Two-tier hydrogel degradation to boost endothelial cell morphogenesis

Karolina Chwalek, Kandice R. Levental, Mikhail V. Tsurkan, Andrea Zieris, Uwe Freudenberg, Carsten Werner*

Leibniz Institute of Polymer Research Dresden (IPF), Max Bergmann Center of Biomaterials Dresden (MBC) & Technische Universität Dresden, Center for Regenerative Therapies Dresden (CRTD), Hohe Str. 6, 01069 Dresden, Germany

Article info
Article history:
Received 6 July 2011
Accepted 26 August 2011
Available online 19 September 2011

Keywords:
Angiogenesis
Hydrogel
Degradation
Endothelial cell
Matrix metalloproteinase

Abstract
Cell-responsive degradation of biofunctional scaffold materials is required in many tissue engineering strategies and commonly achieved by the incorporation of protease-sensitive oligopeptide units. In extension of this approach, we combined protease-sensitive and -insensitive cleavage sites for the far-reaching control over degradation rates of starPEG-heparin hydrogel networks with orthogonally modulated elasticity, RGD presentation and VEGF delivery. Enzymatic cleavage was massively accelerated when the accessibility of the gels for proteases was increased through non-enzymatic cleavage of ester bonds. The impact of gel susceptibility to degradation was explored for the 3-dimensional ingrowth of human endothelial cells. Gels with accelerated degradation and VEGF release resulted in strongly enhanced endothelial cell invasion in vitro as well as blood vessel density in the chicken chorioallantoic membrane assay in vivo. Thus, combination of protease-sensitive and -insensitive cleavage sites can amplify the degradation of bioresponsive gel materials in ways that boost endothelial cell morphogenesis.

© 2011 Elsevier Ltd. All rights reserved.

1. Introduction

Tissue regeneration is based on the ability of cells to expand, degrade, invade, and dynamically remodel the surrounding extra-cellular matrix (ECM) [1] using a sophisticated enzymatic machinery [2]. ECM rearrangement is especially important when cells become hypoxic and the development of new blood vessels is required. Efficient remodeling then becomes a critical prerequisite determining the maintenance of a tissue. Biomaterials promoting tissue healing and regeneration need to mimic and modulate key characteristics of naturally occurring ECM types [3] with respect to various different biochemical and biophysical signals [4]. Degradation rates adjusted to the desired migratory activity of particular cell types define a very important requirement for such materials. The degradation of polymer hydrogels can be engineered through linkages between the building blocks that undergo cleavage upon action of specific stimuli such as light, pH changes or enzymatic activity. Ester bonds are among the most common non-enzymatically cleavable bonds in materials. Being stable over the pH range of 3.0–7.0 many esters slowly become hydrolyzed at physiological conditions (pH 7.4, 37 °C) [5–7] at rates that can be tuned by varying of its molecular environment [8,9], thus allowing simple customization of the degradation properties. Enzymatic degradation is usually integrated into materials through the inclusion of peptide sequences cleaved by specific cell-released enzymes. Among those, matrix metalloproteinases (MMPs) are attracting special attention because of their role in the cell-mediated remodeling of ECM during wound healing and tissue regeneration [10], and MMP-susceptible peptides were successfully applied in the design of a number of bioresponsive materials [11–13].

Herein, we aimed at developing multibiofunctional hydrogels with far-going modulation of their degradation rates at otherwise invariant characteristics. For that purpose, MMP-cleavable peptides were combined with linkers of differing hydrolytic sensitivity (Fig. 1) in the formation of star-shaped poly(ethylene glycol) (starPEG) – heparin hydrogels that offer independently tunable physical and biomolecular properties [14]. The inclusion of a specific linear oligopeptide into the hydrogel network was previously shown to render the material MMP-degradable [15], allowing for the localized cellular invasion into the hydrogel [16]. To modulate the degradation of the peptide-containing biohybrid gel materials we now applied two different chemical linkages between the MMP-cleavable peptide and the starPEG units, i.e. an amide bond which is stable over the considered pH range and an ester bond which slowly degrades under physiological conditions. Using
these two types of gel materials with varied elasticity, RGD conjugation and VEGF delivery we were able to explore the impact of differences in the rate of gel degradation on the morphogenesis of endothelial cells (ECs) in vitro and on the angiogenesis in the chicken chorioallantoic membrane (CAM) assay in vivo.

2. Materials and methods

2.1. Preparation of acryloyl terminated 4-armed PEG

Synthesis of the acryloyl terminated 4-armed PEG was performed as described before [15]. In brief, 0.1 g (1 × 10⁻⁵ mol) of commercially available hydroxyl-terminated 4-armed PEG (PEG-(OH)₄ MW = 10 kDa) was dissolved in 0.5 ml of CH₂Cl₂, next 0.1 ml (6 × 10⁻⁵ mol) of acryloyl chloride was added following by slow addition of 115 ml of TEA. The reaction was stirred overnight under N₂. About 300 mg of dry Na₂CO₃ was added to the reaction mixture and stirred for 2 h. The reaction mixture was filtrated and precipitated from 150 ml of diethyl ether twice. The collected white precipitate was dried under vacuum overnight and kept at −20°C.

1H NMR (500 Mz CDCl₃): δ 6.42 (dd, J = 18.0, 1.4 Hz, 4H), 6.15 (dd, J = 18.0, 1.4 Hz, 4H), 5.85 (dd, J = 18.0, 1.4 Hz, 4H), 4.31 (t, J = 1 Hz, 8H), 3.79 – 3.49 (m, 120H), 3.41 (s, 8H). The conversion rate of the terminal hydroxyl groups on PEG was calculated from the ratio between the signals of the vinyl group residues at δ 5.8 – 6.5 and the PEG core at δ 3.41 (100% corresponds to a ratio of 2:1).

2.2. Preparation of maleimide terminated 4-armed PEG

0.1 g (1 × 10⁻⁵ mol) of amino-terminated 4-armed PEG (PEG-(NH₂)₄ MW = 10 kDa) was dissolved in 0.5 ml of CH₂Cl₂, next 112 mg of (4.2 × 10⁻⁵ mol) 3-Maleimidopropionic acid N-hydroxysuccinimide ester was added. The reaction was stirred overnight under N₂, followed by slow precipitation from 150 ml of diethyl ether. The collected white precipitate was dried under vacuum overnight and kept at −20°C. 1H NMR (500 Mz CDCl₃): δ 6.42 (s, 8H), 6.27 (s, 4H), 3.85 (t, J = 1 Hz, 8H), 3.79 – 3.49 (m, 120H), 3.41 (m, 16H), 2.52 (t, J = 7.5 Hz, 8H). The conversion rate of terminal amino groups of PEG was calculated from the ratio between the signals of the maleimide group residues at δ 6.42 and the PEG core at δ 3.41 (100% corresponds to a ratio of 1:2).

2.3. Preparation of MMP-cleavable peptide

The MMP-cleavable sequence GPQG [YWIGQ was included as a bioactive module into peptide NH₂-GGPQGIWGQGCG-NH₂ (IWGC) which was synthesized using solid-phase methods on an Activo P11 (Activotec, UK) peptide synthesizer by standard Fmoc-chemistry with a C-terminal capping protection strategy. Activation was achieved by O-(Benzotriazol-1-yl)-N,N,N,N-tetramethyluronium tetrafluoroborate (TBTU), and 1-hydroxybenzotriazole (HOBt) in DMF. Deprotection of the amino acid side chains and cleavage from the resin was performed by reaction with a mixture of trifluoroacetic acid (85% v/v), phenol (5% v/v), dithiotreitol (2.5% v/v), tri-isopropyl silane (2.5% v/v) and water (5% v/v) for 2.5 h at room temperature. The crude peptide was precipitated in cold anhydrous diethyl ether, collected by vacuum filtration, extensively washed with diethyl ether and dichloromethane, and dried under vacuum. Final purification was achieved by preparative reversed-phase HPLC (Agilent Technologies 1200 Series; linear gradient H₂O/AcN on XBridge BEH 300 C-18 column 10 µm particle size, 2.1 × 250 mm) equipped with a UV/Vis detector/spectrophotometer. Purity of the peptide and the accuracy of the synthesis were evaluated by a single peak in analytical reversed-phase HPLC (Agilent Technologies 1100 Series; on XBridge BEH 300 C-18 column 5 µm particle size, 2.1 × 250 mm, Waters, USA) and accurate molecular ion mass in ESI-MS (Mariner spectrometer, Applied Biosystems, Germany).

2.4. Preparation of starPEG-MMP conjugates

Both maleimide- or acryloyl-functionalized PEG were converted into their starPEG-MMP conjugates by simple mixing of stoichiometric amounts of the peptide and functionalized starPEG (10% weight/volume) in phosphate buffer pH = 7. A small amount of tris(2-carboxyethyl)phosphine chloride (TCEP) was added to the reaction mixture in order to prevent oxidation of the cysteine residue. The reaction mixture was stirred overnight and purified by dialysis (nitrocellulose membrane with
2.5. Preparation of starPEG-heparin gels and modification with RGD

Biodegradable starPEG-heparin gels were prepared as previously reported for non-enzymatic starPEG-heparin gels using the starPEG-MMP conjugates and EDC/s-NHS activated heparin [15]. Briefly, hydrogels were prepared as planar layers of approximately 200 μm thickness. The liquid gel mixture was placed between an aminofunctionalized and a hydrophobic glass cover slip and allowed to polymerize overnight. After removal of the hydrophobic cover slip, solid gels were washed and swollen in phosphate buffered saline (PBS) for 24 h. For the in vitro and in vivo studies, the hydrogels were functionalized with cyclicRGD (Peptides International, Louisville, KY, USA) using the reaction of EDC/s-NHS-activated carboxylic acids of heparin with 5 or 20 μg/ml cyclicRGD. All samples were extensively washed with PBS before use in cell culture experiments.

2.6. Rheological measurements

Storage moduli of the hydrogels were determined as previously described [14]. Briefly, oscillating measurements on swollen gel disks were carried out on a rotational rheometer (Ares LN2, TA Instruments, Eschborn, Germany) fitted with a 25 mm parallel plate geometry. Frequency sweeps were carried out at 25 °C in a shear frequency range of 10−1−10−1 rad s−1 with the strain amplitude of 2%. Both storage and loss moduli were measured as a function of the shear frequency. Mean values of the storage modulus were calculated. Experiments were performed in triplicate and repeated at least three times.

2.7. Enzymatic degradation studies

5 μl gel droplets were used for degradation experiments. After gelation overnight, the hydrogel drops were washed three times with PBS and swollen in PBS overnight. To determine the kinetics of degradation, gel drops were placed in plastic UV cuvettes (PlastiBrand, Germany) with either 2 ml of PBS or 1.0U/ml collagenase IV (Biochrom AG, Germany). The UV absorption was determined at 278 nm using a spectrophotometer (Beckman Coulter, DU800, USA) and recorded for all samples every 15 min for a time period of 2500 min in total. The samples during the measurements were thermostated at 37 °C.

2.8. Mass swelling degradation studies

In order to visualize the gel materials, Alexa-488 (Invitrogen, USA) labeled heparin was used for the gel formation. 50 μl gel samples were formed in 0.5 ml vials for the degradation experiments. After overnight polymerization the hydrogel samples were washed three times (by 15 min) with PBS and swollen in PBS overnight. The PBS solution was removed by pipetting and the vials with the gel samples were weighed, the measured masses were used as an initial mass by subtraction the mass of the empty vials. To determine the kinetics of degradation, the gel samples were incubated at 37 °C with 400 μl of 10.0 U/ml collagenase IV (Biochrom AG, Germany) solution for 3 days. Fresh solution of collagenase were prepared at least each 12 h. Mass loss of the samples was determined during degradation by precise weighing (Sartorius, Germany). In parallel to the mass determination all samples were imaged in the dark with front illumination by UV lamp (366 nm; Benda NU-8-KL, Germany).

2.9. VEGF uptake and release

Surface-bound gels (n = 3) were placed in custom-made incubation chambers with minimized contact of the solution to additional surfaces [17]. 200 μl of VEGF (1 μg/ml; Peprotech, Hamburg, Germany) solution were added per cm2. Immobilization of VEGF was performed overnight at 22 °C. The VEGF solution was subsequently removed, followed by washing with PBS twice. Each of the solutions was collected and assayed in duplicates using an ELISA Quantikine kit (R&D Systems, Minneapolis, USA). Immunolization under conditions described above resulted in ~ 198 ng VEGF per cm2 gel surface area. Following the loading procedure, VEGF was released from the gels at 22 °C into 250 μl/cm2 of serum-free endothelial cell growth medium (ECGM; Promocell GmbH, Heidelberg, Germany) supplemented with 0.02% sodium azide (Fluka) ± 0.5U collagenase IV (Biochrom AG, Berlin, Germany). Samples taken at intervals were stored at −80 °C until analyzed by ELISA. An equal volume of fresh medium was added at each time point.

2.10. Cell culture experiments

Before cell seeding, the hydrogels were equilibrated in cell culture medium for 1 h at 37 °C. Human umbilical vein endothelial cells (HUVECs), isolated according to the procedure proposed by Weis et al. [18] were used. The cells were cultured up to 80% confluence in ECGM containing 2% calf serum at 37 °C and 5% CO2. Cells from passage 2–6 were used in these studies. Cell morphology, attachment and spreading were monitored daily with light microscopy (Olympus IX50, 10× objective).

2.11. Cell adhesion

Cells were seeded at a density of 1 × 104/cm2 in serum-free medium. After 30 min samples were fixed with 2% paraformaldehyde (PFA) and actin was visualized with AlexaFluor 633-labeled Phalloidin (Invitrogen, Germany). Samples were investigated using a confocal laser scanning microscope (SP5, Leica Microsystems, Germany, 20×/0.70 objective). Projected cell area was measured with ImageJ (NIH) software.

2.12. Viability/proliferation WST-1 assay

Cells were seeded at a density of 1 × 104/cm2. After 7 days cell viability/proliferation was estimated using a colorimetric WST-1 assay (Boche, Germany) according to the manufacturer’s instruction. Briefly, cells were incubated for 30 min with WST-1 reagent diluted 1:10 (w/v) in serum-free medium, followed by a reading of absorbance at 450 nm.

2.13. 3-dimensional migration studies

Hydrogels were created with 0.5% addition of heparin-Alexa-488. Before the cell seeding gels were incubated overnight with 1 μg of recombinant human VEGF165 (Peprotech, Germany) in PBS at room temperature. Cells were seeded at a density of 4 × 104/cm2 and cultured for 24 h in serum-free medium, after which the samples were fixed with 2% PFA and actin was visualized with AlexaFluor 633-labeled Phalloidin (Invitrogen, Germany). Samples were investigated using confocal laser scanning microscopy (SP5, Leica Microsystems, Germany, 20×/0.70 objective).

2.14. Zymography

For the zymography 2 × 105 cells were seeded in 6-well tissue culture treated plates. Reaching 80% of confluence, cells were incubated with serum-free medium for 24 h and VEGF 1 ng/ml was added. After 6 h cell culture medium was collected and concentrated with Amicon Ultra Filters, 10 K (Millipore, Ireland). Protein content of the cell culture medium samples was determined with BCA kit (Pierce, Germany). Aliquots containing 28.5 μg of protein were loaded per each lane. Zymography was performed under non-reducing conditions on 10% polyacrylamide gels, copolymerized with porcine skin gelatin 1 mg/ml (Sigma–Aldrich, Germany). After 2 h of electrophoresis at 120 V, the gel was incubated at 37 °C overnight in freshly prepared development buffer (0.05 M Tris–HCl pH 8.8, 5 mM CaCl2, 0.02% NaN3). Following morning the gel was stained according to the standard procedure with Coomassie Brilliant Blue (Sigma–Aldrich, Germany). To assay gelatin lysis, images of the gel were taken using transmitted light with the Leica Imager (Roche, Germany) and densitometric analysis was performed with ImageJ (NIH) software. The semi-quantitative results were expressed as a ratio of untreated sample.

2.15. CAM assay

Chicken eggs (Erzeugergemeinschaft Pharma-Ei GmbH, Germany) were obtained at embryonic development day (EED) 0. After wiping with 70% ethanol they were incubated at 37 °C, high humidity for 3 days with automatic turning. On EDD3 eggs were opened to sterile weighting boats. The ex-ovo cultures were maintained in a humidified incubator at 37 °C. On EDD8 samples were applied on the outer regions of the choroidalallantoic membrane and cultures were brought back to the incubator. Matrigel (BD, Germany) was used as a positive control. On EDD 12 100 μl Indian Ink was injected into the vasculature of the embryos using a syringe with 30G needle following with immediate imaging with stereomicroscope (Leica Microsystems, SBA0, 25×). The proangiogenic response was evaluated by analyzing the convergence of blood vessels toward the graft as depicted below (see Fig. 2). Blood vessels growing within a distance of 1 mm from the samples were counted, normalized to the number of vessels in the untreated CAM, and expressed as a relative vascularization index. The results were expressed as a ratio of the untreated sample. Pictures with a lower magnification as used for quantification are shown within the paper to point out qualitative differences in vascularization.

2.16. Image analysis and statistics

All images were analyzed using the ImageJ software (NIH). Experiments were performed two times or more with at least triplicate samples in each of the experiments. Statistical analysis was performed with GraphPad Instat software (GraphPad, CA, USA). Analysis of variance (ANOVA) was the primary analysis method using the Turkey post-hoc test or Kruskal–Wallis ANOVA on ranks with Dunn’s post-hoc modification depending on the results of the normality test. In addition, two-tailed Students’ t-test was employed, when appropriate. Levels of significance were determined when *p < 0.05, **p < 0.01, ***p < 0.001.
3. Results and discussion

3.1. Design of degradable starPEG-heparin hydrogels

In this work we sought to investigate the impact of the cleavage rate of MMP-cleavable materials on cell-mediated remodeling. MMP-susceptible peptides were incorporated into starPEG-heparin hydrogels through hydrolytically labile ester bonds or more stable amide bonds, respectively (Fig. 1). The selected MMP-responsive peptide (GPQG \( \text{IWGQ} \)) is recognized and cleaved by several proteases, including MMP1, MMP2, MMP3, MMP7, MMP8 and MMP9 [19]. The ester-linked PEG-peptide conjugate (PE-IWGC) was formed by substitution of all four arms of a hydroxyl-terminated starPEG (Mw = 10000) with acrylate groups, followed by coupling with the thiol group of the cysteine in the peptide-containing the MMP-cleavable sequence (IWGC) [15]. In order to form the amide-linked PEG-peptide conjugate (PA-IWGC), a similar amino-terminated starPEG (Mw = 10000) was functionalized with maleimide groups. The PEG-maleimide conjugate was then coupled to the thiol group of the IWGC peptide as the PEG-acrylate conjugate.

3.2. Hydrogel degradation and growth factor release

Gel materials (AG stands for amide-linked, and EG stands for ester-linked gels) were prepared by converting the N-terminal amino groups of the starPEG-peptide conjugates with carboxylic groups of heparin by carbodiimide chemistry. Utilizing different crosslinking degrees permitted the design of a set of hydrogels with a range of storage moduli (2, 3.5, and 7.5 kPa). As shown in Fig. 3A, this allows to recapitulate similar physical properties in both EG and AG materials. The principal design concept of our hydrogel system permits the independent modulation of physical and biomolecular characteristics [14] which offers most valuable options through the application of secondary biofunctionalization schemes of heparin, including the covalent binding of adhesive peptides and the non-covalent binding of a variety of growth factors.

EG and AG type materials with a similar storage modulus of 2 kPa were evaluated for biodegradation and release of VEGF (Fig. 3B and C). To examine biodegradation the gels were incubated in a solution of bacterial collagenase IV (CLS) which has been shown to be a reliable model of MMP activity [20]. Both materials degraded almost completely within 15 h, however the EG material degraded more rapidly than the AG material. In the absence of enzyme, only minor degradation of both materials occurred, although the initial degradation of EG material was higher than for AG materials. However, EG materials were found to persist for at least several weeks in absence of enzyme, which is in agreement with results from others who had successfully applied ester-linked PEG–PEG [21,22] or PEG-heparin [6,23,24] gels in long term experiments.

The release of VEGF from the materials, a decisive parameter for the functionality of the gels to stimulate endothelial cells, was found to correlate well with the erosion of the compared gel types (Fig. 3C): In PBS the release of VEGF was slow (1.6–4.5 ng/cm\(^2\)) and comparable with previous data of non-cleavable starPEG-heparin gels [14,25] whereas collagenase-induced degradation of the gels significantly increased the release of hydrogel-associated VEGF (to 56–68 ng/cm\(^2\)). After 180 min 54 ng VEGF/cm\(^2\) and 24 ng VEGF/cm\(^2\) were released for EG and AG materials, respectively. This ratio of ~2:3 corresponds to the twofold higher degradation rate of the EG materials.

To analyze the influence of the degree of crosslinking on the degradation rate, EG and AG materials of similar storage moduli 2, 3.5, and 7.5 kPa were degraded in the presence of CLS. The initial degradation rate was linear, as shown before in similar systems [11], and evidently modulated by the crosslinking degree for both EG and AG materials (Fig. 3D).

While degradation of ester-linked, MMP-cleavable PEG–PEG gels was reported to occur across the whole volume of the material (obvious from the initially increased swelling of the degrading sample) [11,12,26] both EG and AG materials do not exhibit this behavior but a primarily surface erosion driven degradation process. Considering the mesh size of the hydrogels (estimated from rubber elasticity theory, see [14] for details, with average mesh sizes of 13 ± 2.5 nm for EG or AG type A gels, 11 ± 1.5 nm for EG and AG type B, and 8 ± 1 nm for EG and AG type C gels) MMPs with molecular weights of 67–92 kDa [19], which were shown to be secreted by endothelial cells in culture (see Suppl. Fig. 1), can be assumed to be restricted from entering the EG and AG materials. (The impact of the mesh size on the uptake of BSA (Mw 68 kDa) into comparable starPEG-heparin hydrogels was previously investigated [14].) However, the decomposition rate of the EG materials exceeds the superposition of the rates of the separated enzymatic (= AG) and non-enzymatic (= EG-scr) degradation processes. We therefore conclude that the non-enzymatic degradation of ester bonds in the EG materials increases the accessibility for MMPs and consequently accelerates MMP-degradation.

In consequence, starPEG-heparin hydrogels can be customized to adjust the degradation rates, including systems that exclusively undergo surface erosion (AG) and others for which this process is
amplified by the non-enzymatic cleavage of ester bonds (EG). The variability of the degradation rate at otherwise constant properties (elasticity, adhesion ligand conjugation and growth factor delivery) defines a valuable advantage when choosing materials to support particular tissue regeneration schemes. With this extension, our platform of cell-instructive biohybrid matrices provides a unique base to investigate the impact of the matrix susceptibility on the cellular response in vitro and in vivo. This was exemplarily studied herein for the morphogenesis of human endothelial cells (ECs).

3.3. Spreading and long term viability of primary endothelial cells

EC morphogenesis is directed by both the physical and biochemical properties of the cell substratum, in particular the material’s viscoelasticity and the availability of adhesive sites and relevant growth factors [27,28]. First, we evaluated the initial cell spreading on different gels of different stiffness at invariant RGD functionalization [14]. The cells efficiently adhered and rapidly spread on the gels and the projected area of the ECs was similar on EG and AG materials, independent of their stiffness (Fig. 3A). Proliferation assays after a prolonged culture time of 7 days showed that the cells responded similarly to both gel types independently of the material stiffness in a range 2–7.5 kPa. However, for the 2 kPa EG material only 50% of cells could be detected after this time period due to the relatively fast degradation of the gel (Figs. 4B and 3D). Thus, the gel properties induced a largely similar initial cell response proving the feasibility of the above-mentioned idea to create materials differing in the degradation characteristics only. ECs are in general thought to be influenced by the susceptibility of the underlying matrix since this process is known to play an important role in vessel formation [31]. Therefore, EC morphology was monitored over a time period of 24 h to explore the proangiogenic potential of our cell-responsive hydrogels with varied degradation dynamics.
3.4. Endothelial cell morphogenesis and hydrogel invasion

Primary ECs are able to keep their phenotype during the in vitro culture and have the ability to form tube-like structures within 24 h after seeding on appropriate substrates [32]. Moreover, tube formation is promoted when EC adhesiveness is reduced by blocking integrins with antibodies or by reducing adhesive ligand concentration [33]. Also, the anchorage of adhesion ligands to polymer surfaces was shown to influence cell morphology and migration were directly in accordance with polymer surface concentration [33]. We therefore studied cell morphology, adhesion, and migration on hydrogels with varying crosslinking degree (3.5 vs. 7.5 kPa), RGD-ligand concentration (5 vs. 20 μg/ml) and presentation of growth factors (loading with 0 vs. 1 μg VEGF per gel sample). In contrast to cell adhesion and proliferation, cell morphology and migration were directly influenced by the degradation of the gel. Cells growing on the faster degrading EG materials were more spindle-shaped in morphology than those on the slower degrading AG materials (Fig. 5A and B). Moreover, EG materials better supported the formation of cord-like structures and the three-dimensional (3D) expansion of the ECs (Fig. 5A and B, arrows for cord-like structures). This might be due to the fact, that for fast degrading EG materials the cellular microenvironment becomes more rapidly adapted by cell-secreted ECM to better promote morphogenesis.

Increasing the RGD functionalization of the gels resulted in a change in the morphology of the cells, making them more spread or even provoked the formation of a confluent layer. Additionally, loading of the gels with VEGF increased the frequency of cord-like structure formation and reduced the overall spreading of the cells (Fig. 5A and B). Various studies have shown that VEGF is a potent chemoattractant for ECs, influences their migration and morphogenetic events, such as tubular sprouting and network formation [39]. Zisch et al. found that PEG matrices lacking VEGF showed less cell invasion than VEGF-incorporated matrices [40]. In line with these findings, we observed a VEGF-stimulated cellular invasion into the hydrogels due to chemotaxis. Quantification of the invasion depth showed that the 3D migration speed of cells plated on the faster degrading EG materials was modulated by both biochemical and mechanical properties of the gel scaffold. The invasion depth decreased when the RGD concentration increased (Fig. 5C), which is in line with results published by Rutolf et al. [11]. Moreover, there was a similar trend if the stiffness of the gel increased from 3.5 to 7.5 kPa (Fig. 5D). ECs did not invade the AG gels within 24 h (Fig. 5D), even in the presence of VEGF and low adhesive ligand concentration, which further proved the observed slower erosion and higher stability of AG materials. Nevertheless, migratory behavior was observed after 48 h in AG gels with low crosslinking degree (Fig. 5E), suggesting that the initial degradation rate of the materials is a crucial determinant in EC invasion into the starPEG-heparin hydrogels. Thus, the degradation characteristics tremendously affect the EC arrangement, morphology, and migration rate.

From the comparison of the two gel types we conclude that the initial cell penetration in EG materials is accelerated due to the combined effect of non-enzymatic and enzymatic surface degradation which is in line with the degradation behavior observed for this material in the presence of CLS (see Fig. 3). Additional effects may result from the faster release of VEGF and the change of the mechanical resistance of the more rapidly degrading materials. In turn, AG materials can be beneficial where mechanical integrity of a scaffold is more important than faster cell/tissue ingrowth.

The importance of proteolysis in EC morphogenesis and angiogenesis is underpinned by the observation that VEGF induces the synthesis of plasminogen activator, collagensases, and other proteases [41,42]. Zymography of the collected cell culture media confirmed the presence of various proteinases within the supernatants and showed a slight upregulation after stimulation by VEGF (e.g. inactive and active forms of gelatinases MMP9 and MMP2, see Suppl. Fig. 1). The detection of MMPs in the conditioned cell culture medium shows that the lack of invasion into AG gels after 24 h has to be attributed to the lower susceptibility of the material but not to the lack of enzymes.

3.5. Angiogenesis in vivo — chicken CAM assay

Having demonstrated the decisive influence of the gel degradation on EC migration in vitro the proangiogenic potential of the biodegradable material was further evaluated in vivo using the CAM assay (Fig. 6). In view of the results discussed above we put emphasis on VEGF-preloaded hydrogels with differing degradation characteristics to stimulate angiogenesis. In comparison with the untreated CAM, slow degrading AG starPEG-heparin hydrogels alone did not induce any angiogenic response, while fast degrading EG materials caused an increase in the density of blood vessels surrounding the implants. On the contrary, VEGF-loaded gels of both types evoked an angiogenic response and increased the vascular density of the CAM. The more rapid release of VEGF from biological cues stimulated angiogenesis even in the absence of an external stimulus.
the fast degrading EG materials correlates well with the less pronounced increase of vascularization compared to the slow degrading AG materials, thus there is a clear link between the VEGF release (Fig. 3C) and the pro-angiogenic response in the CAM assay (Fig. 6A and B). Importantly, the most effective starPEG-heparin gel type, for which the degradation was boosted by the combination of two different degradation mechanisms, was found to be as efficient in promoting angiogenesis as Matrigel, which was used here as a positive control. Considering the drawbacks of the widely used Matrigel, in particular the unavoidable batch to batch variation, the ill-defined composition and the presence of undesired components, our well-defined bihybrid materials can be seen as a very attractive, highly reproducible alternative for many in vitro applications. Further on, the reported results suggest that fast degrading starPEG-heparin hydrogels can be applied to stimulate the formation of new blood vessels in vivo.
4. Conclusions

Bioresponsive starPEG-heparin hydrogels allow for the far-reaching modulation of erosion and cytokine release by combining enzymatic and non-enzymatic degradation mechanisms. With this extension, the versatile hydrogel system becomes suitable to evoke tissue regeneration on cellular level. We demonstrate that the multifactorial adjustment of the defined polymer platform can induce a strong proangiogenic response in vitro and in vivo, opening new perspectives for the use of biomaterials in cardiovascular regenerative therapies and beyond.

Fig. 6. Chicken embryo chorioallantoic membrane (CAM) angiogenesis assay (A) Representative examples of CAM, as observed under a stereomicroscope at 25× magnification. (B) Scored angiogenic response to the applied starPEG-heparin hydrogels shown in arbitrary units.
Acknowledgments

We thank Mareike Roth for valuable technical assistance. U.F., M. T. and C.W. were supported by the Deutsche Forschungsgemeinschaft (DFG) through grants WE 2539-7/1, SFB 655 and FOR/EXC999, and by the Leibniz Association. K.R.L., M. T. and C.W. were supported by the Seventh Framework Programme of the European Union through the Integrated Project ANGIOSCAFF. C.W. was supported by the Bundesministerium für Bildung, Forschung und Technologie (BMBF) through grant 01 GN 0946.

Appendix. Supplementary material

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.biomaterials.2011.08.078.

References


