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

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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.

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.

KEYWORDS

PEG, maleimide, vinylsulfone, gelation, mechanical properties, network architecture

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* 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).

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 $^1\text{H-NMR}$ to be 96.6% and 98.8%, respectively. Enzymatically cleavable end-linking peptide with the sequence Ac-GCRD-VPMS↓MRGG-DRCG-NH₂ [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 μ l 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 minutes 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 less than 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 x 20 indentations on an area of 80 x 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.

2.8 *Small angle x-ray scattering (SAXS).*

SAXS was used to assess structural properties of a PEG-MAL and PEG-VS gel end-linked at similar kinetics (pH 3 and pH 8, respectively). The experiments were performed on a Bruker NANOSTAR equipped with a microfocus X-ray source (IµS Cu, Incoatec, Germany) and a VÅNTEC-2000 detector. Gel samples were swollen for 24 h at 37°C in PBS and placed in a sandwich cell with Kapton windows. Raw scattering data was calibrated to absolute intensity scale using water as a primary standard and radially averaged in order to obtain the 1D scattered intensity profile as a function of the scattering vector ($q = 4\pi/\lambda \cdot \sin(\theta/2)$ with the wavelength $\lambda = 1.54 \text{ Å}$ and θ the scattering angle). Scattering from buffer and empty cell was subtracted using the appropriate scaling factor to take the differences in transmission and concentration of polymer network and buffer into account.

2.9 *In vitro cytocompatibility.*

To assess a possible impact of the used buffers and potentially present unreacted components, commercial primary hOB cells from one donor (Lonza, Basel, Switzerland) were exposed to different gels (PEG-MAL pH 3, 3.5 and 4 and PEG-VS pH 7, 7.5 and 8) in an *in vitro* experiment. Cells were routinely cultured at 37°C in a humidified atmosphere with 5% CO₂ and maintained in osteoblast basal medium (CC-3208, Lonza) supplemented with OGM Single Quots (CC-4193, Lonza) consisting of 10% fetal bovine serum, 0.1% amphotericin/gentamicin and 145 µM ascorbic acid. Upon confluence, each 25 µl of hydrogel were loaded into

membrane inserts (polycarbonate membrane with 0.4 μm pore size, Merck Millipore Corp.), allowed to cure for 20 minutes and introduced to the cell culture. 11 parallels were run per group.

The cytotoxicity was determined with a commercially available LDH quantification kit (Roche Diagnostics GmbH) 1, 6 and 24 h after exposing the cells to gels. As an indicator for cytotoxicity, relative LDH activity is expressed normalised to gel-free controls.

Furthermore, cell culture medium was collected 1 and 6 h and 1, 3 and 7 days after starting the experiment for multianalyte cytokine quantification using the Luminex-200 system (Luminex Corp.). Interleukin-6 (IL-6), osteoprotegerin (OPG) and sclerostin (SOST) concentrations were determined using a human bone metabolism kit (HBNMAG-51K MILLIPLEX MAP Human Bone Magnetic Bead Panel, Merck Millipore Corp.). The fluorescence readings were analysed using the 3.1 xPONENT software (Luminex Corp.). Cytokine quantification was performed on four replicates per group and time point.

2.10 Statistical analysis.

Groups and controls were statistically compared using parametric one-way analysis of variances (ANOVA) with multiple comparisons and post hoc Dunnett test if groups were compared to controls or post hoc Tukey test for pairwise comparison between different groups. When normality test (Shapiro-Wilk) was failed, one-way ANOVA on ranks (Kruskal-Wallis test) and post hoc Dunn's test was performed using SigmaPlot 13.0 (Systat Software Inc.). For all tests, a value of $p \leq 0.05$ was considered significant.

Results and discussion

3.1 Hydrogel formation and pH dependent gelation kinetics.

Immediately after mixing polymer and end-linking peptide under suitable conditions (e.g. an aqueous environment at sufficiently high pH or the presence of a base catalyst), thiol-Michael

addition sets on [14, 32]. The evolution of storage modulus (G') over time after mixing is depicted in **Figure 1**. It is noteworthy to mention that the end-linking reaction instantly commences after mixing. Nevertheless, before a critical cross-link density is reached, leading to percolation, no increase in G' was observed. Hereafter, a steep increase of G' was seen as the macroscopic network gradually grows. It has been proposed that initially tightly cross-linked nanoclusters are formed, which then interconnect into a macroscopic network [33]. Further reactions are increasingly limited by the local availability and diffusion of reaction partners, gradually reducing the growth rate of G' until a plateau is reached. The data shows that longer times than 30 min are required to reach the plateau for the VS pH 7.0 system. Surprisingly, PEG-MAL and PEG-VS gels approach different plateaus at approximately 10 kPa and 5 kPa, respectively (dashed horizontal lines in **Figure 1**). Meanwhile, irrespective of pH, there are no substantial differences for the plateau values of G' for gels of the same functionalisation after complete gelation. This suggests that depending on the polymer functionalisation distinctively different networks are formed.

Figure 1. (*Single column greyscale image*) pH dependence of gelation kinetics for PEG-MAL (triangles) and PEG-VS hydrogels (circles). As a measure for gelation the evolution of G' is shown over time after mixing of gel precursors.

During the addition reaction, thiol groups are deprotonated to thiolate anions by free base and undergo nucleophilic attack on the electron-deficient C=C π -bond of the MAL or VS groups [14]. The resulting carbon-centred anion intermediate then abstracts a proton from the conjugate base, yielding the final thioether bond between polymer and peptide. Despite the lower pH level, the gelation process is generally faster for PEG-MAL compared to PEG-VS under the tested conditions. The same observation has previously been reported [23, 34] and can be attributed to the higher electron deficiency of the C=C π -bond of the MAL group, rendering it more reactive [35]. Furthermore, gelation kinetics increase with increasing pH for both groups. This was expected as a higher concentrations of free base result in an increased thiolate concentrations as previously reported elsewhere [17, 34, 36].

For the tested conditions, all samples gelled within a timeframe suitable for clinical handling, i.e. gelation onset times are ranging from less than two minutes (MAL pH 4.0) to eleven minutes (VS pH 7.0). However, considering its pronounced sensitivity towards slight pH changes, the PEG-VS system might be problematic for gelation *in vivo*, particularly when applied onto inflammatory tissues where pH values as low as 3.6 can be found [37]. Preliminary tests showed that the used PEG-VS system did not form a gel at pH values of 4.0 or lower. The presented PEG-MAL gels in turn effectively gel at pH values as low as 3.0 within few minutes. On the other hand, Darling and colleagues have recently shown that similar PEG-MAL gels could not be homogeneously mixed at physiological pH due to the extremely fast gelation kinetics at these conditions, which conforms to our observations [34]. Based on an earlier study by Lutolf and colleagues [36], they further showed that this can be overcome by introducing negatively charged amino acids in close vicinity to the cysteines on the end-linking peptide. This increases the thiol pKa, reducing the thiolate concentration, and thus, slowing down the reaction kinetics. Only recently, Jansen et al. have investigated further ways to control gelation kinetics of PEG-MAL gels at higher pH values than studied here [38]. They demonstrated that

by lowering polymer weight percentage or buffer concentration and by using more electronegative end-linking peptides, the gelation kinetics can be slowed down and allow PEG-MAL gel formation at physiological pH. However, since the present study focusses on the comparative characterisation of PEG-MAL and PEG-VS gels, such changes to the system would not have been reasonable.

3.2 Physical properties of swollen hydrogels.

3.2.1 Swelling

The extent of swelling depends on the network structure. The higher the density of elastically effective chains (EEC), the less the networks will swell and vice versa. **Figure 2** shows the volume swelling ratio for the different gels. As expected from the increased mechanical properties after gelation (**Figure 1**), PEG-MAL gels swell considerably less than PEG-VS gels indicating a higher cross-linking density of the former. No statistically significant differences were found within the two groups.

Figure 2. (*Single column greyscale image*) Volume swelling ratio versus polymer type and pH.

The measured swelling ratios generally are in good accordance with previously published results for similar PEG-VS gels [17, 21, 39]. Phelps and colleagues also reported approximately

twice as high swelling ratios for PEG-VS gels compared to PEG-MAL gels with 7.5% polymer content [23]. Surprisingly though, they found no difference between the two groups for gels with 10% polymer content. We suspect that this might be due to different reaction conditions resulting in distinct gel architectures.

3.2.2 Rheometry on swollen gels

The viscoelastic response of hydrogels exposed to oscillatory shear is shown in **Figure 3** as a function of frequency. All gels exhibit elastic solid-like behaviour with storage moduli being about two orders of magnitude higher than loss moduli (G''). For later considerations, we thus assume $G \approx G'$. G' and G'' are nearly constant for the individual gels at frequencies up to 4 and 1.5 Hz for PEG-MAL and PEG-VS gels, respectively. In this regime, G' is around 3.5 kPa for PEG-MAL gels and 0.7 kPa, for PEG-VS gels. Again, no statistically significant differences could be found within the PEG-MAL and PEG-VS groups.

Figure 3. (Single column greyscale image) G' (filled symbols) and G'' (open symbols) measured as a function of frequency for PEG-MAL (triangles) and PEG-VS hydrogels (circles) gelled at different pH.

While qualitatively confirming the observation from gelation monitoring, the ratio of the elastic moduli of PEG-MAL and PEG-VS gels increased from about two at reaction conditions

to five in the swollen state. This can be explained by the higher degree of swelling of the PEG-VS gels increasing the discrepancy of cross-link density in the swollen state. Interestingly, the measured G' of PEG-VS gels is about twice as high as reported elsewhere for a comparable gel [17].

3.2.3 AFM force spectroscopy

To gain more information about the stiffness and homogeneity on micro-scale, swollen hydrogels were further characterised using AFM force spectroscopy. **Figure 4** shows exemplary force-indentation curves and representative force maps of PEG-MAL and PEG-VS gels mounted on glass coverslips and unmounted PEG-VS gels. The force-indentation curves show good agreement for the experimental data and the Hertz model (**Figure 4a**). Further, little adhesion can be observed for both polymer functionalisations. The overlay force maps illustrate gel topography and local stiffness. While mounted PEG-MAL gels exhibit both a topographically smooth surface and a homogenous stiffness (**Figure 4b**), the situation is very different for mounted PEG-VS gels (**Figure 4c**). These exhibit wrinkly surfaces, and the gel stiffness depends on the topographical location. The wrinkling is likely caused by the mounting on a rigid substrate, as the concerned PEG-VS gels undergo a large volume expansion during swelling (**Figure 2**). Significantly higher values for E are found in the concave valleys, indicating lower degree of swelling in these regions due to spatial restrictions. Since this is an artefact from sample handling, these areas were neglected for the calculation of mean elastic moduli.

To clarify the origin of the wrinkle formation, we also performed measurements on PEG-VS gels not being mounted on a substrate. Like the PEG-MAL gel, these freely swollen gels show a smooth surface and homogeneous stiffness (**Figure 4d**). We therefore conclude that the wrinkling is an artefact caused by mounting the gels before swelling.

Further, even for the ridge regions of mounted PEG-VS gels elastic moduli were found to be approximately 1.5 times larger than for unmounted gels, suggesting that these gels are globally not swollen to equilibrium. We, therefore, reason that the used mounting method proposed elsewhere for force spectroscopy on polyacrylamide gels [31] is not suitable for highly swelling systems and potentially causes overestimation of gel stiffness. Interestingly, apart from the artefacts from mounting, no substantial inhomogenities could be found for the elastic modulus of neither PEG-MAL nor PEG-VS gels on a micrometre scale.

Figure 4. (Double column colour image) Experimental force-indentation curves (a) coincide well with the Hertz model (dashed lines) for both PEG-MAL and PEG-VS. Horizontal dotted lines indicate the regime used to calculate E. Representative 3D overlay representations of

force maps recorded by AFM force spectroscopy (b to d). Surfaces represent hydrogel topography (10x exaggerated compared to lateral dimensions); colour coding indicates elastic modulus as determined from force-indentation curves. PEG-MAL gelled at pH 3 mounted on glass coverslip (a) and PEG-VS gelled at pH 8 mounted on glass (b) and freely swollen (c).

Mean E values obtained from a total of nine force maps from three individual gels per group are shown in **Figure 5**. Again, two different levels can be identified for PEG-MAL and PEG-VS gels at approximately 7.2 kPa and 4.5 kPa, respectively. One should note that these measurements were taken on mounted gels. As discussed above, the values for the PEG-VS gels correspond to gels not completely swollen to equilibrium. Once more, no statistically significant differences within the polymer groups could be found.

Figure 5. (*Single column greyscale image*) Elastic moduli on μ -scale as measured by AFM force spectroscopy on gels mounted on glass cover slips as previously reported [31]. Each point represents the mean value obtained from 9 force maps of 400 indentations each performed on 3 individual gels per group.

To our surprise, the measured elastic moduli are considerably higher than those previously reported for similar PEG-MAL and PEG-VS gels [23]. Comparing the results of the force spectroscopy measurements with the ones measured for bulk gels in rheometry, one would

ideally expect a relationship obeying the equation $E \approx 3G$. However, for the experimental data on PEG-MAL gels, we observe a relationship of $E \approx 2G$. For PEG-VS gels, we find a relationship of $E \approx 6.2G$. The latter is very high because elastic moduli were measured on mounted gels that do not represent the equilibrium swollen state and are thus too high. Other reasons possibly explaining the discrepancy between theoretically expected and observed relationships are: different material behaviour for indentation and shear, different cross-linking densities on the surface and the bulk of the gels, as well as limitations of the quantitative AFM measurements. The latter are discussed in detail elsewhere [40, 41]. Most importantly the method is highly sensitive to the cantilever calibration and the underlying Hertz model assumes an adhesion-less, ideal linear elastic contact between sample and indenter, which cannot fully be satisfied in the experiment. Despite these limitations, the comparison with the oscillatory rheometry shows that elastic modulus as measured by AFM force spectroscopy is in the right range while the method provides additional information about spatial homogeneity on a length-scale comparable to the size of host tissue cells.

3.2.4 *Gel architecture*

The different physical properties found for PEG-MAL and PEG-VS gels indicate different underlying network architectures. For both gels, we can expect non-ideal networks, meaning that parts of the gel components will not be connected to the macroscopic network (sol fraction) and there are network imperfections such as dangling ends, primary loops and physical entanglements [42]. While dangling ends and primary loops represent network interruptions that significantly weaken the mechanical properties [43, 44], entrapped physical entanglements can partly counteract this effect by acting as pseudo cross-links [45]. Altogether, the weakening effects are dominant though. Matsunaga and colleagues previously reported near ideal hydrogel networks based on four-armed PEG macromeres exhibiting mechanical properties about one order of magnitude higher than those of typical defect-containing end-linked pendants [44, 46].

As a measure for the network architecture, we here focus on the number of EEC per unit volume of the swollen gels ($[\nu]$ in mol/l). As a simple estimate, we calculated $[\nu]$ based on the assumption that each peptide molecule will form one EEC (i.e. an ideal network without any sol fraction or defects is formed). We did not determine the peptide sol fraction in this study and neglected its effect on the idealised estimate. Lutolf and Hubbell previously reported a peptide sol fraction of 8.4% of total peptide for a comparable PEG-VS gel without having a strong effect on the estimate for $[\nu]$ [17]. Considering their higher mechanical properties, it is reasonable to assume that the sol fraction is even smaller for the PEG-MAL gels.

The actual density of EECs can be estimated using theoretical models and the experimentally determined physical properties. Based on Flory's affine network model, Gnanou and colleagues have derived the following correlation for the mechanical properties and $[\nu]$ [47, 48]:

$$G = [\nu]RT \cdot (v_{2,s}/v_{2,r})^{1/3} \quad (1)$$

Here $v_{2,s}$ is the polymer volume fraction in the swollen state ($v_{2,s} = 1/Q_V$) and $v_{2,r}$ the polymer volume fraction during the end-linking reaction. Alternatively, $[\nu]$ can be predicted from the swelling ratio using the Flory-Rehner equation [49]. For gels formed in the presence of solvent, the equation has been modified to [50]:

$$[\nu] = -\frac{v_{2,s}}{V_1} \cdot \frac{\ln(1 - v_{2,s}) + v_{2,s} + \chi \cdot v_{2,s}^2}{v_{2,r} \left((v_{2,s}/v_{2,r})^{1/3} - 1/3 (v_{2,s}/v_{2,r}) \right)} \quad (2)$$

Here V_1 is the specific molar volume of the solvent (18 ml/mol for water) and χ the Flory-Huggins interaction parameter (taken as 0.43 for PEG-water [51], neglecting the presence of buffer and peptides).

Table 1 lists up the experimental results from swelling experiments and mechanical characterisation (Q_V , G and E) together with estimates for $[\nu]$ based on the experimental values and theoretical considerations. The idealised estimate of $[\nu]$ based on the peptide concentration

is much higher than the experimentally derived values indicating a non-negligible amount of network imperfections. Other than reported elsewhere for PEG-VS gels [17], the values obtained for $[\nu]$ based on the experimentally determined values for G and Q_V (using the affine network model or the modified Flory-Rehner equation) are generally in good agreement. The estimates from the swelling ratio generally are about 25% lower. One should consider that both, the affine network model and the modified Flory-Rehner equation do not account for entrapped physical entanglements. These however, might contribute to a different extend to the measured parameters Q_V and G .

Table 1. Summary of mean values of experimentally determined gel properties and theoretical estimates for $[\nu]$.

	ideal ^a	aff. NW ^b	mod. FR ^c
sample	$[\nu]$ (mmol/l)	$[\nu]$ (mmol/l)	$[\nu]$ (mmol/l)
MAL pH 3.0	2.73	2.27	1.19
MAL pH 3.5	3.02	2.02	1.61
MAL pH 4.0	2.77	2.03	1.25
VS pH 7.0	1.64	0.48	0.27
VS pH 7.5	1.78	0.60	0.35
VS pH 8.0	1.81	0.44	0.36

a) Crude theoretical estimate for $[\nu]$ for ideal networks assuming each peptide molecule forms an EEC in the swollen gel. b) $[\nu]$ calculated from G using the affine network (aff. NW) model. c) $[\nu]$ derived from Q_V using the modified Flory-Rehner (mod. FR) equation.

Comparing the differently functionalised gels, both estimates propose an approximately four times higher EEC density for PEG-MAL gels compared to PEG-VS gels. The experimentally derived $[\nu]$ values for PEG-MAL gels are also systematically closer to the ideal estimates suggesting less network defects. Surprisingly, there is no apparent relationship between

gelation kinetics and network architecture which would manifest itself in differences among gels of same functionalisation prepared at different pH. We hypothesise that a competing disulphide-forming reaction between thiol groups of the end-linking peptide at alkaline pH is the reason for the systematically looser PEG-VS networks. The kinetics of both the disulphide formation and the thiol-Michael addition would be increasing with increasing pH. Rate changes of the two reactions with pH may thus just cancel each other out for the different PEG-VS gels prepared at pH 7 to 8, explaining why we cannot observe significant differences in the physical properties of these gels. For the PEG-MAL gels, disulphide formation can be neglected because of the very slow kinetics at acidic conditions. However, it can play a role when PEG-MAL gels are end-linked at physiological pH, possibly explaining the recently published findings of decreased mechanical properties for such gels [34].

3.2.5 SAXS

For two representative samples, the gel architecture was directly studied by SAXS. **Figure 6** shows a double logarithmic plot of the scatter intensity functions, $I(q)$, over the scattering vector q for a PEG-MAL and a PEG-VS gel. $I(q)$ contains contributions from thermal fluctuations of the network mesh and frozen network inhomogeneities [52]. The scattering data was phenomenologically modelled by a linear combination of an Ornstein-Zernike function (OZ) and a Debye-Bueche term (DB) [53, 54]:

$$I(q) = \frac{I_{OZ}(0)}{1 + \xi^2 q^2} + \frac{I_{DB}(0)}{(\xi^2 + q^2)^2} \quad (3)$$

Here, $I_{OZ}(0)$ and $I_{DB}(0)$ are the zero- q intensities, ξ is the correlation length (mesh size) and ξ is the characteristic length of network inhomogeneities. While the typical scattering from the polymer chains forming the network is apparent at high q values following a q^{-2} law (described by the OZ term of the model), larger, frozen inhomogeneities show at low q values (covered by the DB term) [44, 54]. Since SAXS cannot directly detect length scales beyond about 100 nm,

and in order to reduce the number of free parameters, the size of the network inhomogeneities was fixed to an arbitrary value of 100 nm as a typical length scale [33].

Figure 6. (Single column greyscale image) SAXS intensity functions over q for PEG-MAL gels (black triangles) and PEG-VS gels (grey circles) prepared at pH 3 and 8, respectively. Fitted curves (thick lines) represent an OZ model with a DB term to account for structural inhomogeneities. Dashed lines depict an OZ model without a DB term assuming ideal networks free of inhomogeneities.

The intensity functions for both gels are monotonously decreasing over the observed q -range. Below around $0.02 \text{ \AA}^{-1}$, the curves clearly divert from intensity functions of ideal, near defect-free networks (dashed lines in **Figure 6** and as shown elsewhere [44]), again indicating a non-negligible amount of network inhomogeneities. For the PEG-MAL gel, $I(q)$ nicely follows a Lorentz function down to about $0.02 \text{ \AA}^{-1}$ as described by the OZ term of **Equation (3)**. For lower q -values the intensity function turns up again. This excess scattering can be attributed to large-scale immobile network inhomogeneities and is described by a squared Lorentzian function given by the DB term of **Equation (3)** [54]. The intensity function of the PEG-VS gel is horizontally shifted towards lower q -values, indicating a larger mesh size of this network. Around $0.04 \text{ \AA}^{-1}$, the intensity function crosses the one of the PEG-MAL gel. Two regimes

described by the OZ and the DB term cannot clearly be distinguished, suggesting that the two correlation lengths for the mesh size and the inhomogeneities lie closely together.

The mesh sizes fitted by the OZ-DB fits (R^2 values 0.96 and 0.97 for the PEG-MAL and the PEG-VS gel, respectively) are given in **Table 2**. Also shown are theoretical estimates for ξ based on the swelling ratio and the hydrodynamic diameters (D_h) of the macromeres as measured by DLS. Based on the swelling ratio the mesh size of an ideal swollen polymer network can be estimated as [55]:

$$\xi = v_{2,s}^{-1/3}(\bar{r}_0^2)^{1/2} \quad (4)$$

Where $(\bar{r}_0^2)^{1/2}$ is the mean unperturbed end-to-end distance of the polymer and given by:

$$(\bar{r}_0^2)^{1/2} = lC_n^{1/2}(2\bar{M}_c/M_r)^{1/2} \quad (5)$$

Here l is the average bond length (taken as 1.54 Å for vinyl polymers)[56], C_n the characteristic ratio (4.0 for PEG) [51], $\bar{M}_c$ the molecular weight between two cross-links (assumed to be 11500 g/mol) and M_r the molecular weight per repeat unit (44 g/mol). It should be noted that **Equation (4)** and **(5)** give a general estimate of ξ , assuming an even distribution of cross-linking density. The theoretical consideration neither accounts for network interruptions caused by dangling ends and primary loops nor entrapped physical entanglements.

Table 2. Overview of ξ estimated from Q_V for ideal networks, determined via SAXS and D_h as measured by DLS.

	Q_V	SAXS	DLS
Sample	ξ (nm)	ξ (nm)	D_h (nm)
MAL pH 3.0	24	2.7	5.4
VS pH 8.0	28	4.5	5.2

The idealised estimate for ξ agrees well with previously reported values [39] but differs by up to one order of magnitude from the values determined via SAXS. This can be explained by

the fact that the theoretical estimate, which assumes a perfectly homogenous network, represents an average over all correlation lengths (i.e. mesh size and inhomogeneities), while the ξ value determined by SAXS only accounts for the short-range correlation length (larger features such as inhomogeneities are covered by the DB term). The discrepancy is another indicator for pronounced inhomogeneities of the networks. Further, according to SAXS, the PEG-VS gel exhibits an almost twice as large mesh size as the PEG-MAL gel which is in good accordance with the about four times lower values estimated for $[\nu]$.

Interestingly, the hydrodynamic diameter of the polymer macromeres is of the same order as the mesh size in swollen state. This means that, at gelation conditions, the distance between macromeres is smaller than their D_h . In other words, the macromeres potentially overlap, increasing the likeliness of permanent physical entanglements. Summarising the presented results, we can safely conclude that the network architecture of the investigated gels contains a significant amount of inhomogeneities on a typical length scale of 100 nm. On the other hand, we did not see pronounced inhomogeneities on a scale of about 1 μm in the AFM force spectroscopy measurements. This leads us to assume that the characteristic size of the inhomogeneities is approximately on the scale of hundreds of nanometres, as has previously been suggested [33].

While physical entanglements can provide a certain contribution to the mechanical properties of the gel networks [45], particularly the presence of first order defects like primary loops and dangling ends significantly reduces the mechanical properties of the investigated hydrogels compared to ideal networks [43, 44, 57, 58]. Matsunaga and colleagues argue that this is likely due to the high stress concentration in environments of low cross-linking densities [44]. Under mechanical load these fail, reducing the remaining number of elastically active chains, and thus, the mechanical properties of the network. One possible and practical way to overcome

this issue and reduce the possibility of detrimental network interruptions is to increase the functionality of the end-linking molecule [59].

3.3 *Cell compatibility with primary hOB.*

When injected *in vivo*, unreacted gel components and reaction buffer might have negative effects on healthy cells in the adjacent tissues. PEG-VS gels have previously been shown to be highly compatible with a variety of human cells [7, 19, 60]. PEG-MAL gels formed at physiological pH have shown to be non-toxic to murine cells [11, 23]. We here exposed confluent hOB to freshly formed hydrogels to mimic an injection scenario. **Figure 7** shows the relative amount of free LDH for the first day after exposure and concentrations of the cell-markers IL-6, OPG and SOST in the medium for up to one week. Free LDH indicates dead cells [61]. IL-6 can be regarded as an exemplary inflammation marker that has also been reported to suppress osteoblast differentiation [62] and induce bone resorption [63]. OPG is a soluble factor expressed by hOBs to downregulate bone resorption [64], while SOST has been shown to reduce osteoblast proliferation and mineralisation [65] being two important elements in the feedback system of bone homeostasis.

Figure 7. (Double column colour image) Activity of free LDH relative to gel free control samples (a). Values significantly higher than one indicate high cytotoxicity. Levels of cytokines relevant for bone metabolism: IL-6 (b), OPG (c) and SOST (d) as expressed by primary hOB cells exposed to gels based on PEG-MAL (triangles) and PEG-VS (circles) at different pH. For better readability, data points are connected by lines.

Figure 7a shows no elevated LDH concentration for any of the test groups compared to the gel-free control, indicating no cytotoxic effect on hOBs. For the single time points, no statistically significant differences can be found between the groups. The acidic buffer medium of the PEG-MAL gels did not have a toxic effect on the tested cells which is likely due to the

buffer capacity of the cell culture medium. Further, the concentration profiles of the three observed cytokines all follow the same pattern as the control group, suggesting normal cell behaviour (see **Figure 7b to d**). Only the cells exposed to PEG-MAL gel prepared at pH 3 and the PEG-VS gel prepared at pH 7 show statistically significantly elevated values for IL-6 at days one and three and for OPG at day three. There seems to be no recognisable pattern in cytokine expression though. Altogether, the results reveal no notable effect of sol fraction and buffer conditions on cell survival and activity.

Conclusions

Gelation kinetics and physicochemical properties of hydrogels prepared from MAL and VS functionalised tetra-PEG were characterised in comparison. We could demonstrate that the pH and the functional group effectively control the end-linking kinetics. The systems gelled within few minutes around physiological or acidic pH in case of PEG-VS and PEG-MAL gels, respectively. Changes in gelation kinetics induced by pH do not affect the resulting networks but PEG-MAL gels systematically exhibit tighter network architecture and increased mechanical properties compared to PEG-VS gels. While AFM force spectroscopy revealed homogeneous mechanical properties on micro-scale, structural SAXS analysis exposed structural inhomogeneities on sub-micron level as suggested by theoretical considerations. Due to their increased mechanostuctural properties and tolerance to lower pH during gelation, PEG-MAL hydrogels are a promising alternative to the widely-used PEG-VS system for *in situ* gelling applications. Despite the less physiological reaction conditions, we found no negative effect on survival and bone metabolism of hOB cells.

AUTHOR INFORMATION

Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Conflicts of interest

None.

ACKNOWLEDGEMENTS

The authors want to thank Aina-Mari Lian and Janne E. Reseland at the Department of Biomaterials, University of Oslo, for their help with the cell study and performing the cytokine assay.

FUNDING

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

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