Nanostructuring of PEG–fibrinogen polymeric scaffolds

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1. Introduction

One of the common approaches in tissue engineering is based on growing cells in three-dimensional (3-D) porous extracellular matrix (ECM) analogs, known as scaffolds [1,2]. The ECM replacement should mimic the natural organization of the tissue and provide temporary yet necessary structural support for the initial stages of the tissue regeneration process [3]. Many materials used for preparing scaffolds are polymeric, made from either synthetic or natural building blocks. Typically, scaffolds made from synthetic polymers possess good mechanical properties, but lack cellular interactions due to the absence of natural ligands that enable specific communication with cell surface receptors. On the other hand, natural materials, such as hyaluronate, collagen or fibrin, have good interactions with cells but are unable to form mechanically stable constructs. The preparation of a hybrid biomaterial, composed of a combination of natural and synthetic constituents, is one way to overcome the inherent limitations of scaffolds made from either natural or synthetic polymers alone. As an example, polyethylene glycol (PEG), which generally resists protein and cell adhesion [4], can be chemically conjugated to oligo( peptides) derived from cell-adhesion promoting proteins such as fibronectin, laminin and fibrinogen [5–7] in a reaction termed “PEGylation”. Zhang et al. [8] utilized PEGylated fibrinogen as a carrier for isolated mesenchymal stem cells (MSCs) in order to provide a solution in myocardial cell-therapy. Their results suggest that PEGylated fibrin patches increased MSC viability in a rat infarct model and, furthermore, caused phenotypic changes in the MSCs consistent with endothelial cells. Zisch and co-workers [9] developed several synthesis schemes for preparing proteolytically sensitive PEG/peptide hydrogel biomaterials. These include block copolymers of acrylated PEG and oligopeptide domains polymerized by light-induced crosslinking, or end-functionalyzed branched PEG vinylsulfone chains reacted with bioactive thiol-bearing peptides as structural building blocks. Seliktar and co-workers [3,6,10–14] used PEGylated fibrinogen crosslinked in the presence of cells to form a dense cellularized hydrogel network. The fibrin-like scaffold material maintained its biofunctionality through the fibrinogen component, whereas the PEG component provided malleability over the mechanical properties of the material.

Nanostructuring of scaffolds for tissue engineering has recently been suggested as a way of imparting important structural cues...
into non-native scaffold materials in order to make them more like the natural ECM [15]. The nanostructuring of scaffold materials has been shown to induce major alterations in their interactions with cells. For example, nanostructuring by electrospinning, a technique which provides fibers in a broad size range of 50 nm up to 30 μm, provides a large surface area for improved cell attachment. Cells placed on nanofibers tend to preserve their phenotype and direct their growth along the nanofiber orientation [16]. Nanostructuring can also be achieved using the “phase separation method”, which has been traditionally used to create porous membranes and recently adopted for the preparation of 3-D tissue engineering scaffolds [15]. Polymer scaffolds obtained by the phase separation method usually have a porous sponge-like microscale morphology. Another approach for nanoscale design of supported cell matrix is self-assembly, a reversible and cooperative assembly of pre-defined components into an ordered superstructure [15]. Self-assembly can be used to create a variety of structures, such as films, bilayers, membranes, fibers, micelles, multilamellar vesicles and many other structures [17].

The underlying hypothesis of the current investigation is that nanostructuring of a poly(ethylene glycol)–fibrinogen (PF) scaffold could potentially provide an additional means to control both the physical properties of the material and the subsequent interaction between the material and the cells. Currently, the range of fabrication techniques by which nanostructured scaffolds can be prepared is very limited. Moreover, few of these methods allow cell entrapment within a hybrid hydrogel during the preparation step. Therefore, studying the properties and cellular compatibility of PF hydrogels with distinct nanostructures requires the development of an appropriate structuring methodology. In the work described herein, the ability to self-assemble biocompatible nanostructures from amphiphilic block copolymers of poly(ethylene oxide)/ poly(propylene oxide) (Pluronic®) was utilized for preparing PF hydrogels with distinct ultrastructural characteristics. The aggregation of Pluronic®, which is a function of both concentration and temperature [18,19], requires preparation protocols under controlled conditions. Thus, mixing Pluronic with cold PF hydrogel precursor solution and subsequent crosslinking should enable entrapment of nanometric micelles within PF hydrogels.

The overall objective of this study was to create nanostructures in PF hydrogels and understand the structure–property relationship associated with these nanostructures in cell culture applications. Specifically, we attempted to gain additional control over the material properties of the scaffold using nanostructuring without changing their compatibility for 3-D cell culturing. The initial efforts and preliminary results involved the use of a model system based on the block copolymer (EO)100–(PO)55–(EO)100, available under the registered trademark Pluronic® F127 [20]. The results from this research may have direct implications for any number of hydrogel cell scaffold materials that could benefit from self-assembled nanostructures made from co-polymeric components in their precursor solutions.

2. Materials and methods

2.1. PEGylation of fibrinogen

The PEGylated fibrinogen hydrogel precursor was made according to protocols described elsewhere [3,6]. Briefly, acrylation of linear PEG-OH, mol. wt. 10 kDa (Fluka, Buchs, Switzerland), was carried out under argon by reacting a dichloromethane (Aldrich, Sleeze, Germany) solution of PEG-OH with acryloyl chloride.

![Fig. 1. Cryo-TEM micrographs of (A) 10% w/v Pluronic® F127 solution, (B) PF solution with 10% w/v Pluronic® F127, (C) PF solution with 3% w/v Pluronic® F127 and (D) PF solution with 7% w/v Pluronic® F127.](image-url)
Merck, Darmstadt, Germany) and triethylamine (Fluka) at a molar ratio of 150% relative to the –OH groups. The resulting PEG-diacrylate (PEG-DA) was precipitated in ice-cold diethyl ether and dried under vacuum for 48 h. Proton nuclear magnetic resonance imaging (1H NMR) was used to validate the expected product formation. Bovine fibrinogen (Sigma) was dissolved in 150 mM phosphate-buffered saline (PBS) containing 8 M urea to a final concentration of 7 mg ml⁻¹. Tris (2-carboxyethyl) phosphine hydrochloride (TCEP; Sigma) was added to the fibrinogen solution at a molar ratio of 1.5:1 TCEP to fibrinogen. After dissolution, the pH of the solution was adjusted to 8 using 1 M NaOH solution and a solution of PEG-DA (280 mg ml⁻¹) in 150 mM PBS with 8 M urea was added and reacted for 3 h at room temperature in the dark. After the reaction was completed, the PEGylated protein was diluted 1:1 with 150 mM PBS containing 8 M urea and precipitated by adding four volumes of acetone at room temperature in a separation funnel. The precipitate was redissolved, homogenized and dialyzed against 150 mM PBS at 4°C for 24 h with two changes of PBS (Spectrum, 12–14 kDa mol. wt. cutoff) [6].

2.2. Nanostructured PF hydrogels

PEGylated fibrinogen hydrogels containing nanostructures were obtained by thoroughly mixing Pluronic® F127 (BASF) with PEGylated fibrinogen solution (22 mg ml⁻¹) at 4°C, i.e. under conditions in which no micelle formation was expected. The mixture was then mixed with 0.1 vol.% photoinitiator solution, made of 10% w/v Irgacure® 2959 (Ciba Specialty Chemicals, Tarrytown, New York) in 70% ethanol and MiliQ water, for 15 min in the dark. Next, the mixture was placed in a heated water bath at 37°C and equilibrated for an additional 10 min, in order to ensure micelle formation. If the experiments called for cell encapsulation, the cell suspension was added during this step. Finally, an ultraviolet (UV) light (365 nm, 4–5 W cm⁻²) was used to irradiate the solution for 5 min and induce crosslinking of the acrylate end groups.

2.3. Transmission electron microscopy

Transmission electron microscopy (TEM) micrographs were obtained from ultra-fast cooled vitrified cryo-TEM specimens prepared under controlled conditions of 37°C and 100% relative humidity as described elsewhere [21]. Specimens were examined in a Philips CM120 cryo-transmission electron microscope operating at 120 kV, using an Oxford CT3500 cooling-holder system that kept the specimens at about –180°C. Low electron-dose imaging was performed with a Gatan Multiscan 791 CCD camera, using the Gatan Digital Micrograph 3.1 software package.

2.4. Rheometry testing

Stress-sweep measurements were performed at 37°C, using an AR 1000-N TA Instruments parallel plate rheometer with a 20 mm
plate diameter and a 0.6 mm gap. PF – Pluronic® F127 pre-gel mixtures were placed for 5 min incubation at 37 °C, followed by irradiation for 5 min. A parallel plate geometry setup was used for the rheological testing.

2.5. Swelling properties

Disk-shaped gel samples (300 µl volume, 10 mm diameter) were allowed to swell in Petri dishes containing 50 ml of PBS at pH 7.4. Sodium azide (0.2% w/v) was added to prevent bacterial degradation of the hydrogel during the swelling experiment. The samples were kept at 37 °C and weighed periodically. The swelling percentage was calculated using following expression:

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\% \text{ Swelling} = \frac{(m_w - m_d)}{m_d} \times 100
\]

where \( m_w \) is the sample’s weight and \( m_d \) the dry weight, which was determined separately. Each experiment was performed in triplicate.

2.6. Cell-seeded constructs

Cell-seeded hydrogels were prepared by polymerizing PF solutions in the presence of Pluronic® F127 and sheep aortic smooth muscle cells (SMC) according to the published protocols [6]. Briefly, SMCs were cultured up to 4th passage in Dulbecco’s modified Eagle medium (DMEM; Gibco, UK) containing 10% fetal bovine serum (FBS), 1% penicillin–streptomycin and 1% L-glutamine. Cell-seeded constructs (1 × 10⁶ cells ml⁻¹) were made from 400 µl aliquots of the cells in a suspension of block copolymer and PF solution using a flat-bottomed 24-well plate as the molding template. After photopolymerization and consequent hydrogel crosslinking, the cell culture medium was added and changed every other day. The morphology of the seeded SMCs was monitored daily using a light microscope. The cell culture experiments involving the nanostructured PF material were always compared with cellular controls using PF hydrogels without Pluronic® F127.

Cell viability was assessed qualitatively using the calcein and ethidium homodimer live/dead viability assay (Sigma Aldrich, St. Louis, MO, USA) according to the manufacturer’s instructions. Stained cell-seeded hydrogels were visualized using Nikon TE2000 fluorescence microscope and imaged with a Progress C10 camera (Jenoptik, Jena, Germany) for further analysis. Live cells stained positive with green fluorescence, while dead cells were visualized by red-stained nuclei. The live/dead assay was performed on the SMCs after 4 days in culture and the viability of the SMCs was documented at the center of the tissue constructs.

2.7. Cellular outgrowth studies

Outgrowth experiments were performed using dense tissue constructs made from compacted SMC-seeded collagen gels [6]. Smooth muscle tissue constructs were prepared from a solution of 5× DMEM, 10% FBS, reconstituted type-I collagen solution in 0.02 N acetic acid (2 mg ml⁻¹) and 0.1 M NaOH with dispersed SMCs (3 × 10⁶ cells ml⁻¹) [6]. The collagen gels were allowed to contract for 2 days in 600 µl of culture medium in order to reach their final compacted geometry. The seeded collagen gels were then placed in a mixture of PF solution and Pluronic® F127 block copolymer in a multi-well plate and exposed to UV light for 5 min. The constructs were cultured inside the hydrogels in 400 µl of culture medium, which was changed every other day for the duration of the experiment. The cellular penetration into the hydrogel region was observed daily using a phase contrast-enhanced light microscope.

3. Results and discussion

The experiments to determine the effect of the nanostructures on the physical properties of the PF hydrogels included different concentrations of Pluronic® F127 added to the PF hydrogel precursor solution. The crosslinking of the PF hydrogel precursor could not be achieved at a Pluronic® F127 concentration higher than 13% w/v because of the excessive formation of nanostructures at these concentrations. Therefore, only lower concentrations of Pluronic® F127 were used for the characterization experiments. Furthermore, all experiments were performed at either 4 °C, wherein the PF solutions and materials were less affected by the nanostruc-
tures, or at 37 °C, wherein the nanostructures significantly contributed to the structural properties of the materials.

It is well known that, within a certain Pluronic® concentration range, the block copolymers form a micellar crystalline phase as the temperature increases [22]. Pluronic® F127 is unique in its ability to self-assemble into an ordered cubic phase at body temperature (i.e. 37 °C) and at relatively low concentrations (<10% w/v) [22]. Indeed, the cryo-TEM micrographs in Fig. 1A verify the presence of an ordered micellar phase in 10% w/v Pluronic® F127 solutions at 37 °C. The micelles are approximately 14 nm in diameter, in accordance with previously reported values for Pluronic® F127 at these concentrations [22]. Upon introducing the 10% w/v Pluronic® F127 to the PF solution, the cubic arrangement was lost (Fig. 1B); yet individual nanometric aggregates with a large size distribution were clearly visible in the TEM micrographs. Interestingly, the presence of the PF seems to decrease the critical micelle concentration (CMC), i.e. the minimum concentration required for self-assembly at a certain temperature. For example, micelles can be seen in PF solutions containing low concentrations of Pluronic® F127, including 3 and 7% w/v (Fig. 1C and D, respectively). The cryo-TEM micrographs of the pure Pluronic® F127 solution (data not shown) and also reports in the relevant literature indicate that these concentrations are lower than the CMC of Pluronic® F127 at 37 °C [22]. Fig. 1B–D further shows that the density of micelles in PF at 37 °C increases with increasing Pluronic® F127 concentration. A fairly homogeneous dispersion of micelles, with some degree of structural order, can be observed at the highest Pluronic® F127 concentration (Fig. 1B). One possible explanation for the adverse effect of the PF on the otherwise organized Pluronic® F127 phase behavior could be the hydrophobic interactions introduced by the protein precursor together with the poly(propylene oxide) block of the Pluronic® F127. Fibrinogen, like many other proteins, includes several hydrophobic moieties on its backbone [23], which are further exposed following the PEGylation reaction [6]. The hydrophobic interaction between the protein and Pluronic® may destroy the ordinary micellar structures (Fig. 1A) and develop randomly structured aggregates (Fig. 1B and D). The influence of proteins on Pluronic® phase transitions was also reported by Chung et al. [24].

In contrast to PF solutions containing Pluronic® F127, where uniform dispersions of nanometric aggregates can be seen at 37 °C, the crosslinking reaction that forms the PF hydrogel also appears to disturb the homogeneity of the nanostructures (Fig. 2). PF hydrogels containing Pluronic® F127 were characterized by a much larger distribution of aggregate dimensions (characteristic size range between 5 and 50 nm in diameter) compared to the typical aggregate size distribution in the hydrogel precursor solutions (Fig. 1B). It was evident from comparisons between Figs. 1 and 2 that after the crosslinking reaction some regions contained more aggregates than others regions. Nevertheless, the micelles continue to exist within the hydrogel network structure, even after extensive PF crosslinking by photopolymerization.

In earlier studies we demonstrated that the morphological behavior of SMCs in amorphous PF hydrogels was highly influenced by the material properties, including the modulus [6,25]. In particular, increasing the modulus of the PF hydrogels (e.g. by means of increasing PF crosslinking density) was found to be correlated with a decrease in the ability of SMCs to spread and develop a spindled morphology within the amorphous hydrogel network. The presence of nanostructures created by entrapped Pluronic® F127 micelles within the PF hydrogels would presumably increase the mechanical properties of the PF matrix, but would also

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**Fig. 6.** Phase contrast-enhanced light microscope images of cell seeding in PF hydrogels with different Pluronic® F127 concentrations: (A) 0% w/v, (B) 3% w/v, (C) 7% w/v and (D) 10% w/v after 3 days in culture (scale = 10 μm).
alleviate the material’s resistance to cell spreading. It is hypothesized that the nanostructures entrapped within the PF hydrogel could facilitate SMC spreading in the dense amorphous matrix, irrespective of the increase in modulus, by differential mechanical resistance and cell contact guidance. Rheometry measurements showed that the addition of Pluronic® F127 to PF hydrogels was associated with an increase in the storage modulus (Fig. 3). The extent to which the modulus increased compared to pure PF hydrogel controls depended on the Pluronic® F127 concentration. Whereas 3% w/v Pluronic® F127 had only a small influence on the storage modulus of the PF hydrogels, the modulus of the 10% w/v Pluronic® F127 PF hydrogels was four times higher than that of the PF-only controls (at low oscillation stress near 0.1 Pa) and 10 times higher than that of the PF-only controls at the higher stresses (near 1 Pa).

Further characterization of the hydrogels was achieved using the swelling experiment. PF hydrogel without the addition of Pluronic® F127 exhibited swelling of only a few percent (Fig. 4). Since the precursor solution was dialyzed against PBS to remove the urea prior to the crosslinking of the gel, it is probable that the water content was near to its equilibrium value, hence leading to the low degree of swelling. In contrast, hydrogels containing Pluronic® F127 exhibited a large degree of swelling of up to 60%. The swelling reached a maximum after approximately 6 h, followed by a decrease in the sample’s weight, most likely due to diffusion of Pluronic® F127 molecules from the hydrogel into the surrounding medium. The swelling percentage increased with an increase in the Pluronic® F127 content. It is suggested that the enhanced swelling reflects the larger porosity of the nanostructured hydrogels.

In order to ensure that addition of Pluronic® 127 has no adverse effect on the cells, cell viability assays were performed. As can be seen in Fig. 5, SMC seeded within the modified hydrogels were viable after 4 days of incubation at all Pluronic® concentrations tested.

The cell behavior in 3-D culture within PF hydrogels (with and without Pluronic® F127) was evaluated using both a cell seeding assay and a tissue invasion assay. The cell seeding assay detected the ability of individual encapsulated rounded SMCs to spread within all samples (Fig. 6). Although the results from the cell seeding assay were not quantitative, the images show that cells were able to become more spindled within the PF hydrogels containing the highest concentration of Pluronic® F127 (Fig. 6D) when compared to the other hydrogels. In addition, different cell morphologies were apparent within the different samples. Unlike the individual cell invasion with the cell seeding assay, the tissue invasion assay explores the ability of SMCs in a collagen gel construct to invade the hydrogel en masse. In this case, the hydrogel entraps the SMC collagen tissue and gives way to cellular invasion in the form of a moving cellular front originating at the tissue–hydrogel interface (Fig. 7). The results from the tissue invasion assay showed that the higher concentrations of Pluronic® F127 have an impact on the morphology of the invading SMCs. The results of the tissue invasion assay were quantified and are summarized in Fig. 8, which shows the invasion distance of the cells after 4.5 days of culture. The maximum invasion distance was measured in the PF hydrogel containing no Pluronic® F127, which indicates that these more compliant materials pose less resistance to en masse SMC invasion when compared to Pluronic® F127-containing PF hydrogels (Fig. 8). There was difference in the average cell invasion distance among the Pluronic® F127-containing PF hydrogel samples was not significant, despite their very significantly different material moduli (Fig. 3). Thus, modifying PF hydrogels with nanostructural features inherent to Pluronic® F127 helps to increase the modulus of the materials without necessarily limiting 3-D cellular invasion into them.

4. Conclusions

Modification of PF hydrogels by adding Pluronic® F127 at different concentrations enabled the formation of nanostructured materials for 3-D cell culture, characterized by different degrees of

![Fig. 7. Tissue invasion assay after 4.5 days in culture (scale = 50 μm). PF hydrogels with different Pluronic® F127 concentrations: (A) 0% w/v, (B) 3% w/v, (C) 7% w/v and (D) 10% w/v.](image)
order. Evidence from rheological measurements and cell assays have shown that increasing the Pluronic® F127 concentration from 3% to 10% w/v resulted in a 10-fold increase in the modulus. Despite the fact that Pluronic® F127-containing PF hydrogels are stiffer than the PF controls, the inherent ability of cells to spread within or invade the dense amorphous hydrogel milieu was not drastically diminished. Thus, alterations to structure caused by micelles may be used to manipulate the mechanical properties of PF scaffolds while still providing a non-confining environment for cell growth. In future work, this concept will be further explored using nanostructured PF hydrogels that would otherwise be too confining for cell spreading, including PF materials containing a very high PEG-DA crosslinking density. Ultimately, this research should validate the use of nanostructural features, introduced through Pluronic® F127 or other block copolymers, in order to manipulate both the mechanical properties and the cellular compatibility of a hydrogel scaffold material for tissue engineering.

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Appendix. Figures with essential colour discrimination.

Certain figure in this article, particularly Figure 5, is difficult to interpret in black and white. The full colour images can be found in the on-line version, at doi:10.1016/j.actbio.2009.07.015.

References