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Poly(ethylene glycol) Hydrogel System Supports Preadipocyte Viability, Adhesion, and Proliferation

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ABSTRACT

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 present work compares the efficacy of photopolymerizable poly(ethylene glycol) and specific derivatives as a scaffold for preadipocyte (adipocyte precursor cell) viability, adhesion, and proliferation. Four variations of a poly(ethylene glycol) scaffold are prepared and examined. The first scaffold consists of poly(ethylene glycol) diacrylate, which is not susceptible to hydrolysis or enzymatic degradation. Preadipocyte death is observed over 1 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 remain viable in this scaffold system, but do not proliferate in this nondegradable hydrogel. The third scaffold system studied consists of poly(ethylene glycol) modified with the peptide sequence LGPA to permit polymer degradation by cell-secreted collagenase. No adhesion peptide is incorporated into this scaffold system. Cellular proliferation is initially observed, followed by cell death. The previous three scaffold configurations do not permit preadipocyte adhesion and proliferation. In contrast, the fourth system studied, poly(ethylene glycol) modified to incorporate both LGPA and YIGSR, permits 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.

INTRODUCTION

More than 1 million reconstructive, correctional, and cosmetic procedures are performed every year to repair dermal defects. Although many natural, synthetic, and hybrid materials are currently used clinically to correct contour deficiencies, none has proven to be the ideal material for soft tissue repair. Many of these 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.[1] The large number of soft tissue surgeries and the inadequacies of contemporary standard of care to correct soft tissue de-

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fects impact the national healthcare system. Moreover, patients are demanding more optimal outcomes and desire to use autologous cells.

Various adipose tissue-engineering strategies are currently being investigated as a means to repair soft tissue defects. Scaffold materials for tissue-engineering applications must be able to mechanically support and guide tissue formation. Materials must also be biocompatible, biodegradable, easily processed,[2,3] resistant to mechanical strain, and easily shaped to the surgeon's specifications.[4] In addition, materials must permit variability in shape and volume in order to personalize the scaffold to meet the patient's specific contour and volume needs. Ideally, the scaffold material should recapitulate the endogenous extracellular matrix in structure, support, and function.

This article discusses the use of photopolymerizable poly(ethylene glycol) hydrogels as scaffolds for adipose tissue-engineering applications. Poly(ethylene glycol) hydrogels are viscoelastic, three-dimensional polymeric structures that contain a significant volume fraction of water, usually >90%, and are held together primarily by covalent cross-linking. Photopolymerization of the hydrogels involves exposing the precursor polymer solution to an ultraviolet (uv) light source and allowing networks to form via free radical polymerization.[5] Photopolymerizable hydrogels are used extensively in medicine for biomedical applications[6] because of their ability to be cross-linked quickly *in situ*. Poly(ethylene glycol) and its derivatives have already been investigated as potential scaffold materials for bone[7] and cartilage tissue engineering,[8–10] and have been used in wound healing[2] and for the treatment of enzyme deficiencies.[11]

The present *in vitro* work investigates the use of diacrylated poly(ethylene glycol), a photopolymerizable hydrogel, as a scaffold for adipose tissue engineering. Poly(ethylene glycol) was chosen for this application because of its inherent biocompatibility and ability to permit tailored chemical modification, namely the addition of active cell adhesion and degradation sites.[12] Hydrogel systems made of nondegradable and degradable poly(ethylene glycol) with and without cell adhesion ligands were studied in this report to evaluate preadipocyte viability, adhesion, and proliferation within each scaffold type.

MATERIALS AND METHODS

Preadipocyte isolation and culture

Preadipocytes were isolated from epididymal fat pads of male Lewis rats (250 g; Harlan, Indianpolis, IN) via enzymatic digestion and sequential filtering as previously described.[13] Briefly, rats were killed by CO_2 asphyxiation and each prepared harvest site was sterilized with alcohol. Epididymal adipose tissue was aseptically harvested postmortem and placed in 4°C phosphate-buffered solution (PBS) supplemented with penicillin (500 U/mL) and streptomycin (500 μg/mL) (Gibco; Invitrogen, Carlsbad, CA). Harvested adipose tissue was finely minced and enzymatically digested in Ca^{2+}/Mg^{2+}-free PBS supplemented with 2% (w/v) type I collagenase (Sigma, St. Louis, MO) and 5% (w/v) bovine serum albumin for 20 min at 37°C on a shaker. The digested tissue was sequentially filtered through 250- and 40-μm pore size nylon meshes to separate undigested debris and capillary fragments from preadipocytes. The filtered cell suspension was centrifuged at 200 $\times$ *g* for 5 min at 4°C. The resulting pellet of preadipocytes was then plated at one-third confluency onto plastic culture flasks and preadipocytes were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, penicillin (100 U/mL), and streptomycin (100 μg/mL). The preadipocytes were supplied with fresh DMEM every other day. Preadipocytes were used before confluency because contact inhibition initiates adipocyte differentiation and ceases preadipocyte proliferation.[14–17] A cell concentration of 1×10^6 primary preadipocytes per milliliter was used in the following studies.

Synthesis of poly(ethylene glycol) diacrylate

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

Preparation of diacrylated poly(ethylene glycol) derivatives containing degradable sequences

Preadipocytes have been shown to secrete collagenase to remodel their natural environment. Thus, a collagenase-sensitive polymer was prepared. Degradable poly (ethylene glycol) was 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 (GGLGPAGGK) at a 2:1 polymer–peptide molar ratio. The polymer-peptide mixture was then dissolved in 50 mM sodium bicarbonate (pH 8.5) and allowed to react for 2 h. The solution was filter sterilized, lyophilized, and stored frozen under argon. The resulting poly(ethylene glycol) derivative was a block copolymer, ABA, where A is poly(ethylene glycol) and B is the collagenase-sensitive peptide. The poly(ethylene glycol) derivative was terminated with acrylated groups for photocross-linking (Fig. 1A). An *in vitro* degradation assay was used to validate the specific degradation of this polymer.

Preparation of monoacrylated poly(ethylene glycol) derivatives containing cell adhesion ligands

Preadipocyte adhesion sites can also be coupled to poly(ethylene glycol), using the peptide sequence tyrosine-isoleucine-glycine-serine-arginine (YIGSR) (Fig. 1B). YIGSR is one of many cell-binding peptides found on laminin-1. Patrick and Wu have shown that preadipocytes bind preferentially to laminin-1 and that cell adhesion and migration on laminin-1 are mediated by the $\alpha_1\beta_1$ integrin.[18] Acryl-PEG-NHS was combined with YIGSR at a 1:1 polymer:peptide molar ratio and then dissolved in 50 mM sodium bicarbonate (pH 8.5) and allowed to react for 2 h. The solution was filter sterilized, lyophilized, and stored frozen under argon.

Photoinitiator cytocompatibility with preadipocytes

Various photoinitiators can be used to polymerize 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,[9] whereas Irgacure 651 is cytocompatible with endothelial cells[3] and Darocur 2959 is cytocompatible with chondrocytes.[9] These three photoinitiators were tested for cytocompatibility with preadipocytes. Primary preadipocytes were seeded at 40,000 cells per well of 12-well plates in DMEM with serum. Three concentrations of each photoinitiator, varying from 0.025 to 0.1% (w/w), were added to each plate. Control wells contained no photoinitiating system. The number of viable cells was recorded after 2 days, using trypan blue exclusion and a Coulter cell counter ($n = 3$ per test condition).

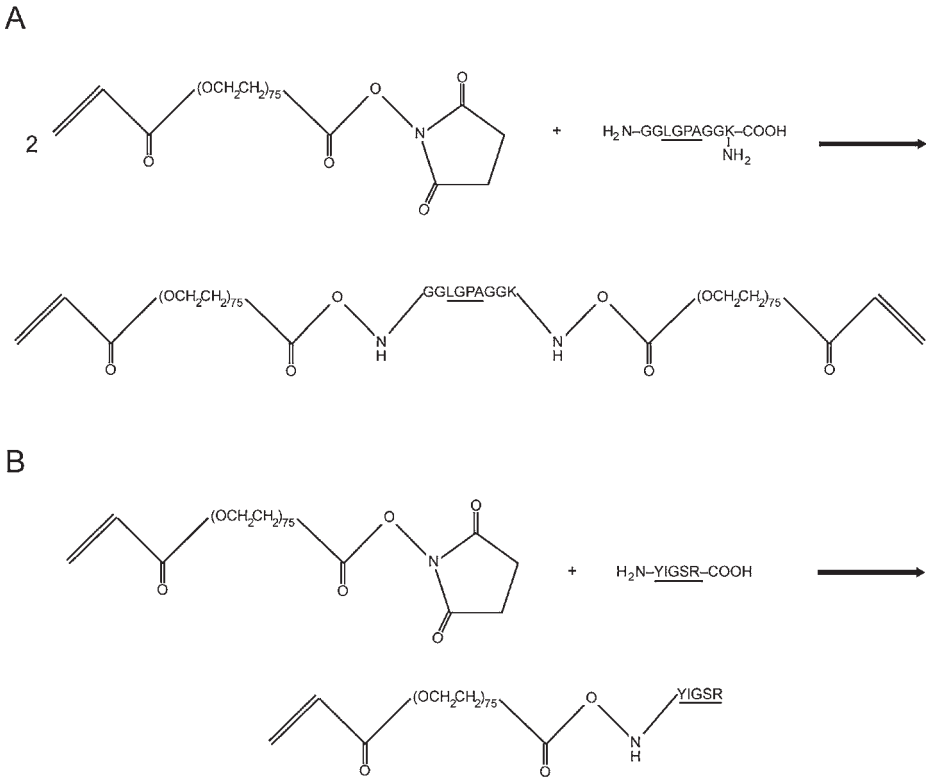

FIG. 1. (**A**) Coupling of collagenase-sensitive peptide, LGPA, with acryl-PEG-NHS to form a degradable hydrogel, ABA block copolymer, terminated in acrylate groups for photopolymerization. (**B**) Coupling of cell-adhesive peptide, YIGSR, with acryl-PEG-NHS to facilitate preadipocyte binding to hydrogel.[21]

Hydrogel formation for adhesion study

To verify preadipocyte adhesion to the YIGSR ligands in the scaffold, poly(ethylene glycol) diacrylate hydrogels were prepared with and without the cell adhesion peptide YIGSR. Poly(ethylene glycol) diacrylate was 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 was also added to achieve a 2.8-μmol/mL polymer solution. Irgacure 184 (60 mg/mL in ethanol) was added to each system such that the photoinitiator concentration was 27 μL/mL of polymer solution. Results from the cytocompatibility study described above showed that Irgacure 184 was nontoxic to preadipocytes at the concentration employed. The polymer solution was placed in a 48-well plate at 200 μL/well and exposed to ultraviolet light (10 mW/cm^2 at 365 nm) for 5 min. Hydrogels were incubated in DMEM with serum and allowed to swell at 37°C, 5% CO_2.

Adhesion study

A solution of preadipocytes in DMEM was placed on the top surface of each system of hydrogels formed for this study. The hydrogel–cell system was then incubated for 3 h at 37°C, 5% CO_2. The hydrogels were removed from incubation and each sample was gently washed twice with sterile buffered saline to remove any free cells. The hydrogels were then viewed for preadipocyte attachment, using an Eclipse E600 upright microscope and $\times$20 objective (Nikon, Tokyo, Japan). Eight images were 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 was then determined to assess the adhesive functionality of the YIGSR ligands used.

Hydrogel formation for viability and proliferation studies

Four configurations of scaffold systems were 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 was 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 was dissolved in HEPES buffered saline at 0.2 g/mL. A volume of solution of phenyl red-free DMEM and preadipocytes (1×10^6 cells/mL) was added equivalent to that of the buffered saline added, such that the final polymer concentration was 10% (w/v). In hydrogel configurations 2 and 4, poly(ethylene glycol) coupled with YIGSR ligands was also added to achieve a 2.8-μmol/mL cell–polymer solution. The photoinitiator, Irgacure 184 (60 mg/mL in ethanol), was added to each system such that the photoinitiator concentration was 27 μL/per mL of cell–polymer solution. The cell–polymer solution was placed in a 96-well plate at 100 μL/well and exposed to ultraviolet light (10 mW/cm^2 at 365 nm) for 5 min. Hydrogels were incubated in DMEM with serum at 37°C, 5% CO_2.

PicoGreen double-stranded DNA assay

Hydrogel samples were analyzed in triplicate for DNA content 0, 2, 4, and 6 days to assess cell proliferation. Samples (described above) were digested at each time point in 250 μL of 0.5 N sodium hydroxide and neutralized, and the DNA content in the gels was determined in a PicoGreen double-stranded DNA assay (P-7581; Molecular Probes, Eugene, OR). 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) diacrylate hydrogels containing no cells were used as the negative control. Hydrogels containing known amounts of calf thymus DNA (D-4522; Sigma) were used as standards to determine the DNA content of the sample gels. Samples were analyzed according to the manufacturer's instructions, using a Bio-Rad (Hercules, CA) fluorometer (excitation, ~480 nm; emission, ~520 nm).

Live/dead viability stain

A two-color fluorescence viability assay (L-3224; Molecular Probes) was used to demonstrate the viability of preadipocytes in all four described hydrogel configurations 6 days subsequent to polymerization. Hydrogels with cells were incubated for 45 min in a combined 2 mM calcein AM and 2 mM ethidium homodimer solution in sterile phosphate-buffered saline. The calcein AM permeates live cells and produces bright green fluorescence (excitation, ~495 nm; emission, ~515 nm), while the ethidium homodimer penetrates cells with injured membranes and produces red fluorescence (excitation, ~495 nm; emission, ~635 nm).[19–20] Samples were viewed with a Retiga EXi Fast Mono Cooled 12-bit camera (QImaging, Burnaby, BC, Canada) and IPLab software (Scanalytics, Fairfax, VA) for image analysis. The number of live and dead/dying cells was recorded for five planes in each of three samples for each hydrogel configuration and the percent live cells was then calculated.

Statistical analysis

When appropriate, the means of data measurements are presented. Error bars were calculated as the standard error of the mean. Statistical significance was defined as $p \leq 0.05$, using a two-tailed, two-sample Student t test, assuming unequal variance.

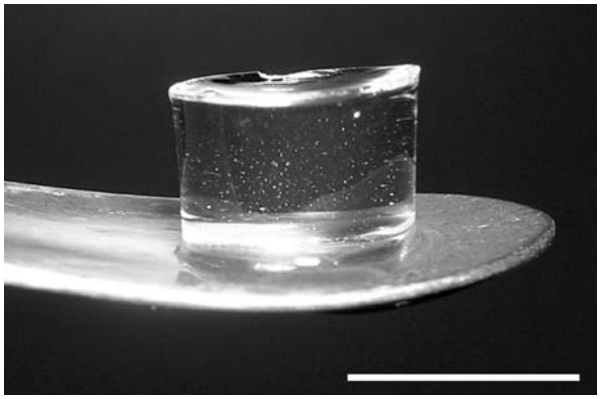

FIG. 2. PEG hydrogel subsequent to gellation. Scale bar: 5 mm.

RESULTS

Overall, hydrogel polymerizations for each of the configurations studied were successful and reproducible. Figure 2 illustrates a hydrogel formed in a 96-well plate. Preadipocytes were found to be cytocompatible with Irgacure 184 and this photoinitiator was used during the gelation process for all polymer chemistry. Specific results from the adhesion, proliferation, and viability studies are discussed below.

Photoinitiator cytocompatibility with preadipocytes

Figure 3 illustrates that Irgacure 651 elicited a significant decrease (70–75%) in the number of preadipocytes compared with the control group. The cytoincompatibility of Irgacure 651 with preadipocytes makes this photoinitiator a poor choice for use in cross-linking preadipocyte-seeded PEG. In contrast, Darocur 2959 and Irgacure 184 are both cytocompatible with preadipocytes for all concentrations tested. Although Darocur 2959 does not adversely affect cell viability, its relatively lengthy cross-linking time (>15 min) makes this photoinitiator an unsuitable choice for use in future clinical settings. However, the cross-linking time of Irgacure 184 is reasonably short (<5 min). Irgacure 184 was selected as the cross-linking agent for all subsequent studies.

Adhesion study

The YIGSR binding peptide used in these studies is functional for preadipocyte adhesion as evidenced by preadipocyte adhesion (Fig. 4). Preadipocytes did not adhere to the surface of PEG hydrogels possessing no adhesion ligands. However, cells did attach to the surface of hydrogels possessing YIGSR, with the surface concentration calculated to be 107 cells/mm^2. Optimization of preadipocyte adhesion was beyond the scope of the present study.

PicoGreen double-stranded DNA assay

Figure 5 compares the DNA content in each hydrogel configuration at various time points. DNA content in nondegradable hydrogels ± YIGSR did not increase over the experimental time course terminating at 6 days. These results suggest that preadipocyte proliferation does not occur in these two polymer configurations. The DNA content in degradable gels with YIGSR increased 4.5-fold

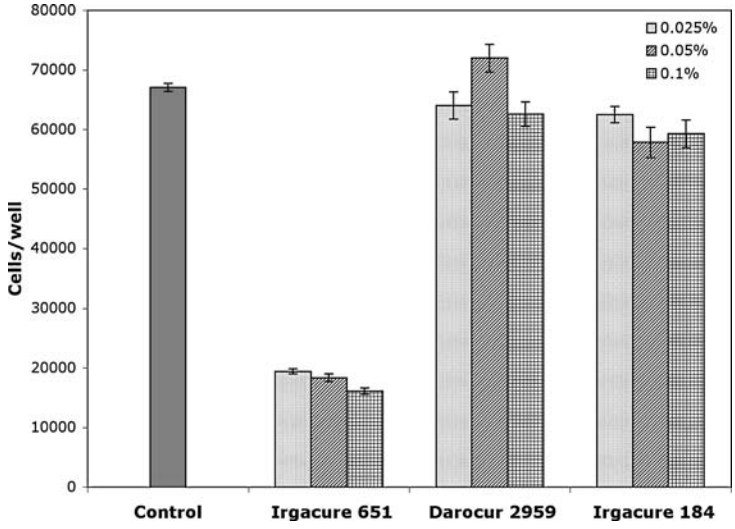

FIG. 3. Preadipocyte response to various concentrations of Irgacure 651, Darocur 2959, and Irgacure 184. Data represent average cell counts ($n = 3$) ± the standard error of the mean (SEM).

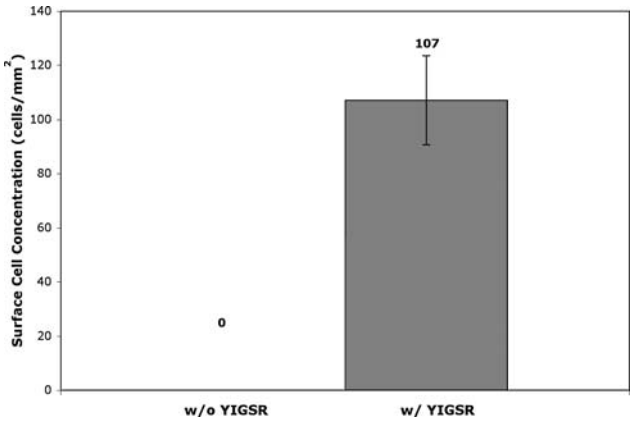

FIG. 4. Average concentration of preadipocytes (±SEM) on the surface of hydrogels with and without the HIGSR adhesion ligand.

over the experimental time course, indicating preadipocyte proliferation. Figure 6 illustrates preadipocytes extending cell processes toward one another through the hydrogel.

Live/dead viability stain

In addition to preadipocyte proliferation, cell viability in the hydrogels was assessed (Fig. 7). Cell viability was minimal (7%) by day 6 for nondegradable hydrogels possessing no YIGSR. The addition of YIGSR increased the viability in the nondegradable hydrogel to 80%. Degradable hydrogels possessing no YIGSR resulted in 61% viability. As described previously, the addition of YIGSR to the degradable hydrogel increased the viability to >80%. As expected, cell adhesion is critical for cell viability for anchorage-dependent cells.

DISCUSSION

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. 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 was initially to provide a material 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.

It is hypothesized that a photopolymerizable derivatized poly(ethylene glycol) hydrogel system can be a three-dimensional scaffold system that aids 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 cell-specific degradation sites and adhesion ligands to accommodate cellular adhesion and proliferation, which is crucial to cell function. The preadipocytes can be homogeneously mixed into the prepolymer solution and then photopolymerized into a hydrogel. Three derivatives of poly(ethylene glycol) were fabricated: diacrylated poly(ethylene glycol), which is nondegradable; poly(ethylene glycol) incorporated with a collagenase-sensitive degradable sequence (LGPA); and poly(ethylene glycol) coupled with cell adhesion ligands (YIGSR). Four scaffold

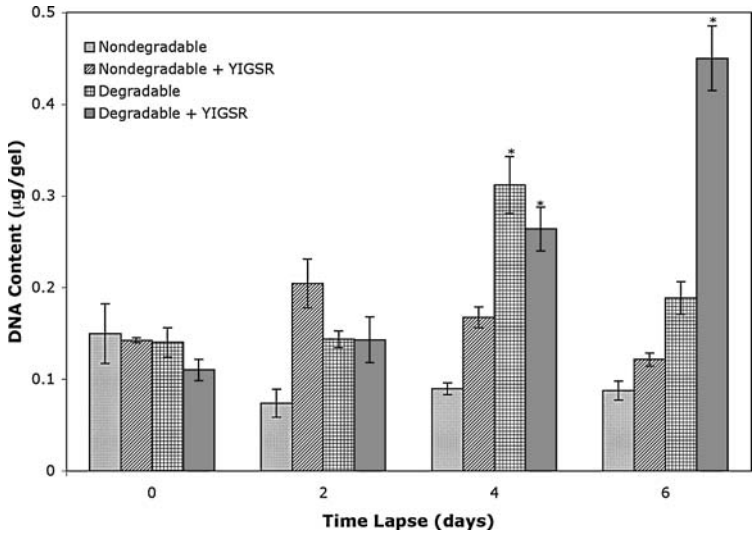

FIG. 5. Average DNA concent (±SEM) in hydrogels versus time. An asterisk (*) denotes statistical significance ($p < 0.05$) when compared with day 0.

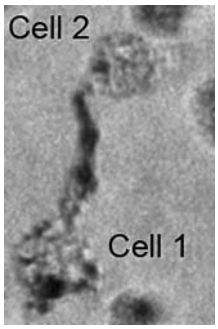

FIG. 6. Two preadipocytes migrating through a degradable PEG hydrogel possessing YIGSR. Numerous small and large cell processes are visible. Note that image resolution is not sharp because the cells are imaged in the interior of the hydrogel rather than at the surface.

configurations were prepared and analyzed: nondegradable scaffold, nondegradable scaffold containing cell adhesion ligands, degradable scaffold, and degradable scaffold containing cell adhesion ligands. Proliferation and viability assays were completed to determine which scaffold configuration is best suited to support preadipocytes.

It was observed that preadipocytes were able to adhere to hydrogels containing YIGSR within 3 h. Preadipocytes did not proliferate while in the nondegradable poly(ethylene glycol) scaffold because of the lack of degradation sites. For cells to undergo morphological changes during proliferation, there must be adequate room in the hydrogel. Nondegradable poly(ethylene glycol) hydrogels do not provide the additional space required for continued cell proliferation (i.e., no available space for cells to migrate or divide). Because of the absence of adhesion sites, cells could adhere to the polymer to provide traction and required adhesion-dependent signals for cell division. 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 because of the nondegradable nature of the network the cells were contact inhibited and no longer proliferated.

In contrast, the DNA content in the degradable scaffold increased initially, but subsequently decreased to initial levels. Serum protein from the DMEM used to form the hydrogels permitted temporary preadipocyte binding and initial degradation of the network by cellular secretion of collagenase resulted in initial proliferation. As degradation continued to occur, the serum protein was no longer entrapped, thus cell proliferation decreased. As a result, there was initial cell proliferation followed by cell death, as the hydrogel could not support continued cell adhesion. Only when poly(ethylene glycol) scaffolds contained both cell adhesion ligands and degradation sequences did the DNA content increase steadily over the course of the study. Cells were able to attach to the supporting network, proliferate, and secrete collagenase to degrade the polymer. Hence a 4- to 5-fold increase in cell growth was observed over the course of the week.

Poly(ethylene glycol) modified with LGPA and YIGSR peptide sequences has proven 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. Future studies will include *in vivo* experimentation and optimization of preadipocyte adhesion, proliferation, and differentiation.

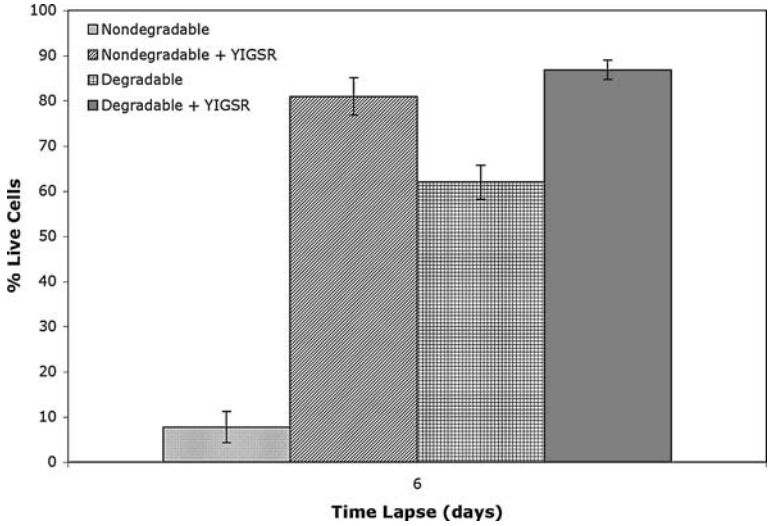

FIG. 7. Average percentage of viable cells (±SEM) in each hydrogel configuration after 6 days.

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