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Protein–polymer conjugates for forming photopolymerizable biomimetic hydrogels for tissue engineering

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Abstract

Collagen, fibrin and albumin are popular proteins for making biological scaffolds for tissue engineering because of their biocompatibility, biodegradability, and availability. A major drawback of biological protein-based biomaterials is the limited control over their physical and biodegradation properties. Our laboratory has been developing new protein-based biomaterials with tunable properties without the use of cytotoxic protein cross-linking techniques. We describe the formation and assembly of photopolymerizable biomimetic hydrogel scaffolds made from protein–polymer conjugates of poly(ethylene glycol) (PEG) and collagen, fibrin or albumin. The conjugation of PEG to these proteins (PEGylation) was verified by SDS-PAGE and the polymerization reaction into a hydrogel network was confirmed by shear rheometry. The differences in rheology and swelling characteristics of the three hydrogel materials underscore the importance of the molecular relationship between the PEG and the protein constituent in this protein–polymer arrangement. The biofunctionality of the PEGylated collagen and fibrinogen hydrogels sustained both cell adhesion and proteolytic degradation that enabled 3-D cell spreading and migration within the hydrogel network. PEG–albumin hydrogels exhibited poor cell spreading and migration by virtue of the fact that the albumin backbone lacks any known cell adhesion sites. Despite differences in the biological and structural composition of the PEGylated fibrinogen and collagen hydrogels, the rate of cellular migration within each material was not significantly different.

Keywords: Albumin; Fibrin; Collagen; Poly(ethylene glycol); Scaffold; Smooth muscle cells

1. Introduction

Natural extracellular matrix (ECM) proteins are widely used to make biomaterial scaffolds for tissue engineering and regenerative medicine. These purified and reconstituted ECM proteins can form hydrogel matrices with unique three-dimensional (3-D) architecture coupled with intrinsic cell signaling that guide remodeling and functional tissue regeneration. Biological scaffolds are easily made from collagen or fibrin, two abundant proteins that have been employed in numerous *in vitro* and *in vivo* applications [1]. Both collagen and fibrin assemble into fibrillar hydrogel networks that are supported by covalent and non-covalent protein interactions [2,3] and can be physically cross-linked with additional factors [3]. The liquid-to-solid transition (gelation) of the soluble protein hydrogel precursors is advantageous for tissue engineering because the matrix can be formed in the presence of cells, soluble growth factors, or living tissues (*in situ* polymerization). The 3-D environment created by protein hydrogels can support cell repopulation and instructive remodeling into hierarchically organized tissues and organs [4]. For this reason, it is important to design the scaffold with pore size, permeability, and material surface properties that favorably affect this remodeling [5,6]. At the same time, the ECM backbone of the material can instruct cells towards important phenotypic transitions [7].

There are some drawbacks to using exogenous or recombinant proteins such as collagen and fibrin as scaffolds for tissue engineering, including a limited control over the physical properties and biodegradation of the polymeric network. For example, collagen gels are made from a network of fibrils that exhibit poor physical strength

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and super-physiological tissue porosity. The specific conformation of fibrils combined with the open pore structure of the interpenetrating network leaves the protein backbone easily accessible and susceptible to freely diffusing proteases from the surrounding host tissue or cell culture system. This often results in uncontrolled and premature deterioration of the scaffold in the presence of cell-secreted proteases [3,8,9]. The discrepancies in structure and proteolytic susceptibility of reconstituted protein hydrogels compared to natural tissues still leaves much to be desired from the biological scaffold systems in many practical tissue engineering applications.

There are a number of techniques used to improve or modify the physicochemical properties of reconstituted protein hydrogels and to protect them from rapid degradation. Techniques for improving the physical properties based on covalent cross-links include the use of aldehydes, carbodiimides, and *N*-hydroxysuccinimides (NHS) [10,11], to name just a few. Many of the cross-linking procedures offer some improvements over the physical stability and reduced enzymatic susceptibility of the scaffold, but do so by introducing a cytotoxic manufacturing step which requires extensive washes and increases the likelihood that residual toxins in the scaffold will affect cellular activity [3,12]. Collagen and fibrin gels can also be processed by freeze-drying to increase the tensile strength and modulus of the protein network [13–15]. However, freeze-drying necessitates a pre-fabrication freezing step, which eliminates the possibility for gelation of the polymer in the presence of cells and also does away with the benefits of *in situ* fixation.

The proteolytic degradation of protein scaffolds can also be delayed by protecting the protein backbone of the polymer network using covalent attachment of a shielding polymer such as poly(ethylene glycol) (PEG). The modification of proteins by attachment of one or more PEG chains (PEGylation) has been applied very successfully to increase the plasma half-life of therapeutic peptides or protein drugs [16]. Based on a similar rationale, PEGylation could be a good strategy for protein-based biomaterial design in as much as the PEG chains can slow down the enzymatic biodegradation of the PEGylated protein scaffold [17–19]. At the same time, the PEG chains are non-toxic, non-immunogenic, highly water soluble, and are already approved by the FDA in a number of different clinical indications [20]. Common proteins used in scaffold design may be readily PEGylated using amine group modifications or thiol modifications of the protein backbone to yield a protein–polymer conjugate [21,22]. The PEG shields the protein from enzymes through steric hindrances without blocking all the natural biological function of the structural protein molecule [20].

In the present investigation, we describe the formation of protein–polymer conjugates and their assembly into biomimetic hydrogel scaffolds for tissue engineering. Three biomedical proteins, including collagen, fibrin(ogen), and albumin were PEGylated and formed into hydrogel networks by free-radical photopolymerization. The three biomaterials were characterized and tested with smooth muscle cells (SMCs) in 2-D and 3-D cultures for up to one week. The viability, mobility and cellular remodeling of the cell-seeded materials were documented and summarized.

2. Methods

2.1. PEG-diacrylate synthesis

PEG-diacrylate (PEG-DA) was prepared from linear PEG-OH MW = 10 kDa (Fluka, Buchs, Switzerland) as described elsewhere [30]. In brief, acrylation of PEG-OH was carried out under Argon by reacting a dichloromethane solution of PEG-OH (Aldrich, Sleeze, Germany) with acryloyl chloride (Merk, Darstadt, Germany) and triethylamine (Fluka) at a molar ratio of 1.5:1 relative to –OH groups. The final product was precipitated in ice-cold diethyl ether and dried under vacuum for 48 h. Proton NMR (^{1}H NMR) was used to validate end-group conversion and to verify purity of the final product.

2.2. Fibrinogen and albumin PEGylation

The PEGylation of fibrinogen and albumin was done according to a PEGylation protocol similar to one described in detail by Dikovsky et al. [18]. Tris (2-carboxyethyl) phosphine hydrochloride (TCEP-HCl) (Sigma-Aldrich) was added to a 7 mg/ml solution of bovine fibrinogen (Sigma-Aldrich) or bovine serum albumin (MP Biomedicals, Ohio, USA) in 150 mM phosphate buffered saline (PBS) with 8 M urea (molar ratio 1.5:1 or 2:1 TCEP to fibrinogen or albumin cysteines, respectively). Linear 10-kDa PEG-DA was reacted for 3 h with the protein at a 4:1 or 2:1 molar ratio of PEG to fibrinogen or albumin cysteines, respectively. The PEGylated protein product was precipitated in acetone and redissolved in PBS containing 8 M urea at 7 mg/ml final fibrinogen concentration and 2 mg/ml final albumin concentration. The protein product was dialyzed against PBS at 4 °C for 2 d (Spectrum, 12–14-kDa MW cutoff, California, USA). The PEGylated albumin was concentrated to 8 mg/ml by lyophilization and reconstitution. The PEGylated product was characterized according to published protocols [18].

2.3. Collagen PEGylation

Collagen (type I) was isolated from rat tail tendon according to published protocols [23]. Thiolation of collagen was accomplished using succinimidylacetyl-thioacetate (SATA, Pierce, Illinois, USA) based on the protocols of Chen et al. [24]. Briefly, collagen was dissolved in 150 mM PBS with 8 M urea at a concentration of 5 mg/ml. SATA was reacted for 2 h at RT with agitation at a concentration of 0.075 mg/mg collagen. The resulting acetylated SH groups on the lysine residues were then deprotected for 2 h by reacting 0.5 M hydroxylamine hydrochloride ($NH_2OH \cdot HCL$, Sigma-Aldrich) at a concentration of 0.125 ml/mg collagen. The product was dialyzed overnight against PBS and 8 M urea and PEGylated with TCEP–HCl (molar ratio 2:1 TCEP to the new collagen thiols), 10 kDa PEG-DA (molar ratio 1.5:1 PEG-DA to new collagen thiols) at pH 8 and RT for 3 h. The PEGylated collagen was precipitated in acetone and redissolved in PBS and 8 M urea, and dialyzed in a Slide-A-Lyzer (10,000 MWCO, Pierce, IL, USA) at 37 °C for 2 d to a final concentration of 4 mg/ml collagen. The PEGylated product was characterized the same as PEG–fibrinogen.

2.4. SDS-PAGE

The PEGylation reaction was confirmed by SDS-PAGE whereby PEGylated and unPEGylated proteins were loaded into 8% gels (5–10 μg of protein in each lane). The gels were stained with Coomassie® blue and digitally imaged using a gel documentation workstation.

2.5. Hydrogel formation

PEGylated proteins were formed into hydrogels by photopolymerization using 0.1% (w/v) Irgacure™2959 (Ciba Specialty Chemicals, Basel, Switzerland) and UV light (365 nm, 4–5 mW/cm^2) exposure for 5 min. All tissue culture experiments were performed with 4 mg/ml PEGylated protein precursor supplemented with 10-kDa linear PEG-DA (10 mg/ml). Hydrogel formation was confirmed by strain-rate controlled shear rheometery. Dynamic time sweep tests in plate–plate geometry were performed at 37 °C, at a constant frequency of 1 rad/s and a sinusoidal 5% strain using an advanced rheometric expansion system (ARES, Rheometer Scientific). The storage (G') and loss (G'') moduli were recorded as a function of time for up to 10 min by the RSI Orchestrator 6.5.8 software. After 120 s the solution was polymerized by photo-initiation using a UV light source (~20 mW/cm^2). The reported shear modulus was taken as the real part of the complex shear modulus $G^* = G' + iG''$ at the conclusion of the time-sweep test.

2.6. Swelling and biodegradation characterization

Swelling experiments were performed on acellular cylindrical plugs (5-mm diameter) polymerized from 100 μl of PEGylated protein hydrogel precursor. The hydrogel plugs were incubated in 10 ml PBS containing 0.1% sodium azide at RT for 24 h. The wet weight of each plug was recorded before being lyophilized overnight. The dry weight was measured and the swelling ratio (Q) was calculated by dividing the wet weight by the dry weight. An additional swelling parameter, the per cent volume ratio, was defined as the per cent increase between the final volume of the plug as compared to the initial volume at the time of casting ($[V_f/V_i] \times 100$). Biodegradation experiments were performed on acellular fluorescently labeled PEGylated protein cylindrical hydrogel plugs according to protocols described by Peled et al. [25]. Briefly, succinimidyl arcidine-9-carboxylate (SAC, Pierce, IL, USA) was reconstituted in PBS and DMSO (0.1 mg/ml) to make a SAC staining solution. Each plug was incubated in the SAC staining solution overnight followed by extensive washing in PBS and 0.1% sodium azide for 3 d. Each plug was incubated in 3 ml of 0.01 mg/ml collagenase type IA solution (Sigma-Aldrich) in PBS and 0.1% sodium azide at 37 °C with continuous agitation. A Varioscan spectral scanning multimode reader (Thermo Electron Corp.) was used to measure fluorescence intensity of the supernatant (excitation 364 nm, emission 460 nm) every 5 min for up to 10 h. The raw data was normalized by the final fluorescence intensity of the fully degraded hydrogel plugs.

2.7. Cell substrate preparation (2-D)

Hydrogels made from PEGylated proteins were used as substrates for 2-D culture of smooth muscle cells (SMCs). For this purpose, sheep aortic SMCs were cultured up to 8th 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 (Biological Industries). Pre-cast hydrogels were made from PEGylated protein solution (4 mg/ml), 10-kDa linear PEG-DA (10 mg/ml) and photoinitiator (0.1% w/v). The gels were cast onto the bottom of a 24-well plate and seeded with a suspension of SMCs (70,000 cells/well) in 1 ml of culture medium. Cell spreading was monitored immediately after seeding using a phase-contrast microscope (Nikon Eclipse TS100) and cell morphology was documented with a digital CCD camera.

2.8. Cellularized construct preparation (3-D)

Cellularized constructs were made by photopolymerizing a PEGylated protein solution in the presence of dispersed sheep aortic SMCs. The passaged cells (P4–P8) were trypsinized and suspended in 300 μl of PEGylated protein solution (0.5×10^6 cells/ml) containing photoinitiator (0.1% w/v). The encapsulated cells in the cylindrical constructs were monitored and documented daily using a phase contrast microscope. Some experiments were continuously monitored using a custom-built time-lapse incubated microscope system (Nikon Eclipse TE-2000). Time-lapse experiments visualized active remodeling of cells in several locations within each hydrogel by sequentially imaging each field every 10 min for up to 96 h (Appendix A).

2.9. Cellular outgrowth studies

Outgrowth experiments were performed using dense tissue constructs made from compacted SMC-seeded collagen gels which were encapsulated inside PEGylated protein hydrogels. The collagen-based tissue was made from a solution of 5X DMEM (Biological Industries), 10% FBS, reconstituted type-I collagen solution in 0.1 N acetic acid (2 mg/ml), and 0.01 M NaOH with dispersed sheep aortic SMCs (0.5×10^6 cells/ml). The cell-seeded collagen gels were cultured for 2 d in 500 μl of medium before the compacted tissue was placed in 300 μl of PEGylated protein solution and photoinitiator in a 48-well plate. After exposure to 5 min of UV light, the encapsulated tissue was cultured inside the hydrogel with 500 μl of culture medium. The cellular outgrowth experiments were monitored daily and the outgrowth results were quantified by measuring the average migration distance of the cells from the margins of the dense tissue into the PEGylated protein hydrogel using phase contrast micrographs of the samples taken at set time intervals.

2.10. Statistical analysis

Statistical analysis was performed using Microsoft® Excel software. Data from independent experiments were quantified and analyzed for each variable. Comparisons between two treatments were made using Student's T-test (two tail, unequal variance) and comparisons between multiple treatments were made with analysis of variance (ANOVA). A p-value of <0.05 was considered to be statistically significant.

3. Results

3.1. Collagen, fibrinogen, and albumin PEGylation

The Michael-type addition reaction between protein and PEG was confirmed by SDS-PAGE and Coomassie® blue staining. The SDS-PAGE profiles of equal amounts of fibrinogen, albumin, and collagen (PEGylated and un-PEGylated) were compared to a molecular weight (MW) marker. The profile of each protein after PEGylation reveals a detectable effect on its mobility in the polyacrylamide due to the grafted PEG chains (Fig. 1). Generally, protein mobility through polyacrylamide gels is relative to MW such that unreacted protein will have a better mobility in the acrylamide gels compared to the PEGylated protein [26,27]. For the fibrinogen and collagen, PEGylation caused a marked increase in the apparent MW, which was visible on the top-most portion of the gel as a continuous smear. Some of the protein remained unPEGylated as indicated by the fibrinogen band around 50 kDa and the collagen band around 105 kDa. The albumin PEGylation reaction caused most of the conjugated protein to immobilize in the loading portion (top) of the polyacrylamide gel and was therefore not observed in the running gel. Rheology data was used to confirm the photopolymerization of the PEGylated protein hydrogel precursors into a hydrogel network. The

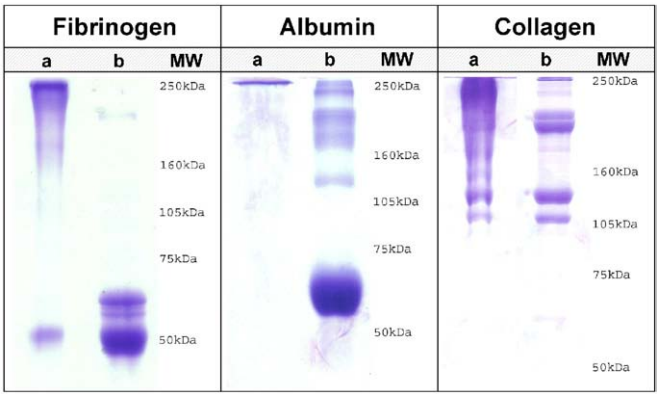

Fig. 1. Protein PEGylation is confirmed by SDS-PAGE. PEGylated and unmodified fibrinogen, albumin and collagen are loaded into 8% polyacrylamide gels and compared to a molecular weight marker (MW). The mobility of the PEGylated proteins (a) in the polyacrylamide gels is markedly reduced when compared to the unmodified proteins (b).

strain-rate rheometry data showed polymerization starting immediately after the UV light was turned on (arrow), and the plateau storage modulus (G') value was reached after less than 3 min, indicating a rapid polymerization reaction (Fig. 2A). The plateau G' values are shown in Table 1 together with the composition of the PEGylated protein hydrogel precursors. The modulus of the PEG–fibrinogen hydrogels was the highest in comparison to the PEG–collagen and PEG–albumin hydrogels, despite having the lowest PEG concentration. The PEG–collagen and PEG–albumin hydrogels contained similar PEG concentrations, yet the PEG–collagen hydrogels exhibited a significantly higher G' value ($p<0.05$, $n\geqslant 6$).

3.2. Swelling and biodegradation properties

The swelling ratio (Q) of the PEG–collagen, PEG–fibrinogen and PEG–albumin hydrogels is shown in Fig. 2B. There was a statistically significant difference in Q among the PEGylated protein materials ($p<0.05$, $n\geqslant 6$), and nearly a two-fold increase in Q of the PEG–fibrinogen hydrogels compared to the PEG–albumin hydrogels. There was also a statistically significant difference in the per cent volume ratio among the PEGylated protein materials ($p<0.05$, $n\geqslant 6$). The volume of the PEG–albumin hydrogels increased by 183% after complete swelling compared to 115% and 55% for the PEG–collagen and PEG–fibrinogen hydrogels, respectively. The biodegradation kinetics of the PEGylated protein hydrogels in a 0.01 mg/ml collagenase solution is reported in terms of percent degradation in Fig. 5. There was a statistically significant difference between the three materials in terms of their biodegradation kinetics ($p<0.05$, $n>6$). The hydrogels made of PEG–collagen degraded the fastest and the hydrogels made of PEG–albumin degraded the slowest in the collagenase solution. The half-life of the PEG–collagen hydrogels in 0.01 mg/ml collagenase was 20 min, compared

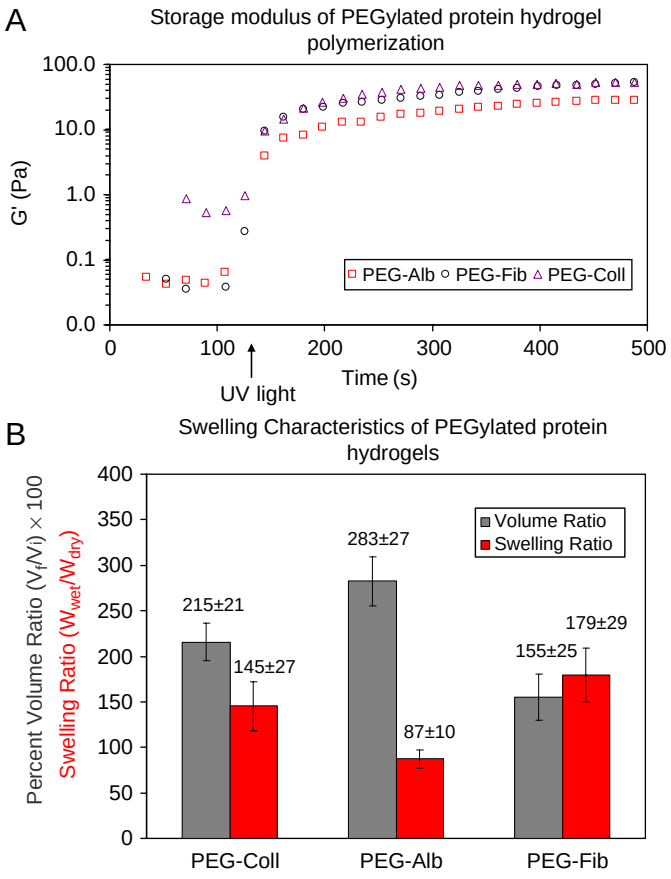

Fig. 2. Physical properties of PEGylated protein hydrogels. (A) shear rheometry data from dynamic time sweep tests were collected during photopolymerization of PEGylated protein precursor with photoinitiator immediately upon activation with a UV light source; the storage (G') modulus of PEG–fibrinogen (○), PEG–collagen and (△) PEG–albumin (□) are shown. (B) The percent volume ratio (V_f/V_i) × 100 and swelling ratio (W_{wet}/W_{dry}) are shown for PEG–collagen, PEG–albumin, and PEG–fibrinogen. Each hydrogel material has significantly different volume and swelling ratios ($p<0.05$, $n\geqslant 6$).

to 40 min for the PEG–fibrinogen hydrogels and 70 min for the PEG–albumin hydrogels (Fig. 3).

3.3. Cell culture experiments

SMCs were cultured on thin layers of hydrogel to assess the ability of the protein backbone of the material to support cell adhesion. A summary of the adhesion experiments is shown in Fig. 4. The cells did not spread on the PEG–albumin hydrogels and remained round 2 h and 24 h after seeding (Fig. 4A and B). In contrast, cell spreading began immediately after seeding on both the PEG–collagen and PEG–fibrinogen hydrogels (Fig. 4C and E), and the cells were completely spread on the surface of the hydrogels after 24 h (Fig. 4D and F). Tissue culture plastic (TCP) and PEG only hydrogels served as positive and negative controls, respectively (data not shown). The 3-D culture experiments were carried out with all three materials by dispersing SMCs into the hydrogel precursor solution and polymerizing the cell suspension to

Table 1
Characterization of PEGylated proteins used for rheological measurements and plateau storage modulus (G')

	Protein conc. (mg/ml)	PEG–protein conc. (mg/ml)	PEG conc. (mg/ml)	% PEGylation [18]	Plateau G' (Pa)	n
PEG–Alb	4	23±1	19±1	89±3	30±7	6
PEG–Fib	4	13±3	9±3	129±47	60±19	10
PEG–Coll	5	27±4	22±4	122±11	52±16	15

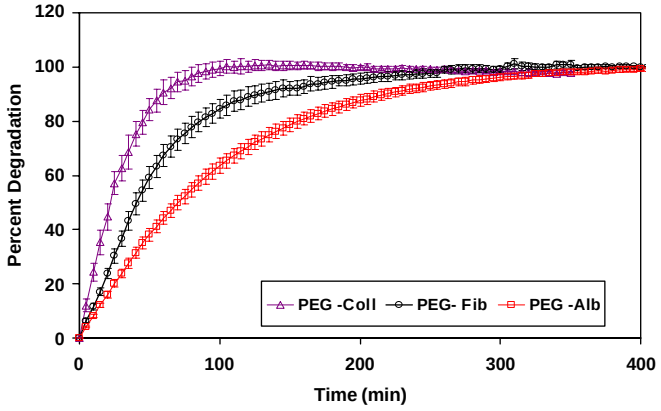

Fig. 3. Degradation kinetics of PEGylated protein hydrogels in collagenase solution. The average rate of biodegradation of at least nine hydrogel samples of PEG–fibrinogen (○), PEG–collagen (△) and PEG–albumin (□) is shown as a function of time with standard error bars. The degradation profiles of the three hydrogel materials are significantly different ($p<0.05$, $n>6$).

encapsulate the cells in the matrix. The initially rounded cells are shown encapsulated by the PEG–albumin, PEG–collagen, and PEG–fibrinogen hydrogel matrix at the onset of the experiment (Fig. 5A, D, G, respectively). After 4 h, the rounded cells in the PEG–collagen and in the PEG–fibrinogen began to form extensions (Fig. 5E and H, respectively), whereas in the PEG–albumin the cells remained rounded (Fig. 5B). After 24 h the cells spread and became spindled in the PEG–collagen and PEG fibrinogen hydrogels (Fig. 5F and I, respectively), whereas in the PEG–albumin hydrogels, the cells remained completely rounded and did not form extensions into the matrix (Fig. 5C). Time-lapse video microscopy of the cell spreading occurring within the hydrogels during the first 24 h is shown in a supplementary video sequence (Appendix A). In the video, the SMCs are documented in their initially rounded morphology as they eventually formed extensions within the dense matrix of the PEG–collagen and PEG-fibrinogen materials. The SMCs in the PEG–albumin hydrogels remain rounded throughout the experimental duration.

3.4. Tissue outgrowth experiments

Compacted SMC-seeded collagen gels were entrapped within the PEG–protein hydrogels and the cellular outgrowth was documented for up to five days (Appendix A). The SMCs began to invade the PEG–fibrinogen matrix almost immediately after casting and continued to uniformly invade the matrix throughout the duration of the experiment (Fig. 6). SMC invasion into PEG–collagen hydrogels was less uniform compared to PEG–fibrinogen. The morphology of the invading cells appeared similar in both the PEG–fibrinogen and the PEG–collagen. There was no SMC invasion observed into the PEG–albumin hydrogels. Quantitative measurements of the average cell migration distance into the PEG–collagen and PEG–fibrinogen hydrogels revealed a similar rate of migration in both materials (Fig. 6, graph).

4. Discussion

Collagen and fibrin are popular biomaterials in tissue regeneration because of their susceptibility to cellular proteases combined with their abundant biological activity. A major drawback of these biological protein-based biomaterials is their limited control over physical and degradation properties that can lead to premature breakdown of the matrix in the presence of cells and tissues. Our laboratory has been developing a new class of protein-based materials using PEGylation to improve structural properties and control biodegradation [28]. We previously reported on PEGylated fibrinogen hydrogels [29], and now we demonstrate the ability to create PEGylated collagen and albumin materials for cell culture. The PEGylation of these three proteins was accomplished by a Michael-type addition reaction in which free thiols present in unpaired cysteine residues in the denatured protein were covalently conjugated to unsaturated double bonds on functionalized PEG-DA [30]. For this purpose, the collagen molecule required modification using a SATA reaction scheme, which introduced acetylated SH groups on the lysine residues [24]. In all of the PEGylation reactions, molar excess of PEG-DA relative to the thiols was used in order to ensure that only a single acrylate on the PEG-DA molecule would react with a single protein thiol. The other acrylate group on the protein-bound PEG was left unreacted in order to allow for free-radical polymerization of the hydrogel in the presence of photoinitiator and UV light. SDS-PAGE was used to confirm the PEGylation reaction of each protein. Shear rheometry was used to verify the formation of a hydrogel network from the PEGylated protein hydrogel precursor in the presence of UV light and photoinitiator.

The best method for PEGylating a protein depends on the desired properties of the protein–polymer conjugate,

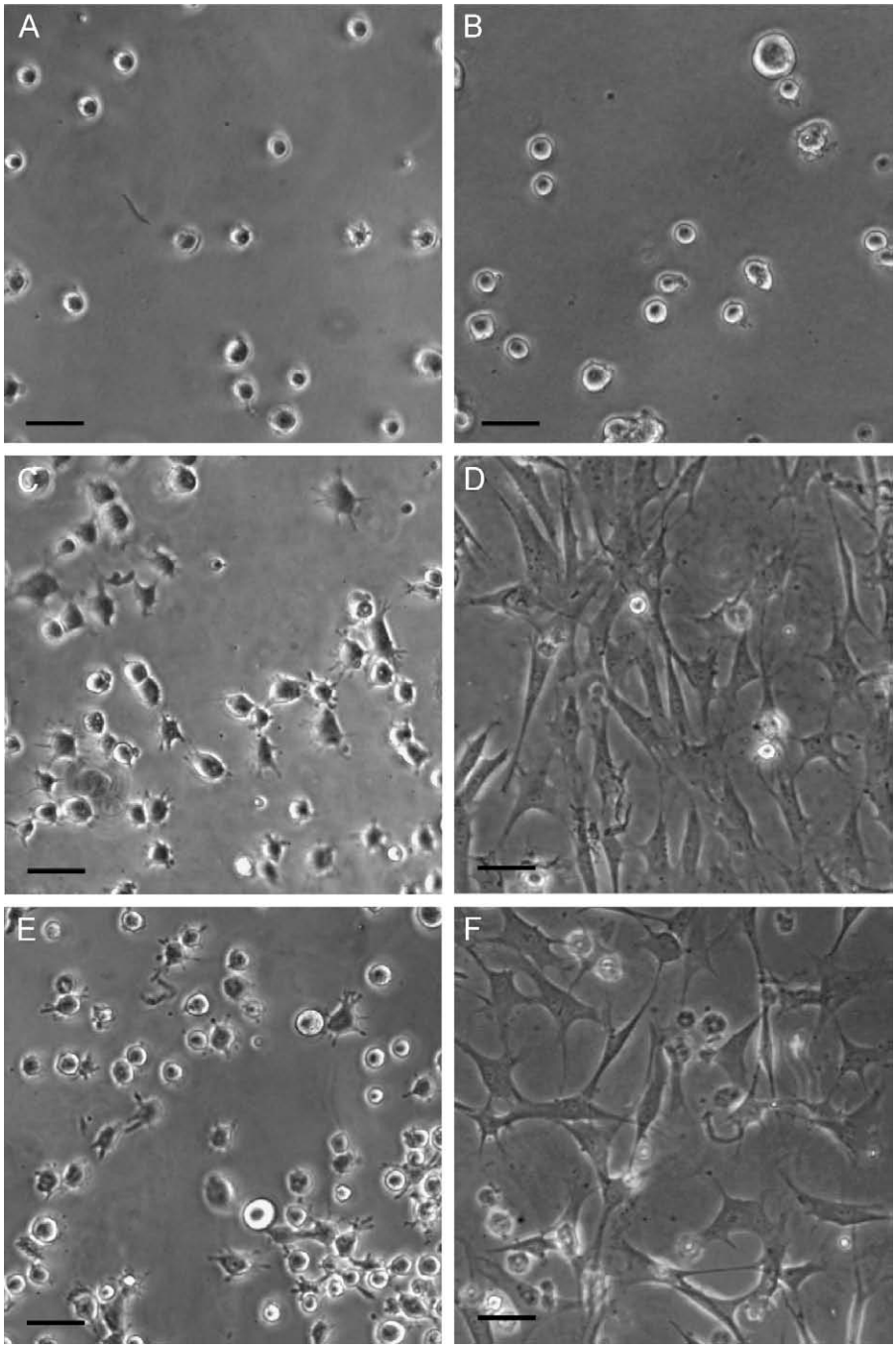

Fig. 4. Smooth muscle cells (SMCs) adhering to PEGylated protein hydrogels. Representative samples show PEG–albumin does not support cell adhesion of SMCs after 2 h (A) or 24 h (B). SMCs adhere to the PEG–collagen and PEG–fibrinogen hydrogels as early as 2 h after seeding (C and E, respectively). The SMCs are completely spread on the surface of the PEG–collagen and PEG–fibrinogen hydrogels 24 h after seeding (D and F, respectively). Phase contrast micrographs are shown with 500 μm scale bar.

which are influenced by the PEG attachment sites, the type of linkage, the reaction conditions, the molecular weight of the PEG chain and the number of PEG chains that are attached [16]. Typically, the biological function of the PEGylated protein conjugate is the most important factor in determining the method of PEGylation. A wide variety of end-group modification chemistries have been developed for coupling PEG to specific amino acids in the protein sequence in order to minimize the inadvertent targeting of active sites by the PEG chains [31]. We chose a thiol modification scheme for PEGylation of our protein in the biosynthetic scaffold system. Unlike therapeutic peptides or protein drugs, the loss of some biological activity as a result of PEGylation of a structural protein such as collagen, fibrin or albumin is less detrimental to the design of a scaffold for tissue engineering. Nevertheless, it is

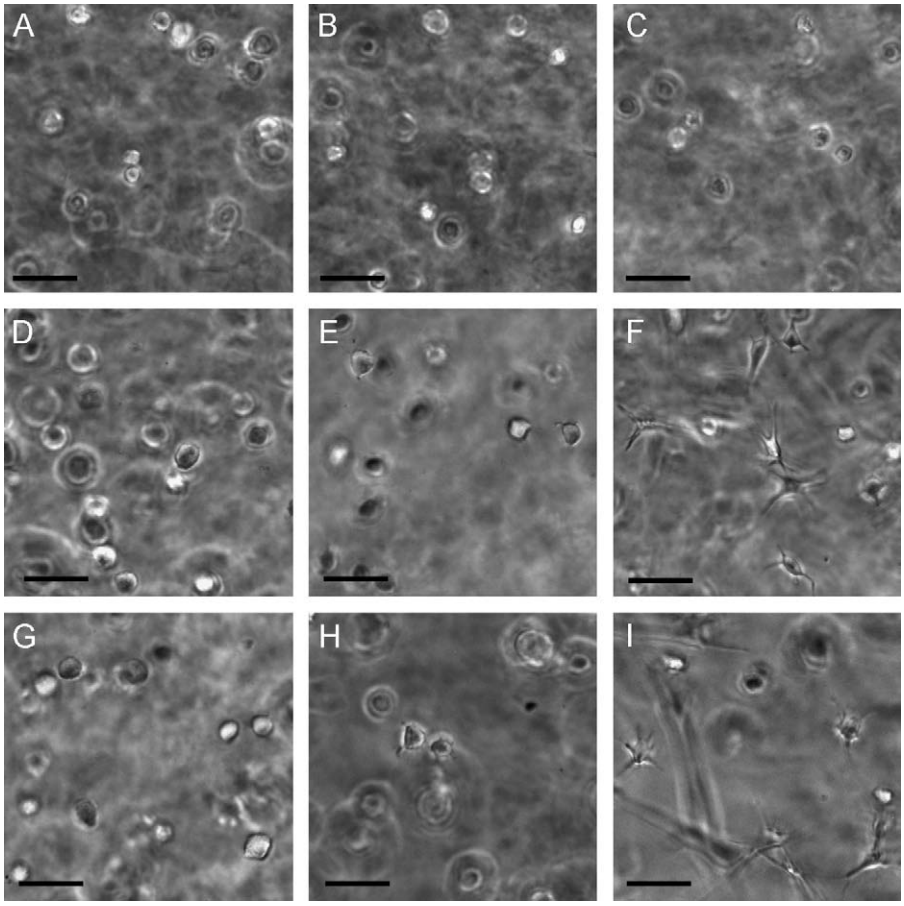

Fig. 5. Morphology of encapsulated smooth muscle cells (SMCs) inside PEGylated protein hydrogels. Representative samples show SMCs are initially rounded and encapsulated by the hydrogel matrix made from PEGylated albumin, collagen and fibrinogen (A, D and G, respectively). After 4 h, the SMCs begin to form protrusions into the PEG–collagen and PEG–fibrinogen matrix (E and H, respectively) but are unable to protrude into the PEG–albumin hydrogel (B). After 24 h, the SMCs are fully extended and highly spindled inside the PEG–collagen and PEG–fibrinogen matrix (F and I, respectively) where as in PEG–albumin they remain rounded (C). Phase contrast micrographs are shown with 500 μm scale bars.

important to recognize that balance between the physicochemical benefits of PEGylation and the loss of bioactivity can be more precisely regulated.

Having shown that the PEGylated protein precursors can be formed into a cell scaffold by *in situ* photopolymerization in the presence of SMCs, we addressed the concern that UV light or the free radicals present during the polymerization may harm the resident cells or alter their viability. We investigated this by exposing the cells to UV light (365 nm, 4–5 mW/cm^2) for 5 min under three different conditions, including in PBS, in PBS with excess photoinitiator (0.3% w/v), and in PBS with excess photoinitiator (0.3% w/v) and PEGylated protein solution (4 mg/ml). SMCs suspended in PBS but not exposed to UV light were used as a negative control. Viability was quantified by counting non-viable cells stained with ethidium homodimer (Sigma-Aldrich) and comparing to the total cell number. The viability of SMCs in the presence of PEG–protein, photoinitiator and UV light was statistically similar to the control group without UV light or photoinitiator (~80%, $p > 0.05$, $n \geqslant 12$). The presence of UV light with or without photoinitiator reduced the SMC viability to 61% and 36% of controls, respectively ($p < 0.05$, $n \geqslant 12$). We speculate that free radicals, formed when exposing photoinitiator to the UV light, were particularly harmful to the cells and that the presence of the acrylate groups on the PEGylated protein precursor has a protective effect on the cells because the acrylates react with free radicals during the polymerization.

In our investigation, the ultimate goal of PEGylating proteins into a hydrogel biomaterial was to provide alternative protein-based biomaterial scaffolds in tissue engineering. Unlike the fibrillar networks formed by reconstituted collagen and fibrin [4,32], the PEGylated protein hydrogels encapsulate the cells with a dense amorphous matrix [18]. There are a number of basic features of encapsulating biomaterials which are essential for cells to remodel the matrix, including proteolytic susceptibility. In this regard, cellular remodeling is defined as the process of resorption of existing matrix followed by replacement with new matrix. Proper cell remodeling requires cell–matrix interactions which are facilitated by cell–surface adhesion molecules. Fibrinogen and collagen are both encoded with cell adhesion sequences, such as

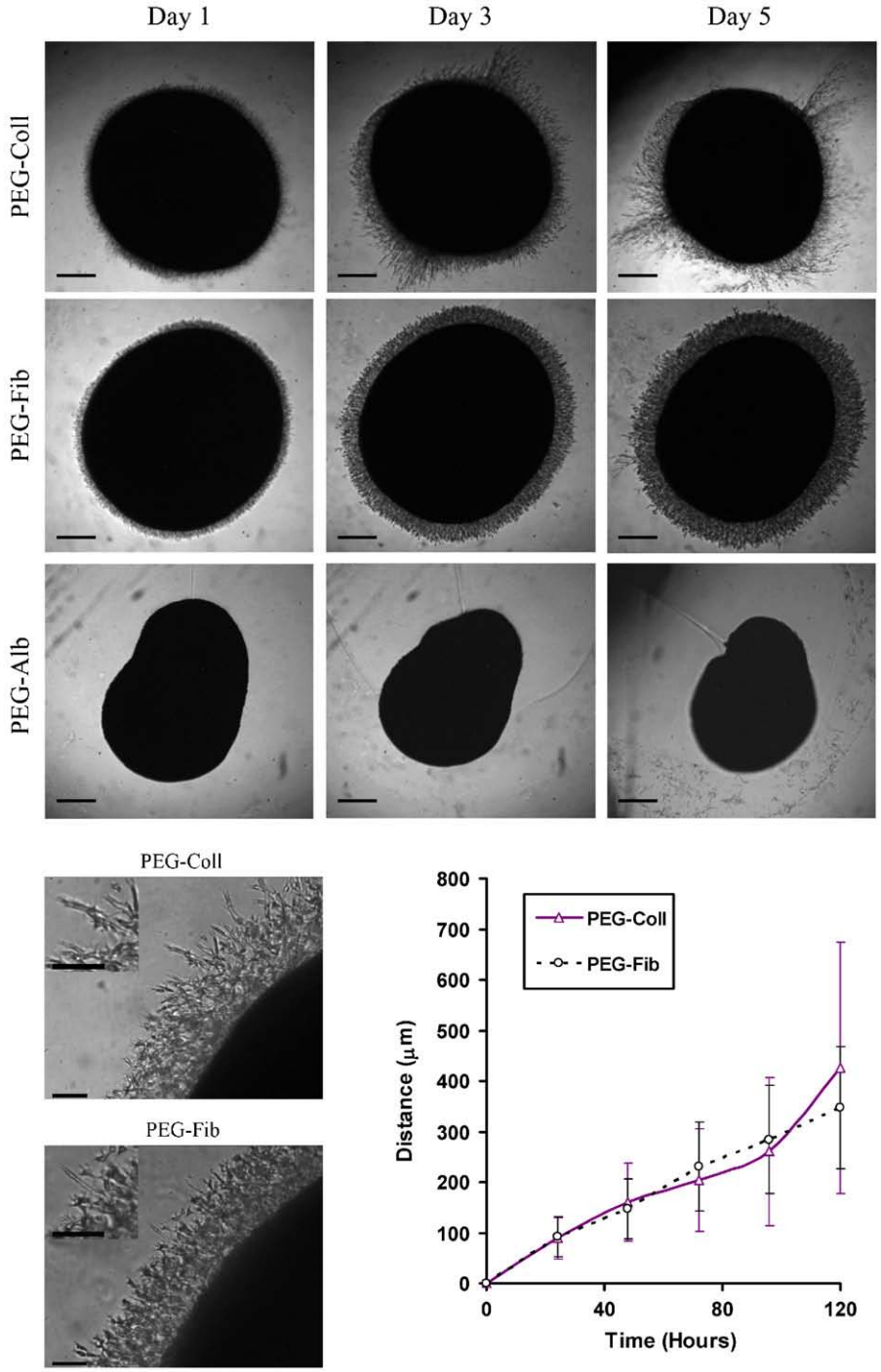

Fig. 6. Cellular outgrowth experiments document smooth muscle cell (SMC) invasion into PEGylated protein hydrogels from a dense tissue construct (dark). The compacted SMC tissue is encapsulated inside hydrogels made from PEG–collagen, PEG–fibrinogen and PEG–albumin. The outgrowth and cellular invasion of the SMCs from the dense tissue into the transparent hydrogel matrix is documented by phase contrast microscopy. The invading SMCs are evident in the PEG–collagen and PEG–fibrinogen hydrogels on the first day after encapsulation. After 5 days, the SMCs have migrated a few hundred microns out from the tissue mass. In the PEG–albumin hydrogels, the SMCs are unable to invade into the matrix. Quantitative data measuring invasion distance directly from the phase contrast images show a similar migration rate in PEG–collagen and PEG–fibrinogen hydrogels. Low magnification phase contrast micrographs (top) are shown with 500 μm scale bars; higher magnification images (bottom) are shown with 100 μm scale bars.

Arg–Gly–Asp (RGD), which are capable of facilitating cell–matrix interactions [33]. PEGylation of these two proteins does not interfere with their ability to support adhesion of SMCs when cultured on top of the PEGylated protein hydrogels (2-D culture). PEG–albumin, on the other hand, does not demonstrate similar ability to support SMC adhesion in 2-D culture. Albumin does not contain any known SMC adhesion domains, which could explain

the lack of cell adhesion (and spreading) in the PEG–albumin hydrogels [34].

Proteolytic degradation is another property of biological materials which facilitates efficient cellular remodeling. In the PEGylated protein biomaterial design, the PEGylation reduces the enzymatic susceptibility of the proteins [22] and provides control over the scaffold biodegradation based on the PEG/protein composition [35]. The proteolytic susceptibility of all three biomaterials was confirmed using a quantitative biodegradation assay in the presence of a collagenase solution, revealing that the degradation rate of each material was affected by the type of protein used. PEG–albumin hydrogels exhibited the longest half-life in collagenase, suggesting that cells may experience more difficulty in remodeling this material when compared to PEG–fibrinogen and PEG–collagen hydrogels.

A morphological assessment of the encapsulated SMCs was performed in each biomaterial to examine how readily cells can manipulate the protein backbone of the dense hydrogel matrix in order to from 3-D cellular extensions. The process of cellular remodeling was captured in time-lapse video microscopy sequences showing the initially rounded cells forming extensions in the dense matrix of PEG–collagen and PEG–fibrinogen (Appendix A). The cells eventually express a spindled morphology based on their ability to adhere to and physically remodel the PEGylated protein backbone of the matrix [35]. The SMCs were not able to spread within the PEG–albumin hydrogels even after several days in culture. The lack of adhesion sites on the PEG–albumin backbone is presumed to limit the ability of SMCs to form extensions.

Matrix porosity and cross-link density may also affect the cellular behavior within these biomaterials. The swelling properties were measured to provide some insight into the matrix porosity of each material. Normally, the relationship between the swelling of a PEG hydrogel and the mesh size of the network is described by a power law and the mesh size is directly related to the degree of hydrogel cross-linking [36]. However, the protein backbone of the polymeric network complicates this straightforward relationship as can be seen when comparing swelling or rheology data among the three PEGylated protein hydrogel materials. For example, there is no direct correlation between the PEG concentration and the plateau storage modulus (G') (Table 1), even though the number of cross-linking acrylate groups is proportional to the PEG concentration. We speculate that the molecular interaction between the hydrophobic protein constituent and the highly hydrophilic PEG chains has a strong influence on the swelling, cross-linking density and mesh size of the PEGylated protein hydrogels. In this regard, the percent volume ratio indicates that the PEG–albumin hydrogels imbibe larger volumes of water and this could suggest that the PEG–albumin is more hydrophilic compared to the other two backbone proteins. Nevertheless, the relative role of the protein backbone and PEG side chains in determining the structural features of the hydrogel network is difficult to ascertain from swelling and rheology data alone and would require further analysis to fully understand.

The uncertainties regarding the mesh size of the hydrogel network not withstanding, the cell migration into the hydrogel biomaterials can be documented using a cellular outgrowth assay which revealed a similar rate of cellular invasion into the PEG–collagen and PEG–fibrinogen hydrogels. The PEG–albumin hydrogels did not support the migration of SMCs into the biosynthetic matrix. It was assumed that SMC migration would proceed through physical and proteolytic remodeling because of the dense amorphous nature of the PEGylated protein hydrogels. In reconstituted collagen biomaterials, the potential of enzyme-independent cell migration arises from the fact that reconstituted collagen (<4 mg/ml) is fibrillar and has a pore size on the length scale of cellular processes and extensions [37]. In contrast to reconstituted collagen gels, cell migration in reconstituted fibrin clots (>30 mg/ml) is more dependent upon cell-associated proteolytic activity, which results from the smaller mesh size of the fibrin's fibrillar matrix. This mesh size is owing to the nature of the fibrin network formation and covalent stabilization of the fibrin fibrils after cross-linking [8,38]. One of the distinct advantages of the PEGylated fibrinogen and PEGylated collagen hydrogels is that the porosity and proteolytic susceptibility of the network can be controlled by the PEG constituent so that there is no longer a porous fibril network controlling the migration and spreading of the cells. Instead, we speculate that cell migration is controlled by proteolytic resistance of the protein backbone as cells tunnel through the dense matrix using proteases to loosen the network, form extensions by cell adhesion interactions, and eventually remodel the provisional scaffold [35]. The mechanism of cell spreading and migration in the PEG–protein hydrogels continues to be thoroughly investigated as part of our ongoing research efforts.

Finally, it is important to note for the purpose of comparison that the PEGylation of collagen, fibrinogen and albumin yielded significantly different hydrogel precursors in terms of protein concentration and PEG concentration (Table 1). A meaningful comparison between the three hydrogel systems required that at least one of the parameters of the material remain constant (i.e. protein concentration or PEG-to-protein ratio). We attempted to normalize the precursor protein concentration of each solution (4–5 mg/ml prior to hydrogel formation) in order to compare the three biomaterials in the cell culture studies. Additional unreacted PEG-DA was also added to the precursor solution (1% w/v) to efficiently cross-link the PEGylated proteins and overcome limitations that may result in poor gelation at these relatively low protein concentrations. The limitation of normalizing the hydrogels with a uniform protein concentration is that the hydrogels contain different PEG-to-protein molar ratios. The relative amount of PEG-to-protein can have a significant impact on the structural properties, cross-link density, bioactivity and biodegradation kinetics of the material.

5. Conclusions

Protein-based biosynthetic materials were developed for the purpose of creating scaffolds that emulate the biological characteristics of the native ECM with added control over structural properties and biodegradation. Using a matrix made from PEGylated fibrinogen, collagen or albumin, we showed that the biomaterial can maintain cell adhesion and support proteolytic degradability based on the specific characteristics of the protein backbone. The biological backbone of these materials also ensures adequate bioactivity and sufficient biocompatibility. The synthetic PEG constituent provides the possibility of controlling the enzymatic biodegradation and structural properties of the materials without introducing additional cross-linking steps. This combination of synthetic polymers and reconstituted proteins for scaffold design may help broaden the usefulness of natural protein-based scaffolds in tissue engineering and regeneration.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.biomaterials.2007.05.005.

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