

Fibrin-based hydrogel scaffolds for controlling cell-matrix interaction in vascular tissue engineering

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Abstract. Hydrogel biomaterials are used as scaffolds in vascular tissue engineering with the purpose of creating tissue mimics as replacement blood vessels. A tissue engineered vascular construct must be capable of supporting the growth and proliferation of different cell types, including endothelial cells, vascular smooth muscle cells and fibroblasts. The purpose of this investigation was to explore the use of a biocomposite hydrogel biomaterial for vascular tissue engineering, made from a network of fibrillar fibrin and amorphous semi-synthetic polyethylene glycol (PEG) – fibrinogen. Both phases of the biocomposite provide distinct biophysical interactions between the material and the cultivated vascular cells. Several properties of the tissue engineered vascular constructs were controlled, including the scaffold composition (fibrin versus PEG-fibrinogen), matrix mechanical properties (shear storage modulus), and cell composition. The constructs were grown for up to 7 days *in vitro*, and examined by fluorescence microscopy for cell viability and cell morphogenesis. The endothelial cells survived poorly in the absence of smooth muscle cells or fibroblasts on the PEG-fibrinogen hydrogels. The fibrillar fibrin constituent promoted better survival and spreading of the endothelial cells on top of the scaffold, even in the absence of smooth muscle cells or fibroblasts. The greatest impact on endothelial cell viability and spreading was observed with the highest fibrin concentrations. The mechanical properties of the composite, as well as different crosslinking protocols had a minor influence on the morphology and longevity of the endothelial cells. However, the addition of PEG-fibrinogen to the fibrin decreased the matrix degradation rate, supporting endothelial cell morphogenesis for longer durations. In conclusion we have demonstrated that a biocomposite fibrin-based material, containing both fibrillar and amorphous phases, may be optimized to enhance endothelial cell survival in vascular tissue engineering.

Keywords: fibrinogen, endothelial cells, hydrogels, biomaterials, vascular grafts, angiogenesis, tissue engineering

1. Introduction

The rate of cardiovascular disease throughout the developed world results in an increasing demand for small diameter blood vessels as replacement grafts [1, 2]. Successful synthetic grafts have been fabricated having diameters larger than 6 mm using polyethylene terephthalate (PET) and expanded polytetrafluoroethylene (ePTFE); however, problems such as thrombogenicity and compliance mismatch still exist with small-diameter (<6 mm) prosthetic vascular grafts [3–6]. An endothelial lining on a small diameter blood vessel graft—one containing a uniform monolayer of endothelial cells (ECs)—could improve its performance by providing a physical blood-contacting barrier with antithrombotic properties [6]. However, *in vitro* endothelialization of prosthetic vascular grafts have been met with various difficulties in terms of the long-term patency in clinical translation [3].

Tissue engineering techniques have been used to overcome some of the limitations of small-diameter prosthetic vascular grafts. The main aim of developing a tissue-engineered vascular graft (TEVG) is to provide a biological blood vessel substitute by combining cellular components with biodegradable scaffolds [1, 7–9]. The engineered substitute must be non-thrombogenic, exhibit vasoactivity and possess appropriate mechanical properties [7, 8, 10]. One possibility for making functional tissue-engineered vascular grafts is to use a cell-seeded biodegradable scaffold made from a semisynthetic biomaterial which incorporates biological macromolecules covalently bonded to structurally versatile synthetic polymers [11]. These more sophisticated biomaterial scaffolds provide a biomimetic cellular environment by balancing the structural and biofunctional requirements of the vascular prosthetic [12]. Synthetic polymer components offer excellent control over the structural properties of a vascular construct [13], whereas the bioactive components afford the material cell signaling, including cell adhesion, proliferation and differentiation cues [14]. The

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bioactive component of a semisynthetic scaffold can consist of macromolecules such as protein fragments [15], growth factors [16–18], or bioactive peptide sequences [19–21] that are found in the native extracellular matrix (ECM).

The design of a semisynthetic scaffold material with biomimetic features is often inspired by structure-function relationships found in the body tissues. For example, interplay between cells and structural features of the vascular extracellular matrix can help guide endothelial cell morphogenesis and help determine barrier and antithrombotic functions. Therefore, artificial mimics of the vascular extracellular matrix have been studied in an effort to devise better tissue-engineered vascular grafts that can similarly mimic both the chemical composition and the physical structure needed for endothelial cells to function properly [3]. Our laboratory has developed an amorphous hydrogel biomaterial from polyethylene glycol (PEG) and fibrinogen that can mimic the provisional fibrin blood clot matrix [22]. Hydrogels made of PEG-fibrinogen (PF) are highly compatible with cells and tissues [23, 24], and exhibit versatile physical characteristics based on their weight percent, molecular chain length, and crosslinking density [25, 26]. Most of the structural properties of the PF hydrogel are controlled through the synthetic PEG constituent, which can produce a crosslinked network by a mild UV light-activated free-radical polymerization reaction [27]. The biological features of PEG-fibrinogen are provided by the fibrinogen component, which contains several cell signaling domains, including a protease degradation substrate and cell-adhesion motifs [28]. Accordingly, the fibrinogen backbone in the PEG-fibrinogen hydrogels endows the semisynthetic PEG-fibrinogen material with inherent degradability by way of cell-activated protease activity and cell-specific adhesivity that are not available in synthetic PEG hydrogels. Despite certain advantages of the semisynthetic PEG-fibrinogen hydrogels, they are completely devoid of microstructural features inherent to fibrin gels and the vascular extracellular matrix [29, 30]. Those features, which include a fibril arrangement of the endothelial cell basement membrane, may be required to support better cell adhesion and morphogenesis.

Fibrin gels have been studied extensively as scaffolds for tissue-engineered vascular grafts, partly because they can mimic the natural tissue architecture of the vascular wall [31]. They can be fashioned together with vascular cells into a fibrillar conduit that results in high cell seeding efficiency, uniform cell distribution and inherent biophysical compatibility that favorably affect cell behavior [32]. Previous studies have shown that vascular cells entrapped in fibrin or PF hydrogels produce collagen and increase the structural integrity of the constructs, suggesting that the fibrin constituent may support cell growth and remodeling, as well as a vascular cell phenotype [33, 34]. However, fibrin gels also degrade much faster than PF hydrogels, and do not possess the mechanical integrity to maintain the de-

sired shape and structure needed for tissue-engineered vascular grafts [32, 33]. In the current study, we tested a bio-composite material combining a PF hydrogel and an insoluble fibrin gel network, in order to create a TEVG scaffold with controllable microarchitecture, mechanical and biological properties. Biocomposites were made with different amounts of PEG-fibrinogen and fibrin, crosslinked by UV light-activated photo-chemistry and enzymatic reactions, and studied using a co-culture of endothelial cells and smooth muscle cells or fibroblasts in order to determine the effects of the material and cell composition on the endothelial cell survival and morphogenesis.

2. Materials and 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 [35]. In brief, acrylation of PEG-OH was carried out under argon by reacting a dichloromethane solution of PEG-OH (Aldrich, Sneeze, Germany) with acryloyl chloride (Merck, 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.

2.2. Fibrinogen PEGylation

The fibrinogen (Bovine Fibrinogen, Sigma Aldrich) was PEGylated according to a protocol similar to the one described elsewhere [25]. Tris (2-carboxyethyl) phosphine hydrochloride (TCEP-HCl) (Sigma-Aldrich) was added to a 7 mg/ml solution of bovine fibrinogen (Sigma-Aldrich) in 150 mM phosphate buffer saline (PBS) with 8 M urea (molar ratio 1.5 : 1 TCEP to fibrinogen cysteines). Linear 10 kDa PEG-diacrylate was reacted for 3 h with the protein at a 4 : 1 molar ratio of PEG to fibrinogen cysteines. The PEGylated fibrinogen was precipitated in acetone and redissolved in PBS containing 8 M urea at 7 mg/ml final fibrinogen concentration. The protein product was dialyzed against PBS at 4 °C for 2 days (Spectrum, 12–14 kDa MW cutoff, California, USA). The PEGylated product was characterized according to published protocols [25].

2.3. PF hydrogels

PF hydrogels were made from a precursor solution of PEGylated fibrinogen with 10 mg/ml protein concentration and 2 % (w/v) additional PEG-DA. The precursor solution was mixed with 10 % (w/v) Irgacure™2959 photoinitiator stock solution containing 70 % ethanol in deionized water (Ciba Specialty Chemicals, Tarrytown, New York), to yield a final Irgacure concentration of 0.1 % (w/v). The precursor solution was then placed into a circular Teflon mold (15 mm diameter and 1 mm height) attached to a glass cover slip (Menzel-Glaser®, Braunschweig, Germany). The PEG-fibrinogen was crosslinked by photopolymerization under

long-wave UV light (365 nm, 4–5 mW/cm²) for 10 min. Cell-seeded constructs were prepared with human foreskin fibroblasts (HFFs) or sheep aortic smooth muscle cells at a final cell concentration of 10⁶ cells/ml. The cells were mixed with the hydrogel precursor solution prior to the light-activated crosslinking reaction. After photopolymerization, the hydrogels were removed from the mold, covered with 1 ml of cell-specific culture medium, and incubated for 12 h at 37 °C prior to endothelial cell seeding.

2.4. Fibrin hydrogels

Fibrinogen was dissolved in PBS to a concentration of 50 mg/ml and filtered through a 0.22 µm disk filter for sterilization. Sterile human thrombin solution (4 IU/ml) in calcium chloride (Tisseel kit, Baxter) was added to an equal volume of fibrinogen solution. The mixture was placed in a mold as before, and left at room temperature for 10 min until polymerized. Cell-seeded constructs were prepared with human foreskin fibroblasts or smooth muscle cells at a cell concentration of 10⁶ cells/ml. The cells were mixed with the fibrinogen solution prior to the addition of thrombin. After polymerization, the hydrogels were removed from the molds and placed in medium with aprotonin (20 µg/ml), as before. The final fibrin concentration in the hydrogel was 25 mg/ml.

2.5. Biocomposite PEG-fibrinogen/collagen hydrogels

Biocomposite PEG-fibrinogen/collagen constructs were made with reconstituted rat-tail type I collagen solution in 0.05 M acetic acid (Becton Dickinson, East Rutherford, New Jersey). The collagen solution was added to a PEG-fibrinogen hydrogel precursor solution with 10 mg/ml fibrinogen concentration and 2 % (v/v) additional PEG-DA, to reach a final collagen concentration of 4 mg/ml. Cell-seeded constructs were prepared with human foreskin fibroblasts or smooth muscle cells at a final cell concentration of 10⁶ cells/ml by adding the cells prior to crosslinking. The solution was mixed with photoinitiator, placed into Teflon molds, photopolymerized for 10 min, and placed in medium, as before.

2.6. Biocomposite PF/fibrin hydrogels

The biocomposites were fabricated by mixing PF precursor solution containing 10 mg/ml protein concentration, 2 % (v/v) PEG-DA, and 0.1 % (w/v) photoinitiator with a 50 mg/ml fibrinogen solution. Thrombin solution (4 IU/ml) was added in an equal volume to the soluble fibrinogen component. The mixture was polymerized in Teflon molds (as before) by one of two protocols: (i) Chemical crosslinking through photopolymerization simultaneous to enzymatic crosslinking (UV light on immediately for 10 min), and (ii) enzymatic crosslinking through thrombin activation followed by chemical crosslinking by photopolymerization (UV light on after 10 min, for 10 min duration). The

crosslinked biocomposite constructs were placed in medium with aprotonin (20 µg/ml), as before. Four biocomposite compositions were tested, including: a) fibrin : PF = 80 : 20, b) fibrin : PF = 60 : 40, c) fibrin : PF = 40 : 60, d) fibrin : PF = 20 : 80. These corresponded to a fibrin : PF protein ratio (w/v) of: a) 20 : 2, b) 15 : 4, c) 10 : 6, d) 5 : 8, respectively. Table 1 summarizes the experimental design for the various biocomposites and control hydrogels that were prepared and tested.

2.7. Endothelial cell cultures

The constructs were seeded with a suspension of bovine aortic endothelial cells (BAECs) or human umbilical vein endothelial cells (HUVECs) in 1 ml of cell-specific culture medium (30 000 cells/construct). The culture medium for BAECs was prepared by mixing low glucose (1000 mg/ml) Dulbecco's modified Eagle's medium (DMEM, Gibco), 10 % fetal bovine serum (FBS, biological industries), 1 % penicillin-streptomycin, 1 % L-glutamine (Biological Industries) and 25 ng/ml human vascular endothelial growth factor (VEGF, PeptoTech, Inc., Rocky Hill, NJ, USA). EGM-2 culture medium for HUVECs was made from 2 % (v/v) FBS in endothelial cell basal medium containing 0.04 % (v/v) hydrocortisone, 0.1 % (v/v) heparin, 0.1 % (v/v) human epidermal growth factor (hEGF), 0.1 % (v/v) long R³-human insulin-like growth factor (IGF-1), 0.1 % (v/v) ascorbic acid, 0.4 % (v/v) human fibroblast growth factor (hFGF)-B, 0.1 % (v/v) vascular endothelial growth factor, 0.05 % (v/v) gentamicin and 0.05 % (v/v) amphotericin-B. The culture medium was changed every other day and endothelial cell spreading was monitored daily using a Nikon T1-SNCP phase contrast microscope. Digital micrographs of the endothelial cells were recorded using a CCD camera attached to the microscope (Jenoptik, Germany). Isolectin B4 and von Willebrand factor (vWF), molecular probes used for labeling endothelial cells, were used for staining the constructs [36]. The constructs were fixed in pH neutralized 4 % formalin in PBS (Gadot, Haifa, Israel) for 20–30 min. After fixation, the constructs were washed three times with PBS. Fluorescein-labeled isolectin B4 (diluted 1 : 100, Vector laboratories, Inc., Burlingame, CA, USA) and DAPI nuclear stain (diluted 1 : 500, Sigma Aldrich) were added to each construct and incubated overnight at 4 °C. For von Willebrand factor staining (vWF), a polyclonal rabbit anti-vWF (diluted 1 : 200, Dako, Glostrup, Denmark) and a Cy2-conjugated anti rabbit IgG (1 : 100) were used together with a DAPI nuclear counterstain (Sigma). After staining, the constructs were washed 3 times with PBS and imaged using fluorescence microscopy with a digital camera. Cell viability, based on membrane integrity, was assessed using a live/dead viability kit (Molecular Probes, Inc) according to the manufacturer instructions. Live cells were stained positive with green while dead cell nuclei were visualized in red.

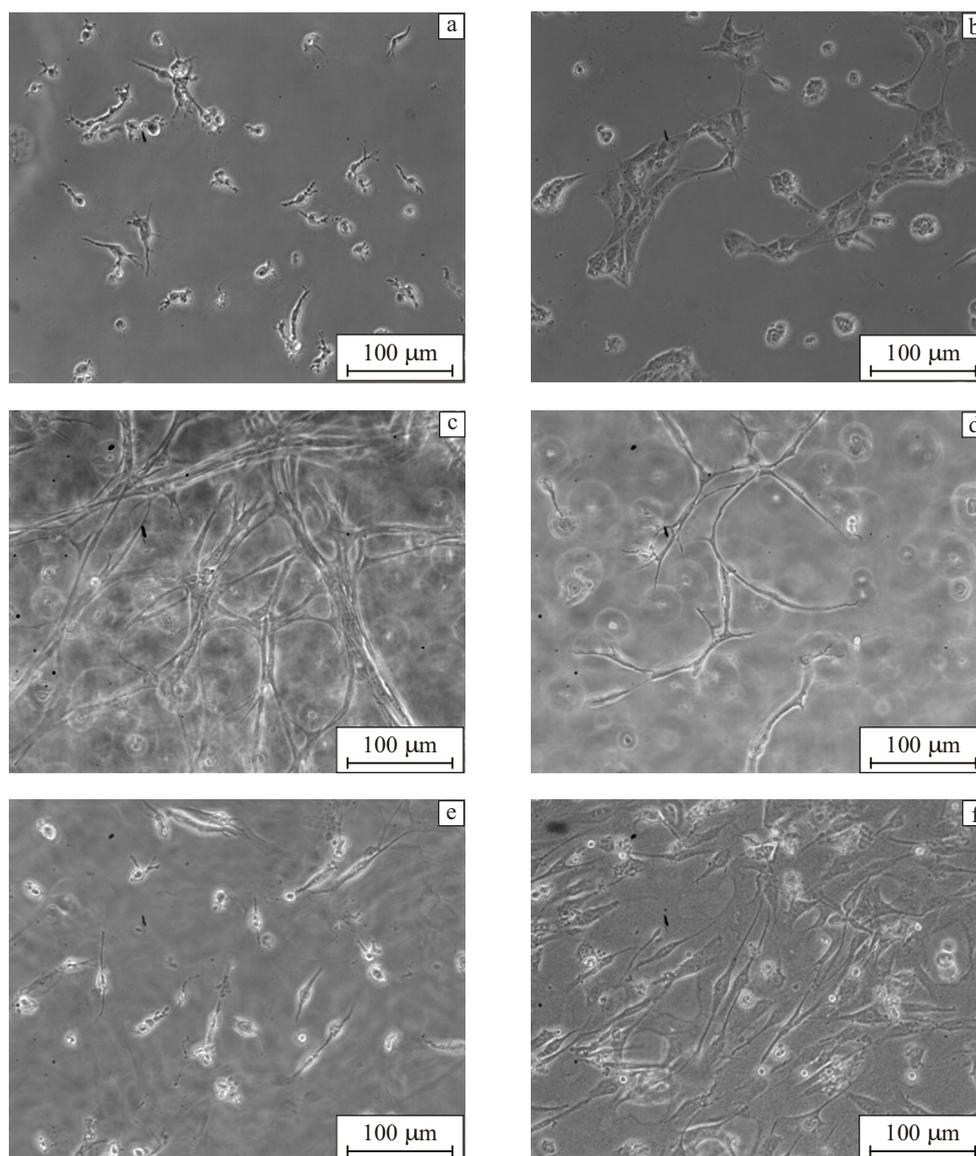


Fig. 1. Endothelial cells (BAECs and HUVECs) adhering to different hydrogel surfaces. Representative images show PEG-fibrinogen alone does not support cell adhesion of BAECs (a) or HUVECs (b) after 2 days in culture. BAECs are spread on the surface of PF hydrogels seeded with HFFs (c) or smooth muscle cells (d) after 6 days, but do not express the typical morphology of endothelial cell monolayers. BAECs spread on the surface of PF with collagen addition but many cells remained round after 2 days (e). BAECs completely spread after 6 days on the surface of a biocomposite made from PF and fibrin (f). All cell types are mentioned by their acronyms except for smooth muscle cells in (d).

2.8. Mechanical properties testing

The in-situ hydrogel formation, mechanical properties, and gelation kinetics were characterized using an AR-G2 shear rheometer (TA Instruments, New Castle, DE, USA) equipped with a UV cell and 20 mm parallel plate geometry. The biocomposites (200 μ l) were subjected to 30 min time-sweep oscillatory tests (2 % sinusoidal strain, 3 rad/s angular frequency) at 25 °C using the two crosslinking protocols, as before. The shear storage and loss modulus values (G' and G'' , respectively) were continuously recorded using Rheological Advantage Instrument Control software (TA Instruments).

2.9. Biodegradation assay

Biodegradation was characterized by colorimetrically labeling the different hydrogels with NHS-fluorescein (0.5 mg/ml). The release of the NHS-fluorescein resulting from enzymatic dissolution of the hydrogels was measured fluorometrically. Cylindrical plugs were cast in 5 mm diameter silicon tubes from 100 ml aliquots of the precursor solutions, and crosslinked according to the two previously mentioned protocols. The hydrogel plugs were stained in 0.5 mg/ml NHS-fluorescein solution in DMSO overnight, washed and transferred into 3 ml of 0.01 mg/ml Trypsin solution containing 50 mM PBS and 0.1 % sodium azide.

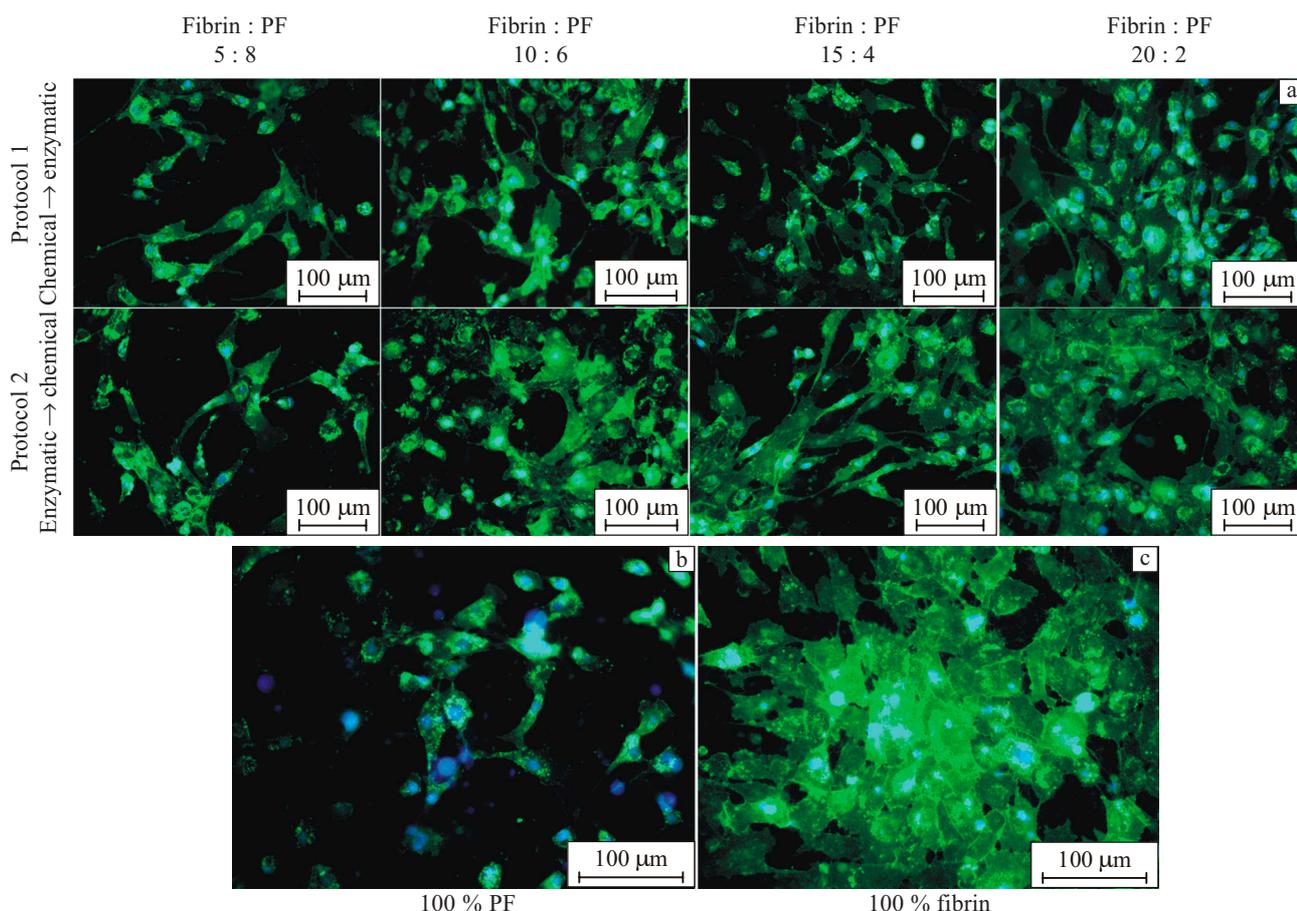


Fig. 2. BAECs grown on the surface of biocomposite hydrogels after 7 days in culture. The hydrogel composition was varied using different ratios of fibrin to PF, including 20 : 2, 15 : 4, 10 : 6, 5 : 8, and different crosslinking methods were used when preparing the biocomposites. The specimens are fluorescently labeled with Isolectin B4 (cell membrane marker, green), and DAPI (nuclei, blue), and imaged using fluorescence microscopy. The images show that BAECs spread on the surface of all composite hydrogels. Cell density was higher on hydrogels that contain more fibrin. There was no observable difference in endothelial cell survival on hydrogels crosslinked with different methods. Hydrogels crosslinked enzymatically and then chemically appear to have more lumen-shaped morphologies (a). Control images show that BAECs do not spread on PEG-fibrinogen hydrogels (10 mg/ml + 2 % (v/v) PEG-diacrylate) after 7 days (b), whereas a high density of BAECs can be seen on the control fibrin hydrogels (25 mg/ml) after 7 days (c).

Emmission values were measured at 494 nm every 5 min for 10 h. All data was normalized with the spectrophotometric measurements of completely degraded hydrogels.

3. Results and discussion

3.1. Endothelial cell adhesion and morphology

BAECs and HUVECs were cultured on the top surface of the PF hydrogels to assess the ability of the hydrogel material to support endothelial cell adhesion. In the absence of fibroblasts or smooth muscle cells in the constructs, the endothelial cells did not exhibit good adhesion or survival, and hardly formed the typical structures associated with endothelial cell morphogenesis [37]. Because endothelial cells did not attach properly to the PF matrix, progressive cell death was observed after 24 h (Figs. 1a and 1b, respec-

tively, for BAECs and HUVECs). The presence of human foreskin fibroblasts or smooth muscle cells in the PF coculture constructs, however, greatly increased the adhesion of the BAECs to the surface of the 3D constructs, and thereby improved endothelial cell survival (Figs. 1c and 1d, respectively, for human foreskin fibroblasts or smooth muscle cells). BAECs were stained with a specific fluorescent marker for von Willebrand factor in order to assure that the cells observed on the surface of the hydrogel were specific endothelial cell (data not shown).

Because vascular grafts require a uniform endothelial cell monolayer, there was a need to find the appropriate PF surface characteristics onto which endothelial cells can adhere and grow into a uniform monolayer. Pompe et al. showed that fibrillar patterns made with fibronectin could substantially improve endothelial cell adhesion onto syn-

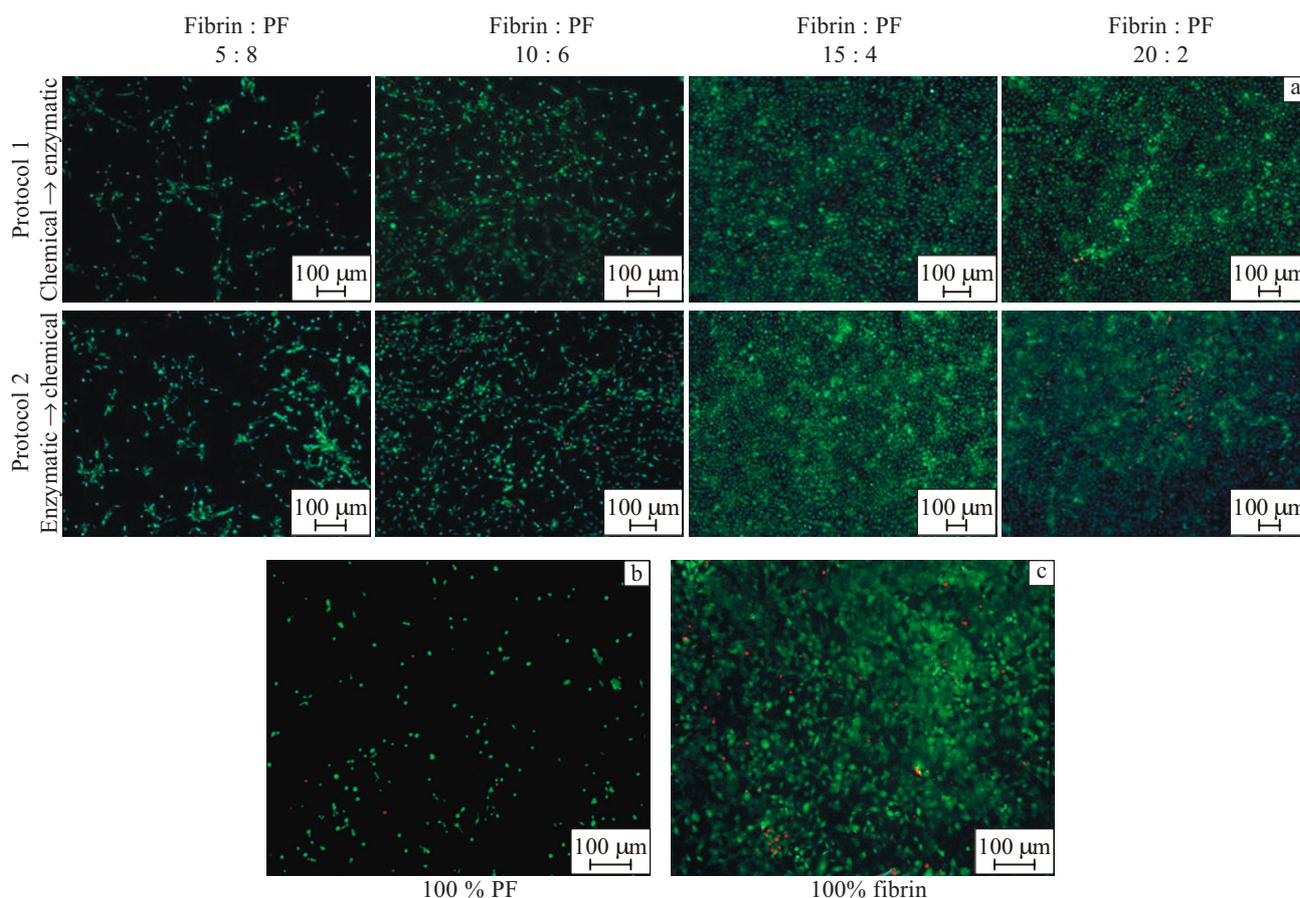


Fig. 3. BAEC viability on the top of biocomposite hydrogels after 7 days. The hydrogel composition was varied using different ratios of fibrin to PF, including 20 : 2, 15 : 4, 10 : 6, 5 : 8, and different crosslinking methods were used when preparing the biocomposites (a). The cell viability, as measured by live/dead staining, was high in all cases and was not affected by the hydrogels crosslinking method. The cell density was higher on hydrogels that contained more fibrin. Control samples are shown of BAECs on the top of PF (10 mg/ml + 2 % (v/v) of PEG-DA (b), and fibrin (25 mg/ml) (c) after 7 days. A significant difference in cell density between these hydrogels was observed. Fluorescence micrographs are shown with live cells in green and dead cell nuclei in red.

thetic polymer substrates [38]. In order to improve the interaction between the endothelial cells and the amorphous PF hydrogels, we added the fibrillar biological constituents collagen and fibrin to the PF precursor solution. The addition of fibrillar collagen to PF showed a moderate increase in BAEC adhesion and lamellipodia formation (Fig. 1e). A much more substantial improvement in BAEC adhesion, spreading and survival was observed on biocomposite hydrogels made with PF and fibrin (Fig. 1f). We speculate that the fibrillar architecture of the fibrin enhanced endothelial cell adhesion and stabilized the monolayer on the amorphous PF material [38]. However, because the fibrillar collagen biocomposite substrates did not provide evidence of a similar role for the collagen fibers, we set out to better understand how fibrin was achieving the endothelial cell stabilization in the biocomposites.

Four types of biocomposite hydrogels were tested, each consisting of a different fibrin and PF ratio (Fibrin : PF = 20 : 2, 15 : 4, 10 : 6, 5 : 8 w/v protein ratios). The bio-

composites were crosslinked by two different protocols and seeded with BAECs on top of the hydrogels. The first protocol enabled simultaneous chemical and enzymatic crosslinking, whereas the second protocol enabled the enzymatic crosslinking to take place without the simultaneous chemical crosslinking for the initial 10 min of the construct formation. All four biocomposites were employed in single-culture endothelial cell experiments in order to study the effect of material composition and mechanical properties on endothelial cell adhesion, viability and morphogenesis after 7 days in culture. Higher concentrations of fibrin increase the protein concentration of the composite, resulting in more cellular adhesion sites. Therefore, BAECs were able to spread on the surface of all hydrogel biocomposites tested, as can be seen in Fig. 2a.

The different crosslinking methods did not appear to affect the endothelial cell survival on the biocomposite substrates and cell viability was high in all biocomposites tested. Nevertheless, on the surface of hydrogels that were first

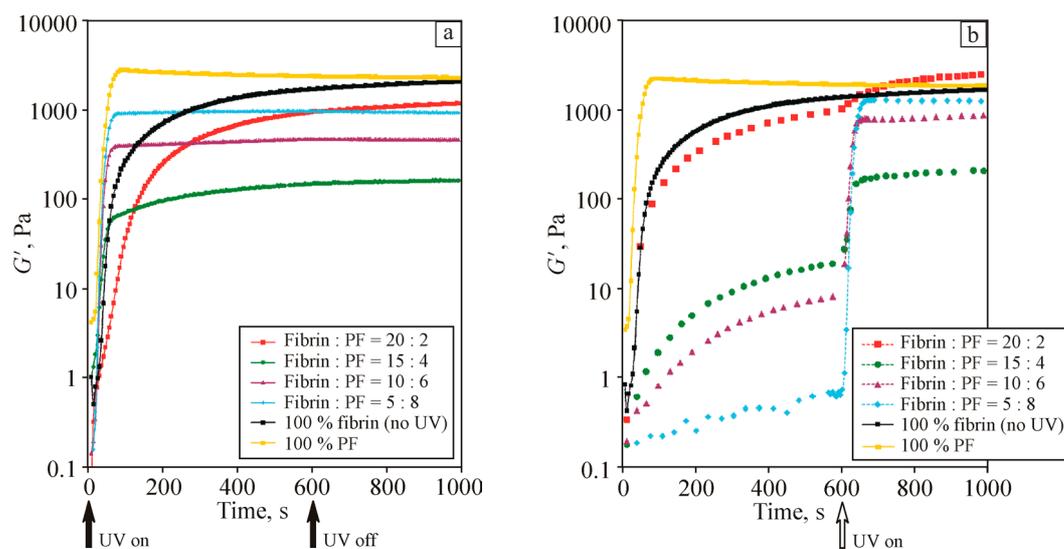


Fig. 4. Shear rheology data from dynamic time-sweep tests were collected during enzymatic and chemical crosslinking of the biocomposite hydrogels. The hydrogel composition was varied using different ratios of fibrin to PF, including 20 : 2, 15 : 4, 10 : 6, 5 : 8, and different crosslinking methods were used when preparing the biocomposites. The shear storage modulus G' of biocomposites made from fibrin : PF of 20 : 2 (red), 15 : 4 (green), 10 : 6 (purple), 5 : 8 (blue), 100 % PF (yellow) and 100 % fibrin (black) are shown. Two different crosslinking methods show differences in the crosslinking kinetics. When chemical crosslinking takes place before enzymatic crosslinking (a), a sharp increase in G' can be seen immediately followed by a plateau. When enzymatic crosslinking occurs first (b), a two-step increase in G' can be observed, leading to higher G'_{\max} values. Control PEG-fibrinogen only (crosslinked using the first protocol whereby UV light was turned on at the beginning of the experiment) and fibrin only hydrogels show characteristic G' crosslinking kinetics of chemical and enzymatic reactions, respectively.

crosslinked biologically, tubular structures formed by the endothelial cells were more apparent, suggesting that cellular morphogenesis may depend on the inner arrangement

of the components that assemble the hydrogel network. Representative images of cell viability results in Fig. 3 show that although viability did not appear to be affected by the

Table 1. Shear storage modulus G' and its maximum value G'_{\max} measured at different time points.

Biocomposition	UV setting	Crosslinking protocol	G' , Pa after 10 min	G'_{\max} , Pa
100 % PF (10 mg/ml + 2 % PEG-DA)	First 10 min	Chemical	2300	2400
100 % fibrin (25 mg/ml)	No UV	Enzymatic	1640	4500
Fibrin : PF 20 : 2	First 10 min	Chemical → Enzymatic	900	1300
Fibrin : PF 15 : 4	First 10 min	Chemical → Enzymatic	150	180
Fibrin : PF 10 : 6	First 10 min	Chemical → Enzymatic	430	460
Fibrin : PF 5 : 8	First 10 min	Chemical → Enzymatic	930	940
Fibrin : PF 20 : 2	After 10 min	Enzymatic → Chemical	1180	3600
Fibrin : PF 15 : 4	After 10 min	Enzymatic → Chemical	25	450
Fibrin : PF 10 : 6	After 10 min	Enzymatic → Chemical	10	1100
Fibrin : PF 5 : 8	After 10 min	Enzymatic → Chemical	0	1550

hydrogel composition, a higher cell density was observed on hydrogels containing larger concentration of fibrin. In fact, as the fibrin concentration in the biocomposite increased, the cellular density was noticeably higher after 7 days (Figs. 2c and 3c). These results support our hypothesis that fibrin addition to PEG-fibrinogen significantly improves endothelial cell adhesion by increasing the total protein concentration and thus the concentration of adhesion sites on the hydrogel surface.

3.2. Mechanical properties

A vascular graft must exhibit enough mechanical strength to withstand the physiological dynamic environments *in vivo*. In order to better understand the influence of each of the components on the material mechanical properties, the shear storage modulus of the biocomposites was analyzed by strain-rate controlled rheometry. In all four treatments, the crosslinking kinetics and the value of the maximum shear storage modulus G'_{\max} , differed according to the crosslinking protocols (Fig. 4a). As can be seen in Fig. 4a, when the first protocol was used (the UV lamp was turned on at the beginning of the experiment), all biocomposites showed a sharp increase in G' , followed by a plateau. Once the UV lamp was turned off, only slight changes in G' were observed. However, the increase in G' over the first 10 min of experiment observed for the 20 : 2 (high fibrin concentration) biocomposite was not as sharp as the increase observed for the other biocomposites, suggesting that this sharp increase is related to the chemical crosslinking of the PF. The crosslinking kinetics for the 100 % PF control material (crosslinked using the first protocol) was consistent with these findings. G'_{\max} and G' values after 10 min of analysis can be seen in Table 1. The crosslinking kinetics from the second protocol, which enabled 10 min of enzymatic crosslinking at the beginning of the experiment, showed a two-step increase in G' across all biocomposites except for the one that contained low fibrin concentration (fibrin : PF = 5 : 8) (Fig. 4b). In general, a moderate increase of G' can be seen for the first time interval followed by a sharp increase once the UV lamp is turned on. The biocomposite that consisted of high PEG-fibrinogen concentration (>80 %) showed a single sharp increase after 10 min, once the UV lamp was turned on.

In contrast to when the first protocol was used, when the second protocol was applied, G' increased steadily during the second 10 min time interval and continued to increase even after the UV lamp was turned off, without reaching a complete plateau. Moreover, higher values of G'_{\max} were obtained for all four biocomposites, suggesting that when the enzymatic crosslinking occurred before the chemical crosslinking, the network mechanical properties of the fibrillar phase was more dominant. The change between the G' value after 10 min and G'_{\max} becomes more significant as the PF concentration is increased (Table 1),

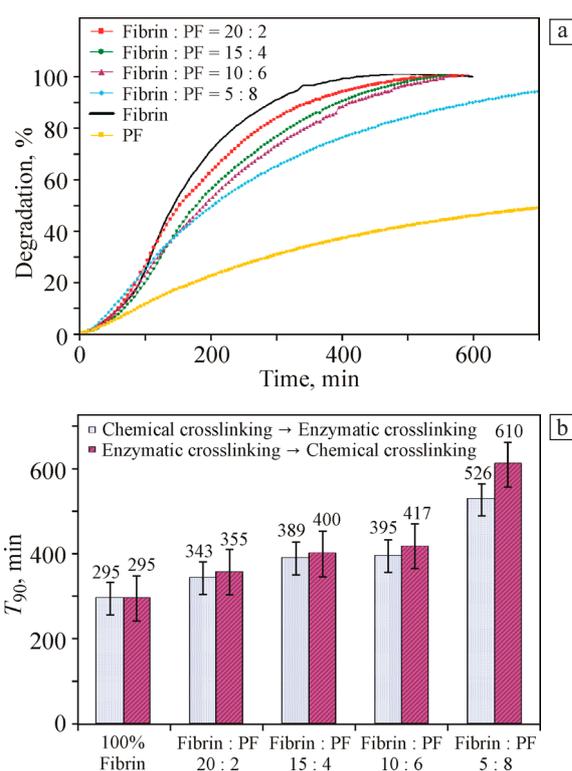


Fig. 5. Biodegradation kinetics of biocomposite hydrogels in trypsin solution (0.01 mg/ml). The average biodegradation rates of eight hydrogel samples (crosslinked enzymatically before chemically) made from fibrin : PF ratios of 20 : 2 (red), 15 : 4 (green), 10 : 6 (purple), 5 : 8 (blue), 100 % PF (yellow) and 100 % fibrin (black) are shown. The rate of enzymatic biodegradation is higher for fibrin hydrogels as compared to PF. As the PF content in the biocomposite hydrogel increases, the enzymatic degradation rate decreases. The characteristic biodegradation time t_{90} for the two different crosslinking methods is calculated for all hydrogels (b) and increases when the PF content in the hydrogel is higher. PF hydrogels (which consist of 10 mg/ml protein and 2 % (v/v) PEG-DA) do not fully degrade by trypsin treatment. There were no significant differences in degradation kinetics between hydrogels that were crosslinked in the different methods.

implying that the G' value obtained after 10 min in this crosslinking method is derived only from the presence of fibrin. Consequently, the fibrin : PF = 15 : 4 biocomposite showed the lowest G'_{\max} using both crosslinking methods. We suspect that none of the components assembling this composite are dominant enough in order to create a highly crosslinked network which causes the high G'_{\max} value that is observed in the other compositions.

When comparing the G' values after 10 min (using both protocols), generally the first protocol (UV is on for the first 10 min) yields significantly higher values than the second protocol due to a faster chemical crosslinking kinetics (except for the biocomposite with the highest fibrin concentration). However, the G'_{\max} values are lower for these samples, thus suggesting that the chemical crosslinking in-

Table 2. Biodegradation analysis of biocomposite hydrogels in trypsin (0.01 mg/ml): half-life t_{50} and t_{90} .

Biocomposite formulation	t_{50} , min		t_{90} , min	
	Protocol 1 Chemical → Enzymatic	Protocol 2 Enzymatic → Chemical	Protocol 1 Chemical → Enzymatic	Protocol 2 Enzymatic → Chemical
100 % fibrin (25 mg/ml)	140		295	
Fibrin : PF = 20 : 2	144	151	343	355
Fibrin : PF = 15 : 4	175	177	389	400
Fibrin : PF = 10 : 6	176	190	395	417
Fibrin : PF = 5 : 8	180	205	526	610
100 % PF (10 mg/ml + 2 % PEG-DA)	Not fully degraded		Not fully degraded	

terrupts the biological crosslinking process, when occurring simultaneously. This phenomenon is more manifested as the relative fibrin composition increases. Hence, it is possible to control the mechanical properties of the scaffold by the interplay between PF and fibrin ratios in the biocomposite materials and by using different crosslinking methods.

3.3. Biodegradation

The degradation analysis of the biocomposite hydrogels in trypsin (0.01 mg/ml) is summarized in Fig. 5. Calculations of half-life (t_{50}) and t_{90} are summarized in Table 2. The kinetics data revealed that hydrogels made from 100 % fibrin degrade faster than fibrin/PF biocomposite hydrogels. As the PF concentration in the hydrogel increases (i.e., PF solution consisting of 10 mg/ml PF + 2 % (v/v) PEG-DA), the degradation rate becomes slower. Hydrogels made from 100 % PF solution (contained 2 % (v/v) additional PEG-DA) remained partially intact in the enzyme solution after the dissolution experiment. There were slight differences in degradation kinetics between the biocomposites that were crosslinked with the different protocols. Materials that were crosslinked enzymatically before chemically showed slower degradation kinetics, as can be seen from Table 2 and Fig. 5b, although these differences were not statistically significant. Consequently, these materials also exhibited higher G'_{max} values. This evidence is consistent with the mechanical properties results, showing that enzymatic crosslinking, which occurs first when utilizing the second crosslinking method, does not prevent the chemical crosslinking from occurring, and may also result in a more efficiently crosslinked interpenetrating network. The differences of degradation kinetics between the biocomposites are evident based on the t_{90} data (Fig. 5b). A strong correlation between the degradation kinetics and the PF content of the material may be observed.

4. Conclusions

Protein-based biomaterials were developed for the purpose of creating vascular scaffolds that mimic the biological

extracellular matrix with added control over structural properties and biodegradation. Using a biocomposite matrix made by combining amorphous PF and fibrillar fibrin, we were able to improve endothelialization of the vascular graft by virtue of the fibrillar fibrin constituent. Specifically, better endothelial cell adhesion was attributed to the addition of fibrin to the PF matrix, which increases the total protein concentration in the hydrogel. It was also possible to control the biodegradation and mechanical properties of the scaffold by changing its biocomposition and crosslinking method. Slower degradation of the biocomposite hydrogel was attributed to the reinforcement effect of the chemically crosslinked PF component, which includes additional synthetic PEG-DA. This approach of combining semi-synthetic PF materials with fibrin is advantageous for the field of tissue-engineered vascular grafts, both in terms of mechanical performance and cell compatibility.

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