The effect of structural alterations of PEG-fibrinogen hydrogel scaffolds on 3-D cellular morphology and cellular migration

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Abstract

The need for alternative scaffolds in tissue engineering has motivated the establishment of advanced biomaterial technologies based on biosynthetic polymers. Networks of synthetic and biologic building blocks are created into a biomimetic environment for enhanced tissue compatibility with precise structural properties. The current investigation describes a unique biosynthetic hybrid scaffold comprised of synthetic polyethylene glycol (PEG) and endogenous fibrinogen precursor molecules. The PEGylated fibrinogen is cross-linked using photoinitation in the presence of cells to form a dense cellularized hydrogel network. The fibrin-like scaffold material maintains its biofunctionality through the fibrinogen backbone, while changes in the molecular architecture of the synthetic precursor are used to alter the nanostructural properties of the scaffold, including mesh size and permeability. The structural properties of 6- and 10-kDa PEG-fibrinogen hydrogels are characterized by measuring the swelling properties and relating them to the degradation kinetics of the scaffold. Increased concentrations of the synthetic PEG are used to further alter the network structure of the PEG-fibrinogen hydrogel. Experiments using smooth muscle cells cultured inside the PEG-fibrinogen scaffold demonstrates a qualitative relationship between the molecular architecture of the matrix and the cellular morphology. A quantitative assessment of cell migration into the hydrogel network demonstrates a strong correlation between rate of cellular invasion and the network structure of the matrix. The ability to regulate cellular characteristics using structural modifications to the PEG-fibrinogen scaffold can be a valuable tool in tissue engineering and tissue regeneration.

Keywords: Tissue engineering; Biosynthetic; Fibrin; Smooth muscle cells; Polyethylene glycol

1. Introduction

Designing an artificial scaffold that can control 3-D cellular remodeling is still a predominant challenge in the field of tissue engineering. Conventional tissue engineering scaffolds that provide temporary structural support for cells during the regeneration process are being replaced with interactive biomaterials that are much more than passive biodegradable constructs. In fact, most modern tissue engineering biomaterials strive to implement a biomimetic design, where biological building blocks are used to create 3-D structures with transient structural stability and built-in biofunctionality. A biomimetic design necessitates not only insight into how biological building blocks in the natural extracellular matrix (ECM) are arranged to function properly, it also requires technical know-how in manipulating these building blocks using man-made technologies, i.e. polymer chemistry.

The advent of biosynthetic hybrid biomaterials signifies a major achievement in the fields of biomimetics and tissue engineering. Hybrid biomaterials incorporate biological macromolecules with structurally versatile synthetic polymers to create a cross-linked network [1–3]. The hybrid biomaterials can be used to create a biomimetic cellular
environment by balancing the structural and biofunctional elements. Control over structural properties, including porosity, compliance, bulk density, and degradability are directed through the synthetic polymer network [4–6], while the biological cell signaling is controlled through the incorporation of biological macromolecules, which may include protein fragments [7], growth factors [8–10], or biologically active peptide sequences [11–13]. These biological factors are used to initiate important cellular remodeling events, including cell migration, proliferation, and guided differentiation.

The manipulation of cell migration by regulated interactions with hybrid hydrogels is just one example of the successful implementation of a biomimetic approach in tissue engineering. Hubbell and co-workers pioneered this technology by introducing cell-adhesive, proteolytically sensitive hybrid biomaterial for tissue regeneration. They use a polyethylene glycol (PEG) hydrogel backbone, modified with the RGD adhesion oligopeptide, and cross-linked with short oligopeptides containing a plasmin or collagenase degradation substrate [12,14]. The cross-linker gives cells the freedom to migrate in the highly dense synthetic PEG network with the aid of their own naturally secreted proteases. West and co-workers also use a proteolytically sensitive PEG-peptide biomaterial modified with adhesion ligands and growth factors for stimulating smooth muscle cell migration, proliferation and new matrix protein production [11,15,16].

The PEG-peptide hydrogels represent an elegant design concept in custom-made biomaterial for tissue engineering. However, there are still considerable challenges in recreating the complexities of the native ECM using this approach. An alternative approach is to create hybrid biomaterials using a multifunctional biological molecule and PEG as the building blocks of the hydrogel matrix. Using a PEG-protein approach, the biological molecule contains all the necessary cell signaling domains within its amino acid sequence, including adhesion and protease degradation substrates. The biological protein serves as the structural backbone of the polymeric network, thereby rendering the hydrogel naturally biodegradable via the degradation sites inherent to the protein sequence. Most of the structural properties of the PEG-protein hydrogel network are controlled through the synthetic PEG constituent.

The PEG-protein approach is already applied in a couple of variations. The work of Halstenberg et al. and Rizzi et al. takes advantage of genetic engineering and recombinant DNA technologies to manufacture custom-made backbone proteins which contains all the desired signaling motifs, including a cell adhesion site and a protease degradation site [17,18]. Another approach which was developed in our laboratory makes use of a naturally occurring protein molecule, fibrinogen, as the biological backbone of the hybrid biomaterial [19]. The hybrid scaffold is made by modifying fibrinogen molecules with di-functional PEG to form a PEGylated fibrinogen precursor; the precursor molecules are cross-linked in the presence of a cell suspension to form a cellularized hydrogel construct.

In the current study, PEG-fibrinogen is used to form a 3-D hydrogel scaffold for cell culture and a provisional hydrogel matrix for cell invasion and tissue regeneration. The focus of the investigation is to characterize the impact of altering the molecular architecture of the PEG-fibrinogen precursor and the structure of the hydrogel network on the cellular response. PEG-diacylates having a molecular weight of 6- or 10-kDa are copolymerized with fibrinogen to create two MW species of the PEGylated fibrinogen precursors. The purified precursors are transformed into a hydrogel network by radical crosslinking induced by a photoinitiator and UV light. Modifications to the hydrogel network structure are made by adding up to 3% (W/V) unreacted PEG-diacylate to the precursor solution before photo-polymerization. A full characterization of the swelling and enzymatic degradability of the hydrogels is performed and correlated to cellular invasion and cell morphology in the PEG-fibrinogen constructs.

2. Methods

2.1. PEG-diacylate synthesis

PEG-diacylate (PEG-DA) was prepared from linear PEG-OH, MW = 6, and 10-kDa (Fluka, Buchs, Switzerland) as described elsewhere [17]. Briefly, acylation of PEG-OH was carried out under Argon by reacting a dichloromethane (Aldrich, Berlin, Germany) solution of PEG-OH with acryloyl chloride (Merck, Darmstadt, Germany) and triethylamine (Fluka) at a molar ratio of 150% relative to the –OH groups. The final product was precipitated in ice-cold diethyl ether and dried under vacuum for 48 h. Proton NMR (1H NMR) was used to validate the expected product formation.

2.2. Fibrinogen PEGylation

To PEGylate the fibrinogen, tris (2-carboxyethyl) phosphine hydrochloride (TCEP HCl) (Sigma) was added to a 3.5 mg/ml solution of fibrinogen in 50 mM PBS with 8 M urea (molar ratio 1:1 TCEP to fibrinogen cysteins). After dialysis, a solution of PEG-DA (280 mg/ml) in 50 mM PBS and 8 M of urea was added and reacted overnight. The molar ratio of PEG to fibrinogen cysteins was 5:1 (linear PEG-DA, 6- and 10-

kDa). The PEGylated protein was precipitated by adding 4 volumes of acetone (Frustrum, Haifa, Israel). The precipitate was redissolved at 10 mg/ml protein concentration in PBS containing 8 M urea and dialyzed against 50 mM PBS at 4 °C for 2 d with twice-daily changes of PBS (Spectrum, 12–14-kDa MW cutoff). To establish the total PEG-fibrinogen concentration, 0.5 ml of the precursor solution were lyophilized overnight and weighed. The net fibrinogen concentration was determined using a standard BCA™ Protein Assay (Pierce Biotechnology, Inc., Rockford, IL) and the relative amounts of total PEGylated product (dry weight) to fibrinogen content (BCA™ result) were compared. A PEGylation efficiency scale was used to determine percent PEGylation of the protein product. The scale assumes 100% PEGylation when all 29 free thiols on the fibrinogen molecule (166 kDa) are bound to the functionalized PEGs. Hence, the PEGylation efficiency (pegylation) is calculated according to Eq. (1). The theoretical MW ratio of fibrinogen to PEG in Eq. (1) is 166-

kDa (for bovine fibrinogen) to 29 x 10-kDa (for 10-kDa PEG).

\[ \text{pegylation} = \left( \frac{\text{PEG}}{\text{Fibrinogen}} \right) \times \text{theoretical} \left( \frac{\text{MW}_{\text{fibrinogen}}}{29 \times \text{MW}_{\text{PEG}}} \right). \] (1)
2.3. Biodegradation and swelling characterization

Acellular cylindrical plugs are cast in 5 mm diameter silicon tubes from 100 µl aliquots of PEG-fibrinogen precursor solution containing photoinitiator. The hydrogel swelling ratio based on mass (QM) is calculated by diving the wet weight (mass after swelling) by the dry weight (mass after lyophilization). The biodegradation of the hydrogels is characterized by colorimetrically labeling the PEGylated fibrinogen with eosin-Y and quantifying the release of protein resulting from enzymatic dissolution of the hydrogel. Briefly, hydrogel plugs are stained in 5 mg/ml eosin-Y solution for 2d, washed, and transferred into 2 ml of either 0.01 mg/ml trypsin or 0.1 mg/ml collagenase solution containing 50 mM PBS and 0.1% sodium azide. Absorbance values are measured spectrophotometrically at 516 nm every 30 min for 3h. After the last time point, each hydrogel is hydrolytically dissociated, and absorbance values are recorded at 100% degradation.

2.4. Cell-seeded construct preparation

The PEG-fibrinogen hydrogels are made from a precursor solution of PEGylated fibrinogen by a radical chain polymerization reaction of acrylate end groups. The purified PEGylated precursor solution (2-3% PEGylated protein—w/v) contains roughly 2 µ free acrylate groups for cross-linking. Additional PEG-DA (6- or 10-kDa) can be added to increase cross-linking density of the PEGylated protein network as well as to minimize steric hindrances that may result in poor gelation [20]. The precursor solution is mixed with 0.1% (v/v) photoinitiator stock solution made of 10% w/v Irgacure™2959 (Ciba Specialty Chemicals, Tarrytown, New York) in 70% ethanol and deionized water. The solution is placed under a UV light (365 nm, 4–5 mW/cm²) for 5 min to polymerize. Cell-seeded hydrogels are polymerized in the presence of smooth muscle cells (SMCs) according to previously described protocols [19]. Briefly, sheep aortic SMCs (SASMCs) from young donors are cultured up to 6th passage in Dulbecco’s Modified Eagle Medium (DMEM) (Gibco, UK) containing 10% fetal bovine serum (FBS) (Biological Industries, Israel), 1% penicillin-streptomycin (Biological Industries), and 1% l-glutamine. Cell-seeded constructs (1 × 10⁶ cells/ml) are made from 400 µl aliquots of the cell and PEG-fibrinogen precursor suspension in a flat bottom 24-well plate. Cell culture medium is added and changed every other day.

2.5. Cellular outgrowth studies

Outgrowth experiments are performed using dense tissue constructs made from compacted SASMC-seeded collagen gels placed inside PEG-fibrinogen hydrogels. The smooth muscle tissue constructs are made from a solution of 5X DMEM, 10% FBS, reconstituted type-I collagen solution in 0.02 N acetic acid (2 mg/ml), and 0.1 M NaOH with dispersed SASMCs (3 × 10⁶ cells/ml) [21]. The collagen gels are allowed to contract for 20 µl of culture medium to compact before being placed in 300 µl of PEG-fibrinogen precursor solution (with photoinitiator) in a 48-well plate and exposed to UV light for 5 min. The constructs are cultured inside the hydrogels in 400 µl of culture medium which is changed every other day during the experiment.

2.6. Cell viability, morphology, and migration

SMC viability is assessed using a live/dead viability kit (Molecular Probes, Inc) according to manufacturer’s instructions. Cell morphology is assessed by phase contrast micrographs and H&E-stained cross-section of cellularized PEG-fibrinogen constructs. The results of cellular outgrowth experiments are quantified by measuring the mean diameter traveled by cells from the dense tissue into the PEG-fibrinogen hydrogels using phase contrast micrographs of the samples taken at set time intervals.

2.7. Statistical analysis

Statistical analysis is performed using the Microsoft Excel statistical analysis software package. Data from at least two independent experiments are quantified and analyzed for each variable. Comparisons between multiple treatments are made with analysis of variance (ANOVA) and comparisons between two treatments are made using a two tail t-test; in either case, P < 0.05 is considered statistically significant.

3. Results

3.1. PEGylation properties

The average PEGylation efficiency is calculated based on data taken from 12 different batches of PEGylated fibrinogen (Table 1). Based on the mean values from at least 6 separate PEGylation reactions, there is no statistical difference between the PEGylation efficiency of 6- and 10-kDa PEG-fibrinogen (n = 6, P > 0.4). Moreover, according to the results from the BCA assay, there is no statistical difference between fibrinogen concentration after PEGylation with 6- or 10-kDa PEG-fibrinogen (n = 6, P > 0.8). Taken together, these data indicate that the concentration of un-reacted acrylate groups (i.e. cross-linking) on the PEGylated fibrinogen precursors is statistically identical for 6- and 10-kDa PEG-fibrinogen. The PEGylation efficiency calculation assumes that one functional group of the PEG-DA molecule binds to a single protein thiol, because of the large excess of PEG-DA in the reaction.

3.2. Swelling properties

The swelling characteristics of the PEG-fibrinogen hydrogels are summarized in Fig. 1. The swelling ratio (QM) of the 10-kDa PEG-fibrinogen gels indicate that they are more hydrated than the 6-kDa gels (n = 6, P < 0.01). The addition of PEG-DA to the precursor solution (6- or 10-kDa) also has a significant impact on the swelling properties of the PEG-fibrinogen hydrogels (n = 6, P < 0.01). Overall, there is an inverse relationship between

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<th>Table 1</th>
<th>PEG-fibrinogen characterization (Mean ± SEM; n = 6)</th>
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<tr>
<td>PEG (M.W.)</td>
<td>Fibrinogen (BCA/mg/ml)</td>
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<tr>
<td>6-kDa</td>
<td>9.5 ± 0.4</td>
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<td>10-kDa</td>
<td>9.5 ± 1.2</td>
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the amount of additional PEG-DA in the hydrogel and $Q_M$. The correlation between swelling and additional PEG-DA evinces an increase in the hydrogel’s cross-linking density and a decrease in the mesh size with the addition of the difunctional PEG prior to the polymerization reaction [20,22].

3.3. Biodegradation studies

The degradation of the PEG-fibrinogen hydrogels in collagenase (0.1 mg/ml) or trypsin (0.01 mg/ml) is summarized in Fig. 2. The kinetics data reveals that hydrogels made with 10-kDa PEG degrade faster than hydrogels made with 6-kDa PEG. The addition of PEG-DA to the precursor solution results in significantly slower degradation kinetics ($n = 5, p < 0.01$). Hydrogels made with more than 1.5% additional PEG-DA remain partially intact in the enzyme solution despite releasing colorimetrically labeled fibrinogen from the hydrogel network into the supernatant.

3.4. 3-D cell culture

SASMCs are entrapped homogeneously inside PEG-fibrinogen hydrogels (6- or 10-kDa) during the polymerization procedure in order to create 3-D cell-seeded constructs. The cell viability is qualitatively assessed using a “live/dead” viability assay after 3 d and 4 wk in culture (Fig. 3a–d). Fig. 3a and b show the majority of cells inside the pure hydrogels are stained green, indicating high viability after 3 d and 4 wks, respectively. Fig. 3c and d...
demonstrate lower viability in PEG-fibrinogen hydrogels made with 2% additional PEG-DA after 3 d and 4 wks, respectively. There was no observable difference in viability between the 10-kDa compared to 6-kDa PEG-fibrinogen hydrogels. In general, cells in the center of the constructs exhibit similar viability to cells situated on the outer rim of the constructs. Based on the qualitative results obtained incrementally during a 4 wk culture period, cell viability did not change with time in culture, though a quantitative assessment would be necessary for a more complete characterization of the viability and cell proliferation.

In the unmodified PEG-fibrinogen hydrogels, cells which are initially rounded begin to spread within the hydrogels and become highly spindled within 24 h. By day three in culture, most of the SASMCs are spindled as demonstrated by phase contrast micrographs (Fig. 4a). Histological cross-sections of the same reveals spindled SASMCs embedded within the dense PEG-fibrinogen ECM (Fig. 4b). There is no observable difference in cell morphology in constructs made from 6- versus 10-kDa PEG-fibrinogen hydrogels (data not shown). SASMC morphology remains spindled in the unmodified PEG-fibrinogen hydrogels for the duration of the time in culture (4 wk). Constructs modified with additional PEG-DA and cultured for 3 d exhibit SASMCs with fewer cellular extensions and a decreased spindled morphology (Fig. 4c–j). Evidently, the SASMCs are less able to form any extension or protrusions in the dense polymer network of constructs as they are modified with additional PEG-DA.
3.5. Cellular outgrowth

Tissue constructs made from compacted SMC-seeded collagen gels are entrapped inside a PEG-fibrinogen hydrogel and cellular outgrowth is observed for a period of up to 1 wk in culture. Initially, the SASMCs are contained within the collagen gel construct (Fig. 5a). Within several hours after casting, the SASMCs begin to invade the dense PEG-fibrinogen matrix surrounding the tissue mass (Fig. 5b) and continue to invade the biosynthetic matrix for the duration of the culture period (Fig. 5c). Throughout this time, the cell cluster becomes integrated within the PEG-fibrinogen hydrogel in the regions of proteolytic invasion (Fig. 5d–e), while elsewhere, the hydrogel maintains its structural stability (Fig. 5f).

During the first 96 h of culture, the rate of cellular invasion is nearly constant and proportional to the molecular weight of the PEG-fibrinogen precursors (Fig. 6a and b). After this period, the SASMCs invade the hydrogel matrix more aggressively as indicated by a sharp increase in the rate of cellular invasion after one week (91% increase, \( n = 5, p < 0.01 \)). Overall, the rate of SASMC invasion (Fig. 6c) into 10-kDa PEG-fibrinogen hydrogels is significantly higher than the rate of invasion into 6-kDa hydrogels (\( n = 4, p < 0.05 \)). The additional PEG-DA used to polymerize the hydrogel network significantly alters the rate of SASMC invasion into the biosynthetic matrix (Fig. 6c). There is a 66% decrease in the distance SASMCs travel in 10-kDa PEG-fibrinogen hydrogels with 2% PEG-DA compared to 0% controls (after 168 h). There is only a 36% decrease in the distance SASMCs travel in 6-kDa PEG-fibrinogen hydrogel with 2% PEG-DA as compared to 0% PEG-DA controls (168 h).

Consequently, the morphological features of invading SASMCs are also affected by the amount of additional PEG-DA used to polymerize the hydrogel network.

**Fig. 5.** Cellular invasion into PEG-fibrinogen hydrogels. Smooth muscle tissue constructs are encapsulated in a PEG-fibrinogen hydrogel and visualized by phase-contrast microscopy. Cellular invasion from the dense tissue (opaque) into the hydrogels (transparent) is seen within several hours after hydrogel casting, and continues throughout the experiment (a–c). Macroscopically, the tissue construct (arrow) becomes integrated within the PEG-fibrinogen hydrogel (d–e), and the hydrogel maintains its structural stability after 1 week in culture (Fig 4f). The morphology of the invading SMCs is affected by the amount of additional PEG-DA in hydrogel network; the cells become more polarized and oriented radially outward from the tissue construct as they invade PEG-fibrinogen matrix containing higher concentrations of 10-kDa PEG-DA (g—0%; h—0.5%; i—1.0%; j—2.0%). Phase contrast micrographs are imaged at 40 × magnification (scale bar = 250 μm).
show a profound difference in the morphologies of cells entering hydrogels made with additional PEG-DA (Fig. 7a and b) as compared to cells entering hydrogels made without additional PEG-DA (Fig. 7c and d).

4. Discussion

4.1. Structural versatility

Biomimetic hydrogel scaffolds comprised of natural and synthetic constituents offer both biocompatibility and structural versatility [6,14,15,17,23]. In this study, we describe a biosynthetic scaffold material made from PEGylated fibrinogen and cross-linked using photoinitation. Other investigators have reported on the use of PEG-protein biomaterials as scaffolds for tissue regeneration; however, most of these studies make use of oligopeptides or recombinantly expressed biological constituents to assemble the PEG-based hydrogel network. The advantages of using a naturally occurring fibrinogen molecule are two fold: (1) The fibrinogen protein is a well-characterized ECM molecule with a central role in tissue remodeling, and (2) large quantities of fibrinogen are readily available and easily purified from human or animal plasma. Similar to other PEG-protein hydrogels, the biodegradability of PEG-fibrinogen matrix is facilitated by substrates for protease digestion; the fibrinogen molecule contains several such protease substrates [24]. The enzymatic dissolution of the cross-linked PEG-fibrinogen network is likely governed by both protease degradation kinetics and protease diffusion through the hydrogel network. In our previous studies, we demonstrate that PEG-fibrinogen hydrogels exhibit proteolytic degradation using a quantitative matrix digestion assays and two different enzymes: collagenase and trypsin [19]. We also demonstrated that cell adhesion to the PEG-fibrinogen matrix is made possible by two RGD sites (97 and 255) in the amino acid sequence of the fibrinogen \( \alpha \)-chain.

Our current research is focused on further characterization of the PEG-fibrinogen scaffold material by studying the relationship between the molecular architecture of the precursor, degradation properties of the hydrogel matrix, and the morphological and migratory behavior of SMCs cultured inside or peripherally invading the matrix. Structural variations in the hydrogel network are instituted through modification to the synthetic PEG constituent, with the biological backbone of the hydrogel maintained constant with an average fibrinogen concentration of \(~9\text{mg/ml}\). Two MW species of PEG-fibrinogen are used as precursors for the hydrogel matrix; one employs a 10-kDa PEG and the other employs a 6-kDa PEG. Table 1 summarizes the PEGylation efficiency of each MW species and confirms that differences in the number of PEG molecules bound to the fibrinogen are negligible. Additional alterations to the hydrogel network structure are implemented through the addition of unreacted PEG-DA to the PEG-fibrinogen precursor solution prior
to photopolymerization. In theory, during polymerization of the precursor solution, the acrylates on the PEG-DA also participate in the free-radical polymerization process and introduce additional cross-links between the PEG-fibrinogen precursor molecules. The increased PEG-DA concentrations, and consequent increased cross-link density, have a profound impact on the swelling properties of the hydrogel network (Fig. 1) as well as on the proteolytic degradation of the hydrogel in the presence of collagenase and trypsin (Fig. 2).

The proteolytic degradation of the PEG-fibrinogen hydrogels is dependent in part on the enzyme diffusion through the dense hydrogel network, as well as the accessibility of fibrinogen protease substrates to fibrinolytic enzymes. According to the swelling data in Fig. 1, the PEGylated fibrinogen hydrogels become more cross-linked with additional PEG-DA, causing an effective decrease in the mesh size of the polymer network. The average molecular weight between cross-links ($M_c$) can be approximated using the hydrogel swelling data together with an equilibrium swelling model developed previously by Flory and Rehner [25] and reported elsewhere [26]. Assuming a large degree of swelling, zero energy of mixing, and the polymer density equal to that of water, PEG-fibrinogen hydrogels possess an average molecular weight between cross-links of 49 kg/mol (6-kDa PEG-fibrinogen) and 89 kg/mol (10-kDa PEG-fibrinogen). As additional PEG-DA is mixed with the precursor solution, the $M_c$ decreases to roughly 13 and 27 kg/mol for the two MW species, respectively (see Table 2). Knowing that the average MW of collagenase ranges between 50 and 120 kDa, and Trypsin’s MW is 24 kDa, it is likely that the hydrogel degradation kinetics are significantly impaired by the increased cross-link density. It is not likely that the transport of oxygen and nutrients is hindered in the hydrogel network given the large mesh size relative to the small size of molecules such as oxygen and individual amino acids.

The degradation data indeed reveals a pattern of decreasing degradation kinetic with increasing percent PEG-DA in the hydrogels. It is speculated that the increased cross-link density in the hydrogel (smaller mesh size increases the network stiffness, making it more difficult for enzymes to penetrate and degrade the hydrogel.)
size) may be retarding enzyme mobility and affecting hydrogel disassembly kinetics. In order to test this hypothesis, the diffusion coefficients for collagenase and trypsin are estimated using the proteolysis data and a simplified kinetic model of protein behavior in hydrogel networks based on previously published work [27,28].

\[
\frac{M_t}{M_\infty} \approx \left( \frac{D t}{\pi \delta^2} \right)^{1/2}
\]

In Eq. (2), \(M_t\) is the mass of degraded gel at a given time \(t\); \(M_\infty\) is the mass of hydrogel degraded at time infinity; \(M_t/M_\infty\) is the fractional mass of degraded hydrogel; \(D\) is the diffusion coefficient, and \(2\delta\) is the hydrogel thickness. The effective diffusion coefficients \((D)\) are calculated directly from the degradation kinetics data (Fig. 2), assuming cylindrical gel geometry with an aspect ratio of 1. These data confirm that protease diffusion decreases with increasing concentrations of PEG-DA in the hydrogel network (Fig. 8). Interestingly, the diffusion coefficients of both trypsin and collagenase are lower in 6-kDa PEG-fibrinogen hydrogels as compared to 10-kDa. Taken together, this data indicates that locally mediated proteolysis of the hydrogel network is significantly affected by the structural changes in the network resulting from alterations in the synthetic PEG constituent. It is possible that additional alterations to the kinetics of hydrogel disassembly are caused by increasing the overall PEG concentration in the hydrogel network, which may reduce accessibility to protease cleavage sites on the fibrinogen molecules and lower the rate of enzymatic cleavage [29]. However, using high enzyme concentrations, it is assumed that the rate of enzymatic cleavage is still greater than the rate of protease diffusion into the hydrogel network.

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<td>Estimated average molecular weight between cross-links in PEG-fibrinogen hydrogels</td>
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<td>Additional PEG-DA</td>
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4.2. Scaffold biofunctionality

Fibrin(ogen)-based biomaterials have been used extensively in tissue engineering for growing cells and reconstituting 3-D tissue structures. Typically, fibrinogen is modified by thrombin to initiate fibrin polymerization into an insoluble network [24]. The unmodified fibrin gels are highly compatible with 3-D cell cultures, based in no small part on fibrin’s inherent biofunctionality. However, fibrin gels are also very sensitive to fibrinolysis because of their highly porous fibrillar network which presents minimal resistance to protease mobility within the protein network. To overcome this problem, enzyme inhibitors can be used; however, inhibitors can easily cause adverse effects with in vitro cell cultures and in vivo application of fibrin gels using abundant enzyme inhibitors can cause undesired side effects. In comparison to fibrin gels, PEG-fibrinogen hydrogels are less sensitive to proteolytic degradation, partly because their biodegradation is heavily influenced by protease accessibility (diffusion) and partly because the hydrophilic PEG protects the fibrinogen from rapid enzymatic cleavage. Finding the correct balance between fibrinogen and PEG can offer an optimization strategy whereby the biodegradation of the biosynthetic matrix is tailored for each specific application. In this context, the PEG-fibrinogen hydrogels are an excellent alternative to fibrin gels, particularly for in vitro 3-D cell culture and in vivo implantation requiring long-term structural stability of the scaffold (greater than one week). Nevertheless, it is important to note that fibrinogen PEGylation can result in blocking important bioactive sites on the fibrinogen molecule and thus render the 3-D matrix less biologically active compared to native fibrin.

While the biofunctional attributes of the PEG-fibrinogen matrix are straightforward [19], the relationship between the molecular architecture of the PEG-fibrinogen network
and the ability of SMCs to proteolytically remodel the matrix is still ambiguous. This is particularly important in PEG-fibrinogen hydrogels because the mesh size of the network is of the same order of magnitude as the size of the proteins that degrade the matrix. Therefore, the network architecture and cell-mediated proteolysis are assumed to be highly interrelated. In order to clarify this relationship, SASMCs are three-dimensionally cultured within the PEG-fibrinogen gels containing up to 2% unreacted PEG-DA (6- and 10-kDa PEG). The cells are physically encapsulated within the hydrogel network during polymerization using two different configurations: homogeneously dispersed single-cells (Figs. 3 and 4) and compacted SASMC tissue constructs (Fig. 5). The idea in using two different configurations is to examine the biosynthetic matrix both as a scaffold for cell culture and as an acellular provisional tissue regeneration matrix; the latter being accessible to cells only by cellular invasion. The single cell configuration enables the evaluation of locally mediated spreading of individual cells, in comparison to the mass cellular invasion typically exhibited with the tissue construct configuration. In both cases, the porosity of the PEG-fibrinogen matrix does not permit cell spreading or migration in the absence of proteolytic hydrogel degradation.

According to 3-D cell culture results, pure PEG-fibrinogen hydrogels (0% PEG-DA) offer little resistance to individual SASMC spreading, as demonstrated by the highly spindled cell morphology (Fig. 4a and b). As the concentration of unreacted PEG-DA increases, so does the resistance to cell spreading, presumably due to the decreased susceptibility of the hydrogel to proteolysis. Hence, the ability of individual SASMCs to spread and migrate inside the network depends on their ability to secrete enough fibrinolytic enzymes to locally break apart the hydrogel network. Individually, SMCs are no longer able to modify the PEG-fibrinogen network if more than 1.5% PEG-DA has been supplemented to it. Interestingly, there is no resolvable difference in SASMC morphology between hydrogels made with the 6- or 10-kDa PEG-fibrinogen species; even though kinetics data demonstrates that 6-kDa hydrogels are less susceptible to proteolysis (Fig. 2).

One alternative explanation to these observations is that the PEG-DA has a cytotoxic effect on the cells, thereby limiting their extensions into the hydrogel network; however, according to several recent studies, cross-linked PEG-DA does not exhibit a cytotoxic effect on encapsulated cells [11,12,14,19,30–32]. The viability data does suggest that fewer cells survive in the modified PEG-fibrinogen hydrogels after 3 d, but this is likely caused by morphological constraints imposed on the cells by their environment, rather than PEG-DA cytotoxicity. Fig. 3 shows viable cells with rounded morphology, thereby confirming that cells remain viable even though they are no longer able to form extensions in the gels. Therefore, it is possible that morphological constraints cause some of the mesenchymal SMCs to undergo transdifferentiation and some cells are induced towards apoptosis because of the harsher environmental conditions [33]. With regards to the latter, it is also likely that increased amounts of PEG-DA in the hydrogel restrict access to adhesion site on the fibrinogen molecule, thus causing the highly anchorage-dependent SMCs to enter a programmed cell death [34].

Nevertheless, the cellular outgrowth data does confirm to some extent the observed relationship between local fibrinolysis and cell spreading, as measured by the invasion distance of SASMCs into the hydrogel network. In contrast to individual SMCs, substantial cellular invasion into PEG-fibrinogen hydrogels is observed even with 2% PEG-DA added to the matrix; although the distance and rate of invasion are significantly lower when compared to pure PEG-fibrinogen hydrogels. This discrepancy is attributed to the presumed increase in enzyme concentration that is active to locally disassemble the PEG-fibrinogen matrix when a large number of SMCs invade it, as compared to individual SMC-mediated proteolysis. Moreover, irrespective of which species of PEG-fibrinogen is used, there is a strong inverse correlation between the rate of cellular invasion and the percent PEG-DA added to the PEG-fibrinogen matrix (Fig. 6c), supporting a relationship between proteolytic susceptibility and cellular remodeling of the matrix.

5. Conclusions

The present study describes a biomimetic scaffold made from endogenous and synthetic building blocks. This material has several advantages over conventional biological scaffold materials, including precise control over density, stiffness, and biodegradability; all are afforded by the synthetic PEG constituent. The endogenous fibrinogen backbone of the material presents ample biofunctional domains for cell-mediated remodeling. The optimal combination of synthetic PEG and endogenous fibrinogen can be customized for each application according to the specified structural requirements. The availability of the endogenous fibrinogen, the simplicity of manufacturing, and the compatibility with living tissues, make the PEG-fibrinogen hydrogel an attractive alternative scaffold for tissue engineering.

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