicate that the cells that are in contact with other cells in these scaffolds begin to lipid load. Hence, once the cells are in a highly concentrated area, they are able to undergo adipogenesis. From the results, we find that preadipocytes are able to differentiate in a modified poly(ethylene glycol) scaffold containing adipogenic factors.
Chapter VI. Concluding Remarks
The ultimate goal of this research is to develop an injectable cell-scaffold system capable of permitting adipogenesis to abrogate soft tissue deficiencies resulting from trauma, tumor resection, and congenital abnormalities. The focus of this thesis is to fabricate a series of hydrogels with adipogenic factors, characterize the polymer and compare the material properties of the hydrogel with adipose tissue to determine whether it is a suitable material for implantation, and demonstrate that this hydrogel system allows for preadipocyte adhesion, proliferation, viability, and differentiation.

Contemporary research includes the use of scaffolds such as poly(L-lactic-co-glycolic-acid) (PLGA) polymer foams, collagen hydrogels, and porous alginate material for adipose tissue engineering. While materials such as PLGA foams are too rigid for soft tissue deficit correction, injectable hydrogel materials that are polymerizable in vivo are more appropriate for this application. The goal of this research is to initially provide a material, poly(ethylene glycol), to act as a support system for preadipocyte adhesion and growth, with the understanding that it will remodel with time and be replaced eventually with adipose tissue. Poly(ethylene glycol) is also relatively cost effective and may ideally provide longer term results than status quo methods of repairing defects. It is easily injectable and can be polymerized and molded to fill the defect volume in vivo, which makes it an ideal material to be used in the clinical setting.

Poly(ethylene glycol) modified with LGPA and YIGSR peptide sequences proves to be a suitable biocompatible material for this application. Poly(ethylene glycol) hydrogels have already been successfully derivatized for other cell systems. For example,
endothelial cells can bind to the peptide sequence arginine-glycine-aspartine-serine (RGDS) found on fibronectin. Other adhesion peptide sequences include VAPG found on elastin and KQAGDV found on fibrinogen. Some examples of degradation sequences are NRV, AAAAAAAA, and AAPF, which are degradable by plasmin, elastase, and cathepsin G, respectively. For preadipocyte adhesion, the peptide sequence YIGSR was used to modify poly(ethylene glycol). To develop a scaffold system degradable by collagenase (which is secreted by preadipocytes), the peptide sequence LGPA was used to derivatize poly(ethylene glycol).

Various photoinitiators can also be used to polymerize the poly(ethylene glycol) hydrogels. The ideal photoinitiator for the system however, is one that is cytocompatible with the cell type used. For example, Irgacure 184 is known to be cytocompatible with fibroblasts, while Irgacure 651 is cytocompatible with endothelial cells and Darocur 2959 with chondrocytes. These three photoinitiators were tested on preadipocytes in vitro. Both Irgacure 184 and Darocur 2959 were found to permit preadipocyte viability, whereas Irgacure 651 caused preadipocyte death immediately upon contact. Based on the shorter crosslinking time (which is ideal for clinical settings), Irgacure 184 was chosen to use as the crosslinking agent.

In order to successfully design a scaffold to correct for soft tissue defects, one must characterize and understand the physical properties of the implantable material. Swelling properties of the hydrogel were calculated to determine the appropriate amount of polymer solution necessary to correct a defect without overfilling. The rheological and
recovery behavior of poly(ethylene glycol) hydrogels and adipose tissue was also investigated using an Advanced Rheometric Expansion System and Creep Indentation Apparatus. Results from these studies indicate that poly(ethylene glycol) is able to withstand daily strains and frequencies more so than adipose tissue, and thus is a suitable material to fill wrinkles and other soft tissue defects.

The present work also compared the efficacy of photopolymerizable poly(ethylene glycol) and specific derivatives as a scaffold for preadipocyte adhesion, viability, and proliferation. Four variations of a poly(ethylene glycol) scaffold were prepared and examined. The first scaffold consisted of poly(ethylene glycol) diacrylate that is not susceptible to hydrolysis or enzymatic degradation. Preadipocyte death was observed over one week in this hydrogel configuration. Adhesion sites, specifically the laminin binding peptide sequence YIGSR, were incorporated into the second scaffold to promote cellular adhesion as a prerequisite for preadipocyte proliferation. Preadipocytes remained viable in this scaffold system, but did not proliferate in this nondegradable hydrogel. The third scaffold system studied consisted of poly(ethylene glycol) modified with the peptide sequence LGPA to permit polymer degradation by cell-secreted collagenase. No cell adhesion peptide was incorporated into this scaffold system. Cellular proliferation was initially observed, followed by cell death. The previous three scaffold configurations did not permit preadipocyte adhesion and proliferation. In contrast, the fourth system studied, poly(ethylene glycol) system modified to incorporate both LGPA and YIGSR, permitted preadipocyte adherence and proliferation subsequent to polymer degradation. Our results indicate that a scaffold system containing specific degradation sites and cell
adhesion ligands permits cells to adhere and proliferate, thus providing a potential cell-scaffold system for adipogenesis.

Bioreactors are being extensively used to culture various cell types in suspension. This thesis also focused on using a Rotating Cell Culture System (RCCS) to monitor preadipocyte differentiation within a poly(ethylene glycol) scaffold modified with adipogenic factors such as adhesion ligands and degradation sites for adipose tissue engineering. Results indicated that the preadipocytes seeded in the nondegradable poly(ethylene glycol) scaffold modified adhesion ligands were able to differentiate, but not able to proliferate over the course of the study. Cells in the polymer system modified with both adhesion ligands and degradation sites were able to proliferate in the scaffold as the polymer chains break down and differentiate once in contact with surrounding cells, hence indicating that the poly(ethylene glycol) scaffold system is a suitable material for adipogenesis.

The generation of adipose tissue using novel biomaterials has the potential to change the way reconstructive surgery is practiced, as well as increase patients’ quality of life. The fabrication of materials specific to adipose tissue is in its infancy. Current scaffolding strategies show promise for adipose tissue growth, but are limited by the level of vascular support of the scaffold. An adequate blood supply must be established to accompany de novo adipose tissue formation. Future studies must also address the long-term maintenance of newly generated adipose tissue as well as the development of appropriate large animal models to assess adipose tissue engineering strategies.
REFERENCES


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