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Injectable synthetic hydrogel for bone regeneration: Physicochemical characterisation of a high and a low pH gelling system

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ABSTRACT

Hybrid poly(ethylene glycol)-*co*-peptide hydrogels are a versatile platform for bone regeneration. For the use as injectable scaffolds, a good understanding of reaction kinetics and physical properties is vital. However, these factors have not yet been comprehensively illuminated. We show that gelation time can be effectively controlled by pH without affecting the elasticity of the formed hydrogels. Maleimide functionalised PEG gels at lower pH and produces more densely cross-linked networks than vinylsulfone functionalised PEG. Both form non-ideal networks. The elastic moduli on the order of a few kPa are in good agreement with the structural characterisation. Primary human osteoblasts cultured in proximity to bulk gels were not adversely affected in vitro. The results demonstrate that hybrid PEG-peptide hydrogels can be tailored to the requirements of in situ gelation. Attributed to their increased structural properties and a higher tolerance towards low pH, maleimide functionalised hydrogels might provide a better alternative for injectable applications.

1. Introduction

Closely mimicking the native extracellular matrix (ECM), hydrogels are of major interest as tissue engineering scaffolds [1,2]. Injectable systems have attracted particular attention for hard tissue regeneration due to their ability to fill complex defect geometries and requiring minimally invasive procedures [3]. Typically, injectable hydrogels are either physically (reversible) or chemically (irreversible) cross-linked in situ. For applications like bone regeneration, requiring stability over several weeks under biological conditions, chemically cross-linked systems are of particular interest [4,5].

In 1999, West and Hubbell introduced a class of fully synthetic, hybrid hydrogels based on vinylsulfone (VS) functionalised branched poly(ethylene glycol) (PEG) macromeres end-linked with enzymatically degradable bis-cysteine peptides [6]. The proteolytic cleavability allows for enzyme-mediated cell migration and degradation of the hydrogel, which serves as a provisional matrix for tissue repair [7]. The degradation kinetics can further be tuned by altering the exact amino acid sequence of the end-linking peptide. In an extensive study, Patterson and co-workers have shown that the particularly degradation-sensitive sequence VPMSMRGG, which is also used in our study, enables increased cell-migration and remodelling [8]. One of the major advantages of this system is its modularity. Furthermore, PEG is approved in many biomedical applications and provokes little inflammatory response [9–12]. Covalent cross-linking occurs via bioorthogonal thiol-ene Michael type addition [13,14], which is highly compatible with living cells and tissues due to the absence of free radicals as well as other toxic reactants and side-products [15,16]. The gelation kinetics of VS functionalised PEG decrease when reducing pH [17,18]. PEG-VS based hydrogels have successfully been used for cell encapsulation [8,19–21] and shown promising in vivo results when gelled ex situ [17].

Bioactive injectable synthetic ECM substitutes may have great potential for repairing damaged tissues [22]. However, the in situ gelation scenario brings along very specific requirements to the hydrogel system. While being well suited for slightly alkaline conditions, slow or incomplete gelation of PEG-VS based hydrogels at slightly acidic pH jeopardises their potential for injectable applications, particularly into inflamed tissues. Phelps and colleagues reported a more robust gelation behaviour and improved mechanical properties when replacing the VS groups by more reactive maleimide (MAL) groups [23,24]. They further demonstrated the suitability of PEG-MAL based hydrogels for in situ

Abbreviations: AFM, atomic force microscopy; DB, Debye-Bueche; DLS, dynamic light scattering; ECM, extracellular matrix; EEC, elastically effective chain; hOB, primary human osteoblasts; IL-6, interleukin 6; MAL, maleimide; LDH, lactase dehydrogenase; OPG, osteoprotegerin; OZ, Ornstein-Zernike; PBS, phosphate buffered saline; PEG, poly(ethylene glycol); SAXS, small angle x-ray scattering; SOST, sclerostin; VS, vinyl sulfone

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gelation by injecting them onto a rat myocardial wall in vivo [24]. In contrast to the PEG-VS system [17,25], the PEG-MAL system has to our knowledge not yet been thoroughly characterised in respect to its physicochemical properties. These, however, are of crucial importance for recruiting and stimulating cells in tissue regeneration [26–28].

The aim of this study was to characterise and compare gelation kinetics and mechanostructural properties of the PEG-MAL and the PEG-VS hydrogel system. Different buffer conditions were used representing either physiological pH for the PEG-VS system or acidic conditions, relevant for injection into inflammation sites, for the more reactive PEG-MAL system. Gelation was quantitatively monitored by rheometry for a range of different pH values. Mechanical properties of swollen gels were assessed in bulk using linear oscillatory shear experiments and on cellular length scale by atomic force microscope (AFM) force spectroscopy. Findings from the mechanical assessment were compared to structural observations from swelling and small angle x-ray scattering (SAXS). Cytocompatibility of the different formulations was tested in a cell culture study with primary human osteoblasts (hOB).

2. Materials and methods

2.1. Reagents

Four-armed PEG macromeres (20 kDa molecular weight) functionalised at each chain-end with either maleimide (PEG-MAL) or vinyl sulfone (PEG-VS) were purchased from JenKem Technologies USA (Plano, TX, USA). The functional substitution was quantified by the supplier via ^{1}H NMR to be 96.6% and 98.8%, respectively. Enzymatically cleavable end-linking peptide with the sequence Ac-G**C**RD-VPMS↓MRGG-DR**C**G-NH_2 [8] (cysteines bearing the reactive thiols for end-linking highlighted in bold and ↓ indicating the site of enzymatic cleavage) was synthesised via solid state synthesis as acetate salt by Pepmic Co. Ltd. (Suzhou, China). Net peptide concentration was determined by the supplier via C/H/N element analysis. All standard reagents were purchased from Sigma-Aldrich Norway AS (Oslo, Norway).

2.2. Hydrogel fabrication

Hydrogels were produced by mixing two precursor solutions containing polymer and end-linking peptide. The polymer was dissolved in 100 mM citrate phosphate buffer (pH 3, 3.5 and 4) in case of PEG-MAL and 300 mM phosphate buffer (pH 7, 7.5 and 8) for the PEG-VS system. Buffer concentrations were determined in initial tests to ensure that the nominal pH was maintained in the presence of the end-linking peptide. Typical stoichiometrically matched hydrogels with 10 wt.-% polymer content were fabricated dissolving 5 mg of PEG-MAL or PEG-VS in 40 µl of buffer. Subsequently, 10 µl of 50 mM end-linking peptide solution in deionised water were added to initiate gelation. Complete mixing was ensured by vigorous vortexing. To assure stoichiometric equilibrium, free thiol concentration in peptide stock solutions was verified using photospectrometric Ellman's assay.

2.3. Rheometry during gelation

The gelation for the different polymer functionalisations and pH values was followed by linear oscillatory shear rheometry using an Anton Paar MCR 301 rheometer equipped with a cone-plate geometry (CP25-4). Since gelation rates can be affected by the shear rate [29], frequency and amplitude were kept constant for all measurements at 0.5 Hz and 5%, respectively. These values were initially verified to be within the linear viscoelastic regime. All measurements were performed at 37 °C. Evaporation was minimised by applying a thin layer of silicone oil onto the free sample edges.

2.4. Swelling

Gel disks were produced by sandwiching 20 µl droplets of freshly mixed gel precursors between two hydrophobic glass slides (coated with Sigmacote® according to manufacturer's instructions) separated by a silicone spacer. Gels were cured for 30 min and subsequently placed in 5 ml deionised water to swell for 24 h at 37 °C. After weighing (m_s), the swollen gels were snap-frozen in liquid nitrogen, lyophilised and weighed again (m_d) giving the mass swelling ratio ($Q_m = m_s/m_d$). The volume swelling ratio (Q_V) was calculated from the mass swelling ratio with help of the densities of polymer (ρ_p, taken as 1.2 g/ml for PEG [30]) and solvent (ρ_s). The density difference of peptide and PEG was neglected at this point. Data was obtained for six replicates.

2.5. Rheometry on swollen hydrogels

Mechanical properties of swollen hydrogels were assessed in linear oscillatory shear measurements using an Anton Paar MCR 301 rheometer equipped with a plate-plate geometry of 25 mm diameter (PP25). Large hydrogel disks with an initial thickness of 1 mm were produced as described in 2.4. After swelling for 24 h in PBS at 37 °C the disks had a typical thickness of about 2.5 mm. Samples were trimmed to 25 mm diameter just before the measurement using a hollow punch. To avoid slip, PEG-MAL and PEG-VS samples were pre-compressed with normal forces of 1 and 0.25 N, respectively. Storage and loss modulus were recorded as a function of frequency, ranging from 100 to 0.01 Hz at fixed amplitude of 1%. The latter was confirmed to be within the linear range of the gels in initial amplitude sweeps performed at a frequency of 1 Hz. Experiments were run in triplicates.

2.6. AFM force spectroscopy

Elastic properties on micro-scale were determined in force spectroscopy experiments using an Asylum MFP 3D AFM equipped with colloidal cantilevers. Tip-less silicon nitride probes (PNP-TR-TL, NanoWorld AG) with 2 µm diameter SiO_2 spheres attached to the long cantilever were purchased from sQube. Before measuring, each cantilever was calibrated in a two-step procedure. First, the optical lever sensitivity was determined by deflecting the cantilever against a hard mica surface. After that, the spring constant was determined via the thermal noise method. Typically, spring constants were around 0.05 N/m.

Furthermore, the setup was validated measuring the elastic modulus (E) of polyacrylamide hydrogels of defined stiffness. Gels with a nominal stiffness of (2.55 ± 0.17) kPa and (19.66 ± 1.19) kPa ($n = 3$) were produced according to a recipe published elsewhere [31]. Measured elastic moduli were deviating < 10% from the reported nominal values.

PEG hydrogels were mounted on cleaned borosilicate coverslips and swollen over 24 h in PBS at 37 °C. Indentations were controlled to a maximal force of 3 nN. Three force-maps of 20 × 20 indentations on an area of 80 × 80 µm^2 each were collected on three different spots on each gel. Three gels were measured per group. E was determined using the Hertz model. A Poisson's ratio of 0.5 (ideal gel) was assumed for all gels. All measurements and calibrations were performed in PBS.

2.7. Dynamic light scattering (DLS)

Hydrodynamic diameters of PEG-MAL and PEG-VS macromeres were determined using a Malvern Zetasizer Nano DLS instrument. Samples were prepared dissolving polymer in deionised water at a concentration of 2 mg/ml and filtered using 0.22 µm pore size filters (Merck Millipore Corp.). Measurements were performed at 37 °C.