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Two-tier hydrogel degradation to boost endothelial cell morphogenesis

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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.

1. Introduction

Tissue regeneration is based on the ability of cells to expand, degrade, invade, and dynamically remodel the surrounding extracellular 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

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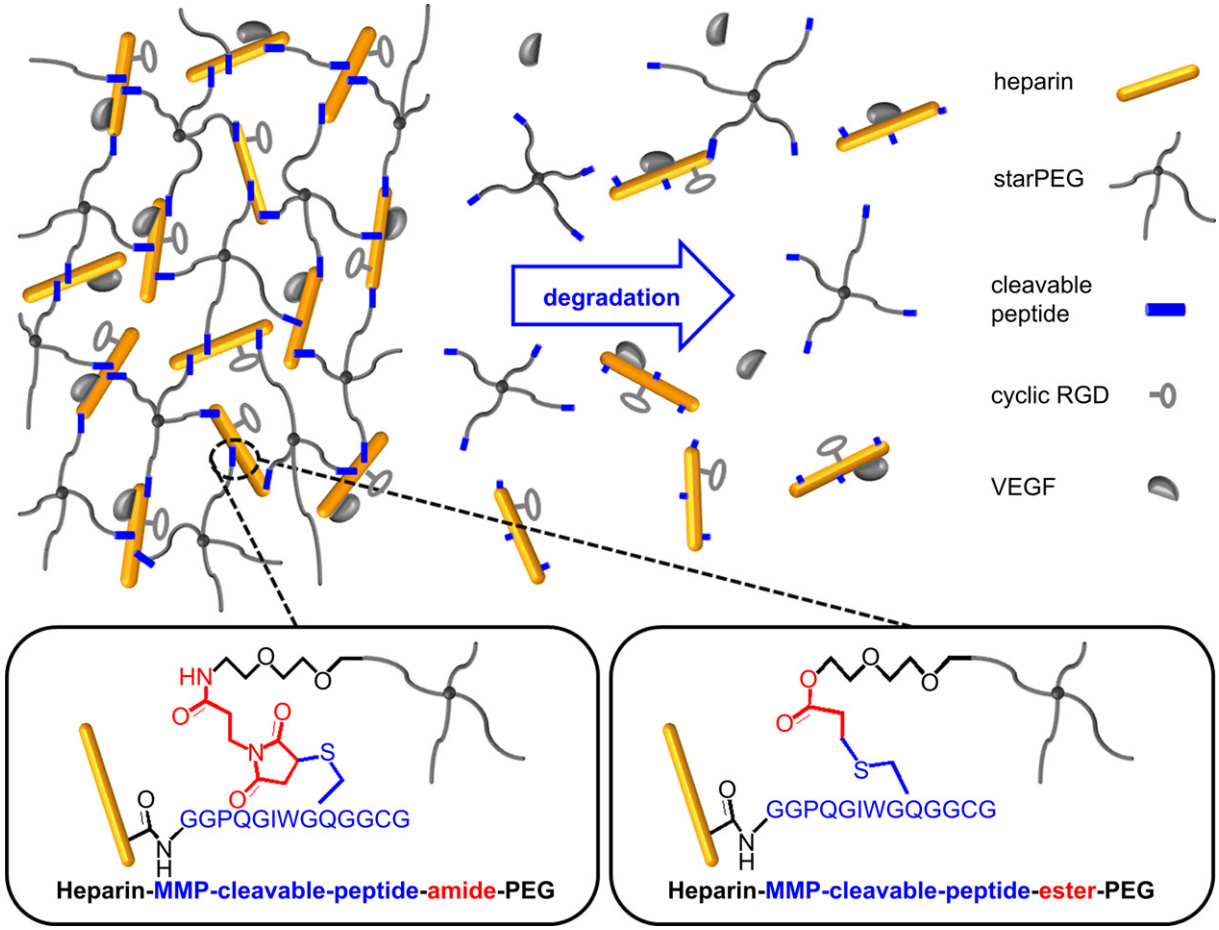

Fig. 1. Schematic representation of degradable starPEG-heparin hydrogels containing either ester (EG) or amide (AG) conjugated MMP-cleavable peptides.

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 CH_2Cl_2, next 90 μl (6×10^{-5} mol) of acryloyl chloride was added following by slow addition of 115 μl of TEA. The reaction was stirred overnight under N_2. About 300 mg of dry Na_2CO_3 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 $CDCl_3$): 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, 1200H), 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-$(NH_2)_4$ MW = 10 kDa) was dissolved in 0.5 ml of CH_2Cl_2, next 112 mg of (4.2×10^{-5} mol) 3-Maleimidopropionic acid N-hydroxysuccinimide ester was added. The reaction was stirred overnight under N_2, following double precipitation from 150 ml of diethyl ether. The collected white precipitate was dried under vacuum overnight and kept at −20 °C. 1H NMR (500Mz $CDCl_3$): δ 6.42 (s 8H), 6.27 (s, 4H), 3.85 (t, J = 7.5, 8H) 3.79–3.49 (m, 1216H), 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↑↓IWGQ was included as a bioactive module into peptide NH_2-GGPQGIWGQGGCG-$CONH_2$ (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 trifluroacetic acid (85% v/v), phenol (5% v/v), dithiothreitol (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 H_2O/AcN on XBridge BEH 300 C-18 column 10 μM particle size, 19 × 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