Enzyme responsive GAG-based natural-synthetic hybrid hydrogel for tunable growth factor delivery and stem cell differentiation

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Abstract:

We describe an enzymatically formed chondroitin sulfate (CS) and poly(ethylene glycol) (PEG) based hybrid hydrogel system, which by tuning the architecture and composition of modular building blocks, allows the application-specific tailoring of growth factor delivery and cellular responses. CS, a negatively charged sulfate-rich glycosaminoglycan of the extracellular matrix (ECM), known for its growth factor binding and stem cell regulatory functions, is used as a starting material for the engineering of this biomimetic materials platform. The functionalization of CS with transglutaminase factor XIII specific substrate sequences is utilized to allow cross-linking of CS with previously described fibrin-mimetic TG-PEG hydrogel precursors. We show that the hydrogel network properties can be tuned by varying the degree of functionalization of CS as well as the ratio and concentrations of PEG and CS precursors. Taking advantage of TG-PEG hydrogel, compatible tagged bio-functional building blocks, including RGD peptides or matrix metalloproteinase sensitive domains, can be incorporated on demand allowing the three-dimensional culture and expansion of human bone marrow mesenchymal stem cells (BM-MSCs). The binding of bone morphogenetic protein-2 (BMP-2) in a CS concentration dependent manner and the

BMP-2 release mediated osteogenic differentiation of BM-MSCs indicate the potential of CS-PEG hybrid hydrogels to promote regeneration of bone tissue. Their modular design allows facile incorporation of additional signalling elements, rendering CS-PEG hydrogels as highly flexible platform with potential for multiple biomedical applications.

Key words: chondroitin sulphate, hydrogels, polyethylene glycol, Factor XIII, matrix metalloproteinase (MMP).

1. Introduction:

Cells in their natural environment reside in an extracellular matrix (ECM) consisting of a three-dimensional (3D) fibrous protein and glycosaminoglycan (GAG) based highly hydrated polymer network, which maintains tissue integrity and provides mechanical cues. Equally important, ECM components establish an intricate network of (temporally and spatially controlled) signalling cues to embedded cells by providing binding sites for cell-surface receptors and allowing matrix-tethering or diffusion of bioactive ligands (growth factors, cytokines). However, cells not only receive signals from their environment, but dependent on the developmental or physiological status of a tissue, continuously modify their surrounding ECM by secretion or degradation of matrix components, resulting in the dynamic adjustment of its composition. Thus, artificial ECM (aECM) mimicking hydrogels should be designed towards the establishment of conditions which closely mimic naturally occurring healing associated ECMs. Towards this direction, multiple protein and GAG components (heparin sulphate, hyaluronic acid, chondroitin sulphate) of naturally occurring ECM have been used as biomaterials in tissue engineering and regenerative medicine approaches.

Protein based hydrogels such as collagen and fibrin, by providing integrin receptor ligands and protease degradable sites, can readily be populated by cells both *in vitro* and upon implantation *in vivo* [1]. Although they greatly support cell viability and tissue regeneration, tailoring the properties of protein-based hydrogels towards specific applications is significantly challenged by their inherent biological properties [2]. In fibrin, the degree of proteolytic degradation, the provision of alternative integrin signals and the controlled release of growth factors could be partially modulated by immobilization of either engineered protease inhibitor molecules, integrin binding domains or by affinity or covalent interactions with trophic growth factors [3–6]. However, for collagen, the complex matrix metalloproteinase (MMP) mediated proteolytic degradation, and integrin-ligand presentation greatly limit the feasibility of modification [7].

Polysaccharides are highly hydrophilic polymers, which under physiological conditions have a large potential for water absorption and can be degraded into non-toxic products. Thus, they provide an interesting basis for the fabrication of hydrogels for cell, drug and gene delivery applications. A variety of animal-derived (chitosan, hyaluronic acid, chondroitin sulphate) as well as plant-derived (cellulose, starch, alginate, agarose) polysaccharides have been investigated for hydrogel fabrication[8–12]. Due to the versatility of their chemical structure, polysaccharides have been covalently modified with acrylamide, methacrylate, acrylates, thiols or maleimides [13–15] allowing their gelation and further functional modification by affinity or chemical cross-linking[16].

Chondroitin sulfate (CS) is an abundant ECM constituting GAG of up to a molecular weight of 50kDa, which provides compressive strength to various connective tissues throughout the body and is also a constituent of the blood serum[17]. Its involvement in enzymatic regulation, cell recognition, and cell signalling, particularly within the stem cell niche [18,19] has inspired scientists to investigate the utility of CS in a range of tissue engineering applications. Among the factors of paramount importance are the capacity of CS to bind to various growth factors and components of extracellular matrix via ionic interactions, its direct binding to cell surface receptors and its ability to degrade into non-cytotoxic disaccharide units. Indeed previous studies have shown that CS binds to bone morphogenetic protein-4 (BMP-4) among multiple other growth factors and thereby can promote osteoblast differentiation and mineralization [20,21]. As a copolymer of *N*-acetyl galactosamine sulfated at 4 or 6 position and D-glucuronic acid, CS contains ample carboxyl and hydroxyl groups on its backbone, permitting its facile and flexible functionalization by covalent conjugation. Thus it is not surprising that CS has been modified by chemical and ionic interaction with reactive functionalities like thiols, acrylates and methacrylate as well as with other polymers such as chitosan [22], gelatin [23], hyaluronic acid [8], collagen [24] and other synthetic counterparts for instance PEG [25] and PLGA [26] for a range of applications in tissue engineering like chondrogenic differentiation of mesenchymal stem cells (MSCs) [27] and tissue adhesives [25].

To overcome the limited control over naturally occurring materials, such as broad integrin signalling, fast degradability, and protein binding properties, a range of synthetic biocompatible polymers have been employed to form hydrogels for the delivery of exogenous cells or cell-instructive factors [28,29]. Hydrogels with controlled, functional architectures and tuneable stiffness have been combined with desired polysaccharide or protein-derived functionalities to specifically tailor their bioactivities on demand, for instance with integrin-specific cell adhesion motives, growth factor binding affinities and enzyme specific degradation sites [30]. With operator controlled site- and time-specific modification of materials properties such as presentation of adhesion sites, material stiffness or release of growth factors, first tools are now emerging, which provide the exciting possibility to control biological processes in four dimensions [31].

In order to integrate the increasing number of available molecular tools in a modular fashion, some of us have developed a PEG-hydrogel platform, which allows for the flexible composition of engineered building blocks [32]. This transglutaminase cross-linked PEG (TG-PEG) hydrogel platform is based on 8-arm PEG-vinyl sulfone (8-PEG-VS) which by a Michael-type addition reaction are functionalized with two complementary short glutamine (TG-Gln; NQEQVSPL-ERCG-NH₂) or lysine peptides (TG-Lys; Ac-FKGG-ERCG-NH₂) that serve as specific substrates for thrombin activated transglutaminase factor XIII (FXIIIa). When using matrix metalloproteinase sensitive lysine peptides (TG-MMP-Lys; Ac-FKGG-GPQGIWGQ-ERCG-NH₂) and the enzymatic incorporation of a Gln-tagged RGD (TG-RGD-Gln; NQEQVSPL-RGDSPG-NH₂) these FXIIIa cross-linked hydrogels have been shown to support growth, migration and differentiation of osteoblasts or MSCs in vitro as well as the bone morphogenetic protein induced healing of bone defects [33,34]. These hydrogels, utilizing FXIIIa-mediated gelation, are highly biocompatible, can be formed under physiological buffer conditions and neither require chemical initiators, UV irradiation, or temperature changes at the site of formation [35]. Additionally, the substrate specificity of the

enzymatic reaction allowed the modular immobilization of growth factors and therefore the localized functionalization of the hydrogels and the site- and concentration-dependent specific differentiation of MSCs [36,37].

Currently, properties from naturally occurring materials and tools developed for synthetic materials cannot be easily combined due to incompatibility of the systems (different cross-linking mechanisms and conditions). Therefore, the development of rationally adjustable biomaterial platforms that would allow the use of transposable modular building blocks is highly desirable for tissue engineering and regenerative medicine.

In the present work, a modularly assembled, TG-PEG and MMP-Lys-peptide grafted CS precursor based CS-PEG hybrid hydrogel platform was developed. This platform, by taking advantage of a mild and highly specific FXIIIa-mediated cross-linking mechanism is designed to generate hydrogels with defined physical and chemical properties as well as to provide localized and slow released biological cues. CS grafted with MMP-Lys peptides upon addition of FXIIIa readily formed hydrogels with the corresponding TG-PEG precursor PEG-Gln. By evaluation of swelling behaviour, storage moduli or fluorescence recovery after photobleaching (FRAP), hydrogel properties were shown to be dependent on the degree of CS grafting with MMP-Lys as well as concentrations and stoichiometric ratios between the components of the hydrogel system. Although CS-PEGs were shown to be degradable by chondroitinase, encapsulated human BM-MSCs mainly relied on MMP-mediated hydrogel degradation to remain viable, proliferate and migrate. Finally, CS-PEG hybrid hydrogels bound bone morphogenetic protein 2 (BMP-2) in a CS concentration-dependent manner. Such tunable BMP-2 releasing osteogenic environments supported robust osteogenic differentiation of embedded BM-MSCs, as shown by induction of their alkaline phosphatase (ALP) expression. CS-PEG hybrid hydrogels by their modular design could be used to flexibly integrate various available molecular tools and matrix properties to generate an unprecedented platform, which allows the rational design of next generation regeneration promoting materials.

2. Materials and Methods

2.1 Materials

CS, mol. wt. 50kDA (108 repeating units) was purchased from LKT Labs (St. Paul MN), N-(2-Aminoethyl) maleimide, trifluoroacetate salt (AEM), 2-(N-morpholino) ethanesulfonic acid (MES), N-(3-Dimethylaminopropyl)-N-ethylcarbodiimide hydrochloride (EDC.HCl), N-hydroxysuccinimide (NHS) were obtained from Sigma (Oakville, Ontario Canada). The earlier described matrix metalloproteinase sensitive factor XIIIa substrate peptides TG-MMP-Lys (mol. wt. 1717.9 g/mol), TG-Gln (mol wt. 1358.5 g/mol), and the adhesion ligand TG-RGD-Gln (mol wt. 1539.6 g/mol) were obtained from NeoMPS (Strasbourg, France) [38]. Eight-arm PEG-OH (8-PEG-OH, mol wt. 40000) was purchased from Nektar (Huntsville, AL). Divinyl sulfone was purchased from Aldrich (Buchs, Switzerland).

2.2 Synthesis of maleimide functionalized Chondroitin Sulfate

CS was dissolved in deionized water (1.0 g in 50.0 mL) and filtered to remove insoluble impurities. To remove the low molecular weight moieties the filtrate was dialyzed for 24 hours with periodic bath changes using dialysis

membranes (Spectrum Labs MWCO 3.5). The dialyzed sample was lyophilized for 48 h to obtain purified CS. The synthesis of CS functionalized with *n* groups of maleimide (CS_n-mal) was performed as described previously [39]. Briefly, 1.0g CS (2.14 mmol -COOH) was dissolved in 50.0 mL of MES buffer (0.1M, pH 6.5) followed by the addition of 409.8 mg of EDC (1 molar ratio to free -COOH), 246.0 mg of NHS (-COOH:NHS, 1:1) and 544.0 mg of AEM (-COOH:AEM, 1:1). The mixture was allowed to react overnight with constant stirring at room temperature. The product was then dialyzed using dialysis membranes (MWCO 3.5 kDa) first against 1M NaCl then against deionized water for 3 days. The freeze-dried sample was characterized by ¹H NMR (Bruker 400 MHz in D₂O) to determine the degree of functionalization using characteristic peaks at δ = 7.4 ppm (2H maleimide), 3.5-3.8 ppm (-CH₂-CH₂-NH-), and 5.3 ppm corresponding to proton 1 of the disaccharide unit. (Further details can be found in supplementary information).

2.2.1 Coupling of maleimide functionalized CS with TG-MMP-Lys peptide

Two hundred mg CS_n-mal were dissolved in 20.0 mL of triethanolamine (TEA) buffer (0.3 M, pH 6.5-7.5). In a separate vial a 1.2 molar excess (cysteine over maleimide) of TG-MMP-Lys was dissolved in 5.0 mL of TEA buffer and the two solutions were mixed, vortexed and incubated at 37°C for 3 hours with mild shaking. The solution was then dialyzed using MWCO 3.5 kDa dialysis membranes (Spectrum Labs.) for 4 days. The lyophilized material was characterized via ¹H NMR on a Bruker Avance 400 MHz spectrometer. The samples were prepared in deuterium oxide before spectra of CS and its derivatives were recorded at 80°C for better peak separation. To confirm complete conversion of maleimides, UV absorbance spectra of CS and its derivatives were analyzed (Supporting Figure S-4).

2.2.2 Synthesis of TG-PEG precursors

Eight-arm PEG precursors containing either the glutamine acceptor substrate (8-PEG-Gln) or a lysine donor substrate containing an MMP-sensitive linker (8-PEG-MMP-Lys) were produced and characterized as described previously [32].

2.3 Formation of CS-PEG hydrogels

Hydrogels were formed by FXIIIa mediated crosslinking of 8-PEG-Gln and CS_n-MMP-Lys. For this, FXIIIa (200 U/mL, CSL Behring, Switzerland) was activated with thrombin (2 U/mL, Sigma–Aldrich, Switzerland) for 30 min at 37 °C and stored in small aliquots at -80 °C. Precursor solutions consisting of varying concentrations and ratios of 8-PEG-Gln and CS_n-MMP-Lys were prepared leaving a spare volume of ~10% for the addition of RGD, growth factors or cells. If not stated differently the crosslinking reaction was performed in Tris buffer (50 mM, pH 7.6) containing 50 mM calcium chloride by the addition of 20 U/mL thrombin activated FXIIIa at 37°C. Hydrogel discs were obtained by injecting the reaction mixture between two silanized microscopic glass slides separated by silicone spacers (~1mm). The crosslinking reaction was allowed to proceed for 1 h under humidified atmosphere.

2.4 Swelling & Network Characterization

Immediately after crosslinking, hydrogels were frozen at -20°C, lyophilized and the initial dry mass (*w_{di}*) recorded. Then the hydrogels were soaked in Tris buffer (50 mM, pH 7.6) with a change of medium at least three times over a period of 24 hours to reach equilibrium. The swollen mass (*w_s*) of hydrogels was measured at 2, 4, 6, 12, 24 and 48

hours post incubation after gently wiping the surface with a dry paper towel. The swelling ratio Q_m was calculated by using formula w_s/w_d . The volumetric swelling (Q_v) was calculated from the mass swelling (Q_m) using the densities of polymers and Tris buffer [40].

$$Q_v = 1 + \frac{\rho_p}{\rho_s}(Q_m - 1) \text{ ---- (1)}$$

Where, ρ_p and ρ_s are densities of polymer and solvent used for gelation (water = 1.0 g/cm³). The density of the polymer blend was calculated by taking the percentage of the two polymers in precursor solutions: PEG (1.12 g/cm³) and CS (0.67 g/cm³) [40,41]. The network parameters like polymer volume fraction in swollen state ($v_{2,s}$), effective crosslink density (ρ_x), molecular weight between crosslinks (M_c) and network mesh (ξ) were evaluated using Q_v according to modified Flory-Rehner theory [42,43] accounting for the ionic character of the hydrogel components as described by Peppas et al. [44]. The detailed method and employed equations is provided in supplementary information.

2.5 Rheology of swollen hydrogels

Hydrogels were prepared as described above and equilibrated for 24 h in Tris buffer, pH 7.6 at 37°C. To determine hydrogel mechanical properties, storage modulus (G') and loss modulus (G'') were measured at 25°C in a small strain oscillatory shear with an Anton Par Rheometer (Buchs/AG, Switzerland) in parallel plate geometry and a 20 mm diameter upper plate. The gap was adjusted starting from the original swollen hydrogel height and compressing the sample to reach a normal 0.15 N. Gap sizes for swollen hydrogels were generally between 1.0 and 1.5 mm. An amplitude sweep (G' as a function of strain) was performed in order to confirm the linear viscoelastic regime and the measurements were conducted before shear thinning phase. Measurements were conducted at a frequency of 0.05 to 10 Hz and constant 2% strain to obtain mechanical spectra.

2.6 Fluorescent recovery after photobleaching (FRAP)

Stock solutions of FITC-dextran of 20, 150 and 2000 kDa (FD-20, FD-150 and FD-2000) were prepared at a concentration of 10.0 mg/ml. Hydrogels of different composition were formed in presence of 1.0 μ L of FITC-dextran stock solutions and equilibrated for 4 hours in Tris buffer containing same concentration of FITC-dextran. FITC-dextran loaded hydrogels were transferred to a microscopy slide and recovery curves were taken with a Leica SP5 confocal microscope with 20x magnification objective and argon laser at 488 nm. A bleach spot of 25 μ m diameter was selected for all measurements. The area was monitored by 10 pre-bleach image scans at laser intensity of 2-5% and bleached by 50 image scans at 100% laser intensity. The fluorescent recovery was detected by 200 post-bleach image scans (~500 ms interval) at low intensity. Further details of the FRAP analysis are described in supplementary information.

Diffusion coefficients for 3D samples were calculated as described previously by Brandl et al.[45]. In brief, normalized fluorescence intensity $f(t)$ values were further normalized to the full scale $F(t)$ using equation (2) where $f(\text{pre})$ is the normalized fluorescence intensity before bleaching and $f(0)$ the normalized fluorescence intensity immediately after bleaching.

$$F(t) = \frac{f(t)-f(0)}{f(pre)-f(0)} \text{ ---- (2)}$$

The mobile fraction k and the diffusion time τ_D were then obtained by least-squares fitting of equation (3) to the fully normalized experimental recovery curves where I_0 and I_1 are the modified Bessel functions of the first kind of zero and first order, respectively.

$$F(t) = k * e^{-\frac{\tau_D}{2t}} \left[I_0 \left(\frac{\tau_D}{2t} \right) + I_1 \left(\frac{\tau_D}{2t} \right) \right] \text{ ---- (3)}$$

The diffusion coefficient D was then calculated using equation 4 where w was the radius of the bleached area (25 μm).

$$D = \frac{w^2}{\tau_D} \text{ ---- (4)}$$

Free volume theory proposed by Lustig and Peppas [46] was employed to relate the diffusivity of incorporated molecules to the hydrogel network structure as:

$$D_g/D_o = (1 - r_H/\xi) \cdot \exp(-Y(v_{2s}/1-v_{2s})) \text{ ---- (5)}$$

In the equation Y represents the ratio of critical volume required for translational movement of the encapsulated molecule and the average free volume per solvent molecule. A good approximation of Y , for correlation purpose is unity [47]. Diffusion coefficients of FITC-dextran were estimated using this equation, based on determined values of mesh size (from swelling data) and are presented in Table 3.

2.7 Degradation of hydrogels

Hydrogels were equilibrated for 8 hours in chondroitinase buffer (0.02% BSA in 50 mM Tris, 60 mM sodium acetate and pH 8) according to manufacturer's specifications. After swelling each hydrogel was incubated in 1 mL buffer (50 mM Tris, 60 mM sodium acetate and 0.02% BSA) containing no or 2 mU/gel of chondroitinase ABC (Sigma Buchs, Switzerland). At different time points, the hydrogels were washed with ddH₂O and weight loss was measured. The kinetic activity of chondroitinase ABC was also monitored by quantifying the release of the chondroitin sulfate degradation product, which due to unsaturated disaccharide units shows an absorbance at 232 nm.

2.8 Cell encapsulation within hydrogels

If not indicated differently, hydrogels for cell encapsulation experiments were formulated in presence of 50 μM cell adhesion ligand TG-RGD-Gln. Cells were added to hydrogels precursor solutions prior to the addition of FXIIIa and vortexing. Hydrogels were allowed to polymerize for 1 hour at 37°C under humidified atmosphere and 5% CO₂ before they were immersed in cell culture medium.

2.8.1 Cell viability and proliferation

Bone-marrow-derived mesenchymal stem cells (BM-MSCs) were isolated according to a previously published protocol [48]. Bone marrow aspirates of healthy donors were obtained during surgical procedures on informed consent that is in accordance with the local ethical committee of University Hospital Basel (Prof. Dr. Kummer; approval data 26/03/2007 Ref Number 78/07). The cells were cultured in MEM α supplemented with FCS (10%), penicillin–streptomycin (1%), and bFGF (5 ng mL⁻¹). The viability of hydrogel-encapsulated cells was examined after 3, 7 and 14 days of culture using a LIVE/DEAD viability kit (Molecular Probes). At pre-determined time points media was removed, hydrogels were 3 times washed with PBS and incubated for 20 min in diluted staining solution of ethidium homodimer-1 (4 μ M) and calcein AM (2 μ M). The number of viable cells versus dead cells was assessed using ImageJ. The data is presented as mean $\pm$ SD.

The DNA content of cells was measured by CyQuant NF cell proliferation assay kit (Molecular Probes). At 0, 1, 3 and 7 days of culture period, hydrogels were washed with PBS and frozen at -80 °C until measurements. After thawing, the hydrogels were digested with collagenase A (2 mg/mL in PBS) for 10 min and released cells were stained with CyQuant dye for 1 hr at 37 °C. Fluorescent intensity was measured at 520 nm using a microplate reader.

2.9 Cell migration (time-lapse microscopy)

Cell migration of encapsulated cells within the hydrogels was conducted under high relative humidity at 37°C and 5 % CO₂. After gelation was completed, cell-laden hydrogels were equilibrated in cell culture medium overnight and placed at the bottom of 12-well culture plates before 2.0 mL of complete medium (α MEM, 10% FCS, 1% PenStrep and 5 ng/mL FGF) was added. Random positions were selected inside the hydrogels using an inverse wide field microscope (DM IRBE; Leica, Wetzlar, Germany), equipped with a motorized stage and focus, and a black and white camera. Cell migration and spreading were followed up to 24 hours at 10 min interval, by software controlled image acquisition.

Projections of 3D cell migration tracks were automatically obtained using the program CellTracker with the MATLAB GUI [49]. Analysis of migration was performed applying a correlated random walk model, as described previously by Raeber et al. and Ehrbar et al.[33,50]. In brief, this model assumes the exponential decay of cell speed in time with the characteristic time constant P, termed directional persistence time [51,52]. P and the root-mean-squared Speed (S) are thereby related to the mean-squared displacement of a cell $\langle d^2(t) \rangle$ during a time interval Δt by the following equation:

$$\langle d^2(t) \rangle = 2S^2P[t - P(1 - e^{-t/P})] \text{---- (6)}$$

Since no preferential migration direction was observed when plotting migrated distance against the migration direction in a polar plot (Fig. 6A), isotropic cell movement was assumed. Therefore, the cell speed calculated from the 2D projections could be corrected by a geometry factor of $\sqrt{3/2}$ in order to obtain an estimate of cell speed in 3D. Mean-squared displacements were calculated from the experimental cell tracking data using the overlapping interval method [53] and were also dimension-corrected to predict 3D displacements. To obtain an estimate of the persistence time of each cell, its dimension-corrected cell speed was then inserted into the random walk model

(equation 5) and fit to the dimension-corrected displacement data using a nonlinear least-squares regression. Persistence time values resulting from regression analysis with R^2 below 0.6 were excluded from the analysis.

2.10 Growth factor binding assay

To investigate the binding capacity of CS to proteins and growth factors in the hydrogels, BMP-2 was encapsulated within hydrogels at a concentration of 250 ng/mL. The hydrogels were then immersed in 1 mL of Tris buffer (50 mM, pH 7.6) containing 0.1 % BSA and incubated at 37 °C under a humidified atmosphere. During or after 7 days of incubation, the release of BMP-2 to the immersion solution (50 mM Tris pH 7.6 containing 0.1% BSA) was monitored by human BMP-2 ELISA development kit (Peprotech, NJ, USA) according to supplier's protocol. The release profile of BMP-2 was also studied at various time points till day 7 for (1)CS22/8PEG.

2.10.1 MSCs differentiation

MSCs (2×10^3 cells) were 3D encapsulated in hydrogels (20.0 μ L) and cultured in 1 mL cell culture medium (DMEM, 10% FCS) under standard cell culture conditions (humidified atmosphere, 5% CO₂ and 37°C). For osteogenic stimulation, the cell culture medium was complemented with 250 ng/mL BMP-2. New medium without BMP-2 was given every second day and samples were evaluated at day 7. To quantify ALP activity hydrogels were washed three times with PBS before they were digested with collagenase A to release encapsulated MSCs. Harvested MSCs were lysed with 500.0 μ L of cell lysis buffer for 30 min at room temperature. 50.0 μ L of p-nitrophenyl phosphate (NPP) was added to 50.0 μ L of cell lysate and incubated for 30 min at 37 °C in dark followed by addition of 100.0 μ L of stop solution. The absorbance (410 nm) was measured using a microplate reader.

2.11 Statistical analysis

All statistical analysis was undertaken using GraphPad Prism (v6.0). Viability and DNA quantification data was compared by two way ANOVA followed by Tukey's test with $n=3$ and with three internal replicates. Persistence time and speed mean values were compared by one-way ANOVA with MATLAB (R2013a, MathWorks Inc, USA) followed by a Tukey–Kramer post hoc test to judge statistical significance. A t-test was performed to compare BMP-2 binding and ALP activity within different hydrogels. A P-value of < 0.05 was considered significant.

3. Results

3.1 Synthesis of gel precursors

To allow the enzymatic FXIIIa mediated crosslinking of CS with PEG, both precursors have to be modified with enzyme specific substrate sequences (Fig. 1). Substrate sequences containing either an α -carboxamide group of glutaminy (Gln) residues or the ϵ -amino group of lysyl (Lys) residues and allowing the transglutaminase FXIIIa catalyzed formation of $\epsilon(\alpha$ -glutamyl)lysine isopeptide side-chain cross-links as well as a cysteine for their reaction with vinylsulfone or maleimide functionalized polymers have been earlier described [32,38].

The transglutaminase acceptor substrate containing peptide TG-Gln was grafted via the free thiol of its cysteine residue to 8-PEG-VS as earlier described [32,38] (Fig. 1A). ¹H NMR of peptide-conjugated PEG (8-PEG-Gln) indicates the disappearance of vinyl peak, which is evidence of reaction completion (Fig. S-3).

In order to graft TG-MMP-Lys peptides onto CS, in a first step a fraction of carboxyl groups (–COOH) of CS had to be functionalized with maleimide (Fig. 1B and Fig. S-1). For this, CS (1mmol; total concentration of –COOH) was reacted with EDC (1 mmol), NHS (1 mmol) and AEM (1 mmol). ¹H NMR spectra of the resulting maleimide functionalized CS (CS-mal) show a peak at 7.4 ppm (80 °C, 6.9 ppm at room temperature) corresponding to the double bond of maleimide and a peak at 5.25 ppm corresponding to proton-1 of a disaccharide unit allowing the determination of functionalization by comparing the integrals of the two characteristic peaks (Fig. S-3). The degree of maleimide substitution was determined by the integration of maleimide peak with respect to proton-1 peak of disaccharide unit. While the initial ratio of –COOH to AEM of 1:1 resulted in CS functionalized with 22 maleimides (CS22-mal) lowering the ratio to 1:0.8 resulted in CS functionalized with 12 maleimides (CS12-mal).

To obtain CS functionalized with 12 or 22 MMP-Lys peptides (CS12-MMP-Lys or CS22-MMP-Lys) CS12-mal or CS22-mal were reacted with a 1.2 molar excess TG-MMP-Lys peptide (reaction scheme and details of reaction are shown in Fig. S-1 and supplementary information). The complete disappearance of the maleimide peak (at 7.4 ppm) and the appearance of peaks between 7.7-7.9 ppm corresponding to aromatic protons (due to phenyl alanine and tryptophan residues) in NMR spectrum of CS-MMP-Lys indicate the successful grafting of TG-MMP-Lys peptide to CS-mal via Michael addition and the full consumption of maleimide groups (Fig. S-3). In order to further characterize the MMP-Lys functionalization, the complete absence of a characteristic UV absorbance peak of maleimide at 292 nm was confirmed (Fig. S-4).

3.2 Network properties of CS-PEG hybrid hydrogels

Hydrogels can take up significant amounts of water as a function of the precursor hydrophilic properties. However, this water uptake is counterbalanced by cross-linking mediated tensional forces, resulting from backbone architecture and cross-linking efficiency. Thus, in order to establish optimized CS-PEG hybrid hydrogel properties, parameters influencing cross-linking efficiency as well as backbone architecture were varied and influences on hydrogel swelling, mechanical properties and mesh size as well as diffusion coefficients were determined. Starting conditions were 50 mM CaCl₂, 50 mM Tris HCl, pH 7.6 and the presence of 10 U/mL FXIIIa, as earlier established for the enzymatic formation of low stiffness (1.5%) pure TG-PEG hydrogels [33]. We studied the network architecture in relation to i) degree of Lys functionalization, ii) stoichiometric ratio between the two reactive functionalities (Lys & Gln) of gel precursors and iii) total percentage of gel precursors.

3.3 Enzymatic formation of CS-PEG hybrid hydrogels

To optimize the needed FXIIIa concentration for the formation of CS-PEG hybrid hydrogels, stoichiometrically balanced (Lys:Gln) blends of CS22-MMP-Lys and 8-PEG-Gln precursor solutions were used. The nomenclature employed throughout this paper for such hydrogels will be: (1)CS22/8PEG, where the first digit within parenthesis

here (1) represents the molar ratio between Lys and Gln functional groups, the digit after CS (here 22) the degree of CS functionalization with MMP-Lys, and the digit before PEG (here 8) the number of Gln groups on PEG.

Under standard reaction conditions (1)CS22/8PEG hydrogels made with 6 wt% of precursors took 10 min to reach polymerization under equal FXIII concentrations. The resulting gels were not very stable and did not allow for studying the swelling properties (Fig. 2a). While in presence of 8% precursors, stable hydrogels were formed in 6 min on average. Therefore, to optimize the enzymatic formation of hydrogels, 8 % (1)CS22/8PEG hydrogels were formed in presence of 20, 30 and 50 U/mL FXIIIa (Fig. 2b and c). While increasing the FXIIIa concentrations from 10 to 20 U/mL resulted in the formation of stiffer hydrogels and also significantly increased the speed of cross-linking, usage of FXIIIa concentrations higher than 20 U/ml decreased the crosslinking time but had only a marginal effect on swelling and stiffness. Therefore throughout this study we kept the FXIIIa enzyme concentration for the crosslinking of hydrogels constant at 20 U/mL.

Figure 1: Factor XIII mediated CS-PEG hydrogel formation.

The functionalization of A) 8-PEG-VS with TG-Gln peptides resulted in 8-PEG-Gln and B) CS-maleimide with CS-MMP-Lys in CSn-MMP-Lys. C) Formulations of 8-PEG-Gln, CS-MMP-Lys and the cell adhesion ligand TG-Lys-RGD are crosslinked by FXIIIa in presence of cells to form 3D cellular microenvironments.

Figure 2: Effect of FXIIIa concentration on hydrogel formation. The onset of hydrogel polymerization is determined in response to a) initial concentration of hydrogel precursors b) concentration of FXIIIa addition. c) Shows there is no significant effect on equilibrium swelling ratio of hydrogels with increase in concentration of FXIIIa. Data represents mean $\pm$ SD for $n=3$, ** $p < 0.05$, *** $p < 0.001$ in two tailed t-test.

3.3.1 Effect of molar ratios of gel precursors

The functionality and accessibility of both reciprocal FXIIIa reactive groups on the hydrogel precursors can significantly influence the cross-linking efficiency and consequently hydrogel properties. Thus, the influence of molar ratios of reciprocal functional groups on swelling and mechanical properties of CS-PEG hybrid hydrogels was determined. Hydrogels were formed with Lys:Gln ratios of 1:1, 1.5:1 or 2:1 and swollen in Tris buffer before their swollen mass was determined and storage moduli were probed through shear rheology. Storage moduli and equilibrium swelling ratios are reported in Table 1a&b. It was found that 8 % hydrogels that were formed by stoichiometrically balanced precursor solutions of CS22-MMP-Lys and 8-PEG-Gln [(1)CS22/8PEG] have lower storage moduli of 348.57 ± 28.8 Pa as compared to hydrogels obtained with 0.5 molar excess of CS22-MMP-Lys [(1.5)CS22/8PEG] that showed moduli of 526.53 ± 47.4 Pa. However, further increasing the excess of CS22-MMP-Lys to 1 molar [(2)CS22/8PEG] again showed a decrease in modulus to 345.94 ± 10.6 Pa. This behavior is more prominent in 10 % hydrogels made from same proportions of the same precursors showing the maximum value of storage modulus at a 1.5:1 ratio [(1.5)CS22/8PEG] of 1122.08 ± 15.1 (Table 1a).

3.3.2 Effect of concentration of hydrogel precursors

In order to test the influence of total precursor concentrations on hydrogel properties, hydrogels with identical molar ratios were compared. CS-PEG hydrogels made with less than 8 % of total hydrogel precursors formed but disintegrated after 24 hours of incubation, indicating low crosslinking efficiency and the presence of non-covalent interactions which are slowly disrupted in aqueous buffers (data not shown). In contrast, both (1.5)CS12/8PEG and (1.5)CS22/8PEG hydrogels with total concentration of 8% precursors formed efficiently, remained stable and showed storage moduli of 371.5 ± 21.7 Pa (Table 1a) and 526.53 ± 47.4 Pa, respectively (Table 1b). Increasing the total precursor concentration from 8 to 10% resulted in hydrogels with higher stiffness, which for (1.5)CS22/8PEG

was more pronounced (113% increase) than for (1.5)CS12/8PEG (23% increase). The increase of stiffness was accompanied by a small and non-significant lower degree of swelling.

3.3.3 Effect of degree of functionalization of CS with Lys-peptide

The amount of absorbed water and the storage modulus of hydrogels are a function of the hydrogel chemical properties and the crosslink density. In order to test the influence of the degree of CS functionalization on CS-PEG hybrid hydrogel properties, CSs with two different densities of grafted MMP-Lys peptide were employed. In hydrogels with optimal (1.5:1) Lys : Gln ratios, increasing the number of MMP-Lys groups per CS molecule from 12 to 22 resulted in a significant increase in the storage moduli of 371.5 ± 21.7 Pa (Table 1a and b) to 526.53 ± 47.4 Pa (42% increase) for 8% hydrogels and 458.8 ± 46.7 Pa to 1122.1 ± 15.1 Pa (144% increase) for 10% hydrogels. The increase in mechanical properties due to higher functionalization was again reflected in significantly reduced hydrogel swelling.

3.3.4 Mesh size and Crosslink density

According to the Flory-Rehner theory, hydrogel network characteristics can be calculated based on equilibrium swelling and rheology data as well as on characteristic polymer parameters and network parameters (values indicated in Table 1 are based on parameters related to PEG, values based on parameter related to CS are given in supplementary info). These evaluations show that Lys:Gln ratios of 1.5:1 for all tested CS/PEG hydrogels result in smallest mesh sizes which can be further decreased by increasing the hydrogel concentration (Table 1a&b). However, the largest reduction of hydrogel mesh size from 37.2 ± 0.4 nm to 16.5 ± 0.4 nm (55% decrease) can be observed by increasing the degree of CS functionalization from 12 to 22 MMP-Lys peptides per molecule. Together this data indicate that among the analyzed CS/PEG hybrid hydrogel formulations tested, (1.5)CS22/8PEG at relatively low precursor concentrations (8%) could provide stable biomaterials with least hydrogel network defects for the 3D growth of cells. Thus subsequent biological functionality was mainly focused on this formulation.

Table 1a: Network properties of CS-PEG hydrogels based on CS12-MMP-Lys

% of gel precursors	8			10		
Molar ratio (Lys/Gln)	1.0	1.5	2.0	1.0	1.5	2.0
Swelling (Q_M)	47.3 ± 0.9	39.3 ± 1.8	41.4 ± 1.4	45.5 ± 0.5	38.3 ± 1.5	42.0 ± 1.3
Storage modulus (Pa)	224 ± 17	371 ± 21	201 ± 32	310 ± 28	458 ± 46	285 ± 33
Crosslink density ($\rho_x, \text{mol/cm}^3$) $\times 10^{-5}$	6.7	7.8	7.7	7.3	8.1	7.4
Molecular wt. between crosslinks M_c (g/mol)	16721 ± 209	14330 ± 546	14436 ± 447	15291 ± 206	13851 ± 212	15027 ± 13
Mesh size (ξ) (nm)	42.7 ± 0.4	37.1 ± 0.6	37.9 ± 0.8	39.4 ± 0.3	36.2 ± 0.4	38.9 ± 0.1

Table 2b: Network properties of CS-PEG hydrogels based on CS22-MMP-Lys

% of gel precursors	8			10		
Molar ratio (Lys/Gln)	1.0	1.5	2.0	1.0	1.5	2.0
Swelling (Q_M)	23.4 ± 0.7	18.9 ± 0.7	22.0 ± 1.0	20.8 ± 0.6	16.3 ± 0.5	20.2 ± 0.8
Storage modulus (Pa)	348 ± 28	526 ± 47	345 ± 10	708 ± 8	1122 ± 15	385 ± 12
Crosslink density (ρ_x , mol/cm ³) $\times 10^4$	1.2	1.7	1.4	1.5	2.2	1.5
Molecular wt. between crosslinks	$8936 \pm$	$6472 \pm$	$7538 \pm$	$7454 \pm$	$5086 \pm$	$7117 \pm$
M_c (g/mol)	476	744	596	216	179	428
Mesh size (ξ) (nm)	24.6 ± 0.9	19.5 ± 1.3	22.2 ± 1.2	21.6 ± 0.4	16.5 ± 0.4	21.0 ± 0.9

($\chi = 0.42$, Mr ~44, Cn = 8)

Q_M degree of swelling, M_c molecular weight between crosslinks

(χ - polymer-solvent interaction parameter, Mr- molecular weight of repeating unit, Cn- characteristic ratio of polymer)

3.4 Diffusion of macromolecules within hydrogel networks

To confirm the calculated mesh size of the CS-PEG hydrogels, diffusion coefficients (D) of hydrogel entrapped FITC-labelled dextran probes (FD-20, FD-150 and FD-2000 with molecular weights of 20, 150 and 2000 kDa) were determined by FRAP. Fluorescent species that were homogeneously distributed within the hydrogels were photobleached within a defined area and the fluorescent recovery was monitored as a function of time, as shown for a typical experiment in Fig. 4a. The resulting data was fitted using equation 3 to determine the diffusion coefficient of respective species (Table 2 and Fig. 3c). For both 8% hydrogels (1.5)CS12/8PEG and (1.5)CS22/8PEG the calculated mesh sizes of 31.1 and 18.1nm are larger than the hydrodynamic diameter of FD-20 (6.6nm) and fall within the same range or are even smaller than FD-2000 (53.78 nm). Diffusion coefficients of approximately $17 \mu\text{m}^2 \text{sec}^{-1}$ for FD-20 and $2 \mu\text{m}^2 \text{sec}^{-1}$ for FD-2000 indicate that in both hydrogel formulations FD-20 diffuses with relatively good efficiency whereas diffusion of FD-2000 is largely restricted. While for FD-150 with a hydrodynamic diameter of 17 nm relatively good diffusion was obtained in 8% (1.5)CS12/8PEG hydrogels, diffusion was almost absent ($D_0 = 4.9 \pm 0.7$) in 8% (1.5)CS22/8PEG hydrogels. In general, for the small FITC-dextran the measured diffusion coefficients (D_0) were smaller than the calculated diffusion coefficients (D_g), whereas for the large FITC-dextran (FD-2000) the opposite was observed.

Figure 3: Diffusion properties in (1.5)CS12/8PEG hybrid hydrogels. a) Confocal microscopic images of a FRAP experiment with FD-20, b) Full scale normalized fluorescent recovery ($f(t)$) of 20 kDa FITC-dextran (in gray) and fitted data using equation 3 (black). c) Diffusion coefficient (D_0) ($\mu\text{m}^2/\text{s}$) of FITC-dextrans measured by FRAP. Data shown is mean $\pm$ standard deviation (n=3).

Table 2: Diffusion coefficient ($\mu\text{m}^2/\text{s}$) and mobile fraction of FITC-dextran incorporated in CS-PEG hydrogels

FITC-Dextran	20 kDa		150 kDa		2000 kDa	
Lys functionalization	12	22	12	22	12	22
Estimated diffusion coefficient, D_g (calculated from mesh size) ²	58.6	51.9	18.8	13.5	1.1	--
Mobile Fraction (κ)	0.89 $\pm$ 0.05	0.79 $\pm$ 0.04	0.81 $\pm$ 0.04	0.85 $\pm$ 0.03	0.54 $\pm$ 0.04	0.28 $\pm$ 0.06

¹ Experimentally determined after swelling in Tris buffer (pH 7.6) for 24 hours.

² Calculated from average mesh size using equation 5

3.5 Degradation of hydrogels

The hydrogels are degradable by two enzymatic mechanisms, proteolytic through secreted MMPs by breaking peptides and sugar based degradation by breaking glycosidic bonds of disaccharides. In order to determine whether the modification with functional groups or the incorporation of CS into hydrogels would influence their susceptibility towards chondroitinase digestion, native or peptide modified CS and CS/PEG gels were degraded with chondroitinase. The resulting unsaturated disaccharide unit degradation products as continuously measured by UV absorbance at 220 nm indicated that native CS is faster degraded than CS-MMP-Lys (Figure S-5a). Although, accumulation of degradation measurable disaccharide units is significantly slower than from soluble CS precursors, these CS/PEG hydrogels are clearly degradable by chondroitinase (Figure S-5a).

An alternative method to determine the changes of hydrogel properties and their degradation is the evaluation of the hydrogel weight. While preformed hybrid hydrogels incubated in enzyme buffer did not degrade, the addition of chondroitinase leads to a dose-dependent degradation within 24 hours at 37 °C (Figure S-5b). Degradation experiments using different formulations of CS22/PEG hydrogels at low (2 mU per mL) chondroitinase concentration indicate that hydrogels with smaller pore size degrade slower than hydrogels with larger pore size. The initial increase of hydrogel mass followed by a continuous loss of mass indicates that digestion involves both bulk as well as surface erosion (Fig. 4).

Figure 4: Chondroitinase mediated degradation of CS/PEG hybrid hydrogels. CS/PEG hybrid hydrogels of identical initial weight (mg) but different ratios of CS22-MMP-Lys and 8-PEG-Gln were formed and incubated with (2 mU/mL conc.) Chondroitinase. Changes of the hydrogel weight were followed over 24 hours for hydrogels with a) 8% or b) 10% initial dry mass content. Data represents mean $\pm$ SD for n=3.

3.6 Cell viability and proliferation

To assess cell viability in various conditions of hybrid hydrogels, (1.5×10^6 cells/mL) BM-MSCs were encapsulated and cultured in MEM α including 10% FBS, 1% PenStrep and 5 ng/mL fibroblast growth factor 2 (FGF-2) for 3, 7 and 14 days before they were stained with Live/Dead cytotoxicity kit. After 2 weeks of culture fluorescence images taken along the diameter of the hydrogel and at a depth of 2/3 from the top surface show that approximately 90% of MSCs remained viable within the hybrid hydrogels. In contrast in hydrogels without RGD peptide the cell viability drops below 80% after the same period (Figure S7).

To evaluate cell proliferation, DNA content of BM-MSCs encapsulated in hydrogels was evaluated after 1, 3 and 7 days of culture. For all conditions upon BM-MSC encapsulation the DNA content increased by 3-5 fold between day 1 and day 7. Hydrogels made with 8 % dry weight of precursors (1.5)CS22/8PEG (Fig. 5a) and 10% dry weight of precursors (2)CS22/8PEG (Fig. 5b) showed maximum proliferation with 5 and 6 folds increase in DNA at 7 days, respectively. Together this data show that BM-MSCs remain viable and can proliferate upon encapsulation into CS-PEG hybrid hydrogels.

Figure 5: BM-MSCs proliferation in CS/PEG hydrogels. BM-MSCs were encapsulated within hybrid hydrogels and after 1, 3 and 7 days DNA content was measured by fluorescent intensity in a) 8% hydrogels, b) 10 % hydrogels. Data represents mean $\pm$ SD for n=3, ** p < 0.05, *** p < 0.001

3.7 Cell migration

To elucidate 3D cell migration behavior in CS-PEG hydrogels, BM-MSCs were embedded in 8% (1.5)CS22/8PEG hydrogels and were monitored for 24 hours by time-lapse microscopy (supplementary movies S-1, S-2 and S-3 showing, cell migration in (1)CS22/8PEG, (1.5)CS22/8PEG and (1.5)CS22/8PEG without RGD hydrogels respectively). Polar plots of overlaid cell tracks indicate that 3D BM-MSCs migration is taking place with directional independence and that BM-MSCs migrate $382 \pm 164 \mu\text{m}$ within 24 hours (Figure 6A). To further assess the mechanism of 3D cell migration occurring in CS-PEG hydrogels, the same experiment was performed in hydrogels lacking RGD peptides as well as in presence of the MMP inhibitor GM6001. Albeit the mean cell migration distance was slightly reduced to $321 \pm 163 \mu\text{m}$ in absence of RGD peptides, RGD presence clearly was not a prerequisite for 3D cell migration in these hydrogels. Since PEG polymers are biologically inert, this result suggests that BM-MSCs are able to adhere to the sugar molecules or the peptide grafted onto CS chain of the CS-PEG backbone. On the other hand, inhibition of MMPs by GM6001 led to almost complete repression of 3D cell migration with a mean cell migration of $105 \pm 100 \mu\text{m}$.

To further substantiate these findings, we quantified cell speed and persistence time by applying an unbiased random walk model [33,50]. The histograms of the speed of migrating cells showed a rather broad distribution for all conditions in 8% and 10% CS22/8PEG hydrogels (Figure 6B and Supplementary Figure S-6). The median value of cell speed in 8% (1.5)CS22/8PEG hydrogels was in absence of RGD peptides and presence of GM6001 reduced from $0.63 \mu\text{m min}^{-1}$ to $0.44 \mu\text{m min}^{-1}$ and $0.18 \mu\text{m min}^{-1}$, respectively (Figure 6C). Whereas in 8% (1)CS22/8PEG and 10% (1)CS22/8PEG hydrogels cell speed was similar, increasing the initial polymer concentration from 8% to 10% in (1.5)CS22/8PEG hydrogels resulted in a decrease of median cell speed from $0.63 \mu\text{m min}^{-1}$ to $0.50 \mu\text{m min}^{-1}$ (Supplementary Figure S-6). Histograms of the cell persistence time showed a positively skewed distribution for all conditions (Figure 6D and Supplementary Figure S-6). However, among the 8% (1.5)CS22/8PEG conditions a clearly broader distribution was seen for the standard CS-PEG hydrogel and the variant lacking the RGD peptide. Thus, there was no significant difference seen between these two conditions and they showed a similar median value

of persistence time of 24 and 28 min, respectively (Figure 6E). However, presence of GM6001 drastically lowered the persistence time and reduced the median value to 6 min.

Figure 6: Cell migration in CS-PEG hydrogels is MMP but not RGD dependent. A) Polar plots showing overlaid projections of 60 cell tracks with a common starting point. Tracks represent a period of 24 hours (72 x 20 min). Histograms depicting the cell population's cell speed (B) and persistence time (D). Cell speed (C) and persistence time (E) of all cells of a group depicted as boxplots ranging from the 25th to 75th percentile including the whiskers from the 10th to the 90th percentile. *, $p < 0.05$.

3.8 Growth factor binding and osteogenic differentiation of BM-MSCs

CS is known to bind various different growth factors by electrostatic interactions and thus can contribute to the regulation of tempo-spatial availability of growth factors in tissues. In order to modulate the binding efficiency and release of the osteogenic growth factor BMP-2 from CS, CS/PEG hybrid hydrogels were formed using decreasing amounts of CS22-MMP-Lys and in the presence of 250 ng of BMP-2. As controls fully synthetic TG-PEG hydrogels with corresponding dry weight of 1.5% were formed in presence of equal quantities of BMP-2 [32]. Hydrogels were incubated for 7 days in 1.0 mL of a physiological wash buffer and the amount of released BMP-2 was determined by ELISA-based quantification. Samples collected from the wash buffer of (1.5)CS22/8PEG hydrogels indicate that BMP-2 is continuously released over the 7 days of the experiment (Fig. 7a). After one weeks of incubation in wash buffer, hydrogels containing high concentrations of CS, for example (1.5)CS22/8PEG, still very efficiently retained BMP-2 (ca. 70% of the initial amount) (Fig. 7b). Reducing the amount of CS in hydrogels resulted in decreased binding and in pure TG-PEG hydrogels bound BMP-2 was almost negligible (10%).

Since the binding of BMP-2 to CS-PEG hybrid hydrogels could be controlled by varying the concentration of CS, this system could serve as a tunable growth factor repository for *in vitro* and *in vivo* applications. Therefore, we aimed at demonstrating the ability of CS-PEG hybrid hydrogels to bind and provide biologically active BMP-2 to cells, which are in contact with or invade the hydrogel. It has been shown that BMP-2 promotes the osteogenic differentiation of BM-MSCs, which can be readily quantified by the expression of the early osteogenic marker alkaline phosphatase (ALP) [54]. To test the hydrogel assisted provision of BMP-2, BM-MSCs were encapsulated in 20 μ L (1.5)CS22/8PEG or pure TG-PEG hydrogels which contained 0 or 250 ng BMP-2. Hydrogels were cultured for 7 days in 1 mL of BMP-free differentiation medium, which was exchanged every other day. Additionally, control hydrogels were cultured in 1 mL differentiation medium that contained 250 ng BMP-2 and was also exchanged every other day. Measurements of ALP activity showed that ALP activity in (1.5)CS22/8PEG or pure TG-PEG hydrogels containing no BMP-2 is minimal (Figure 7c). Consistent with a sustained release, BM-MSCs differentiated more efficiently when encapsulated in (1.5)CS22/8PEG than in pure PEG, that both were formed in presence of BMP-2. Interestingly, while BMP-2 provided in the cell culture medium could efficiently promote the ALP activity of encapsulated BM-MSCs in TG-PEG hydrogels, in (1.5)CS22/8PEG hydrogels this response was largely blocked.

Figure 7: CS mediated BMP-2 binding and presentation. a) Cumulative release profile of BMP-2 in (1)CS22/8PEG as representative hydrogel. b) Amount of bound BMP-2 encapsulated within hydrogels, 7 days post incubation in Tris buffer measured by ELISA. The data is compared with PEG hydrogels without CS blending. c) MSC differentiation within hydrogels investigated by measuring absorbance at 410 nm using *p*-nitrophenyl phosphate, as a product of ALP activity. Data represents mean $\pm$ SD for $n=6$, ** $p < 0.05$, *** $p < 0.001$ in two tailed t-test.

4. Discussion

We report on the development of a modular designed, enzymatically cross-linked CS-PEG hybrid hydrogel platform, which enables the flexible tailoring of hydrogel properties towards chondroitinase or protease degradability as well as mechanical, cell adhesion and growth factor binding properties. This platform builds on a FXIIIa catalyzed glutaminating reaction, which has previously been shown to allow the cross-linking of glutamine acceptor (NQEVSPL) and lysine donor (Ac-FKCG) functionalized PEG precursors to form fully synthetic fibrin mimetic TG-PEG hydrogels under physiological buffer conditions. To substitute or supplement the backbone of PEG-based hydrogels with CS, we modified parts of the abundant $-\text{COOH}$ groups (108 per molecule) of CS with maleimide by earlier described approaches using EDC coupling chemistry [55]. The initial feed ratio between $-\text{COOH}$ and AEM controlled the different degree of CS functionalization with maleimide (CS12-mal or CS22-mal) as shown by

quantification via integration of maleimide and proton 1 of the disaccharide unit specific peaks at 3.5-3.8 and 5.3 ppm respectively by NMR. At physiological pH, polymeric Michael acceptors bearing a maleimide functionality were earlier shown to couple significantly faster with sulfhydryl groups than with amine nucleophiles, resulting in the efficient formation of non-reversible thioester bonds [56]. Here the reaction of CS-mal with 1.2mol excess Lys-peptides resulted in the formation CS12-MMP-Lys or CS22-MMP-Lys, which are devoid of any residual maleimide groups as confirmed by NMR analysis (Fig. S-3).

In order to test the ability of Lys functionalized CS to act as a substrate for FXIIIa, blends of CS22-MMP-Lys and 8-PEG-Gln containing equimolar concentrations (80 μ mol) of Gln and Lys groups were cross-linked under reaction conditions which were earlier optimized for soft (20 nmol Lys and Gln containing) TG-PEG hydrogels (Fig. 2). Although the use of hydrogel precursors with higher functionalization is expected to result in the formation of hydrogels with higher cross-link density [57], the highly functionalized CS22-MMP-Lys in combination with 8-PEG-Gln resulted in CS-PEG hybrid hydrogels with only very weak mechanical properties. The need of higher concentrations of FXIIIa for the fast formation of stable hydrogels indicates that either the availability or the efficiency of the enzyme in the CS containing reaction mixture is compromised (Fig. 2). It is therefore likely that temporary binding of the enzyme to sulfate groups of CS is partially responsible for the inefficient reaction. The minor increase in swelling as a results of higher FXII concentration might be due to the fact that crosslinking reaches saturation and any additional amount of FXIII would only decrease the polymerization time and doesn't have stronger effect on swelling.

Additionally, experiments using different molar ratios of Lys and Gln functional groups provided by CS-MMP-Lys and PEG-Gln show that 1.5 fold excess of Lys leads to CS-PEG hydrogels with higher cross-link density than both 2-fold excess or identical concentrations of Lys (Table 1a&b). Similar observations were earlier made with PEG hydrogels made of PEG-VS and bi-cysteine oligopeptide linkers. These hydrogels were most efficiently formed in presence of 25% excess of thiol groups over vinyl-sulfone groups [58]. However, in contrast to uncharged, star-shaped PEG, CS is a negatively charged linear polymer [59]. Therefore, both the architecture as well as the high negative charge could affect the accessibility of grafted positively charged Lys substrates due to sterical hindrance or electrostatic interactions, leaving unreacted groups and thus contributing to the formation of network imperfections and consequently weaker hydrogels. Furthermore, differences in backbone architecture could also be reflected in reduced numbers of entanglements, which especially at higher polymer concentrations could contribute to the cross-link density of hydrogels [16].

Differences in swelling properties are due to both polymer dry mass and crosslinking density. As can be seen throughout the experimental conditions (Table 1 a & b), hydrogels with a 0.5 fold excess of CS-MMP-Lys over Gln-PEG swell less than hydrogels with higher or lower excess. Using a constant total polymer concentration to form the hydrogel, this is rather expected, given the assumption that both CS and PEG have a similar capacity to take up water. However, increasing the concentration of polymer precursors from an initial concentration of 8% to 10% rather results in a moderate decrease of hydrogel swelling, for example in (1.5)CS22/8PEG hydrogels. This decrease in swelling can be explained by a large increase in the crosslinking density from 1.7 to 2.2 and can also be appreciated from large increases in storage moduli, decrease in molecular weights between the crosslinks and mesh size.

Hydrogel network properties are strongly influencing the diffusion of molecules as well as the behavior of cells both *in vitro* and *in vivo* [60]. It can for example influence the distribution of signaling molecules or ECM components. It has been reported that loosely crosslinked hydrogels resulted in the greater production of ECM of encapsulated chondrocytes by allowing diffusion of ECM components [61,62]. Likewise the diffusion of signaling molecules such as growth factors is affected by the hydrogel mesh size [63–65]. CS with a MW of 50 kDa and 108 available –COOH groups provides means to generate hydrogel precursors with different degrees of functionalization, while chemical properties are minimally changed. Therefore, the combination of CS and PEG precursors that are grafted with reciprocal substrate sequences for FXIIIa provides the possibility to form a broad spectrum of CS-PEG hybrid hydrogels, which contain variable molar ratios of CS and PEG building blocks as well as different hydrogel architectures. In agreement with earlier studies on different hydrogel materials [66], increasing the degree of CS functionalization as well as initial hydrogel precursor concentrations results in CS-PEG hydrogels with higher stiffness. In order to determine mesh size, crosslink density and molecular weight between crosslinks of hydrogels, information on hydrogel architecture and solvent interactions together with equilibrium degree of swelling and shear modulus can be applied in Flory-Rehner theory based equations.

For our hybrid hydrogels consisting of precursors with very different architecture and chemistry we conducted calculations using values either for CS or PEG precursors. These values of polymer-solvent interaction parameter (χ), characteristic ratio of polymer (C_n), molecular weight of precursor (M_n), the molecular weight of repeating units and bulk density of dry polymer for PEG and CS precursors are given in supplementary information. For all values derived from calculations considering CS, ionic contribution from CS acidic groups as well as ionic strength and nature of ions present in the gelation medium was considered in contrast to the neutral nature of PEG precursor. Mesh size values based on equilibrium swelling measurements and using either parameters specific for PEG or CS are significantly different, demonstrated strong dependency on the nature of hydrogel backbone. Also mesh size values based on the parameters of both gel precursors are significantly different, but it is assumed that the actual values should be intermediate which was confirmed by diffusivity of fluorescently tagged biomolecules via FRAP.

In FRAP experiments with (1.5)CS22/8PEG hydrogels and 8% initial precursor concentration with a calculated mesh size of 19.5nm (Table 1a) the model molecule FD-20 with a hydrodynamic diameter of 6.6 nm showed fast diffusion and a large mobile fraction, while for FD-150 and FD-2000 with a hydrodynamic diameter of 17 nm and 53.78 nm, respectively diffusion was largely inhibited (Fig. 3c). In (1.5)CS12/8PEG hydrogels with a calculated mesh size of 37.1 nm FD-150 showed high diffusion efficiency while for FD-2000 diffusion was largely inhibited, indicating that the by Flory-Rehner based calculated mesh sizes as shown in Tables 1a&b are in the correct range.

Although CS-PEG hydrogels were shown to be degradable by chondroitinase (Fig. 4), degradation studies with chondroitinase suggest that the degradability of CS upon Lys functionalization and hydrogel formation become less efficient (suppl Fig. S-5a & b). However, this effect could likely be due to sterical restrictions, experiments supporting this notion have not been conducted. MMP-Lys peptides in the context of PEG hydrogels have been shown to be degradable with MMP-1.[28] Earlier studies have shown that cells encapsulated at low density in hydrogel biomaterials with mesh sizes in the low nanometer range can spread, migrate and survive only if hydrogel bonds are being actively degraded by cell-mediated proteolysis [50,67]. Consistent with these studies BM-MSCs encapsulated in CS-PEG hydrogels spread within a few hours after seeding, remained viable (suppl Fig. S-7) and

even proliferated over the course of 3D culture (Fig. 5). Cell migration studies conducted during the initial 24 hours of encapsulation indeed confirmed that in CS-PEG hybrid hydrogels BM-MSCs migrate in an almost exclusive MMP dependent manner and with a comparable speed as earlier described for pure PEG hydrogels [33] (Fig. 6). Interestingly, in CS/PEG hybrid hydrogels the availability of the prototypic integrin ligand RGD improves cell behavior but is no longer absolutely required to establish 3D cultures. Indeed CS has been shown to have selective protein binding resistance resulting in low blood platelet adhesion, while at the same time it promoted the adhesion of endothelial cells as well as mesenchymal cells on CS coated surfaces in presence of FBS [68,69]. These studies indicate that such cell-material interactions could be mediated by CS-adherent FBS derived protein components. However, the direct cell-surface receptor based recognition of the CS, comparable to interactions of HA with the cell surface receptor CD44 cannot be excluded. Additionally, cell secreted matrix component, which by ionic and hydrogen bonding interactions can bind to the abundant number of negatively charged sulfate groups as well as MMP-Lys or Gln peptides grafted onto CS or PEG precursors could also contribute to the observed cell migration behavior. However, the exact mechanisms of cell attachment and matrix deposition have not been evaluated in this study and need further investigation.

Chondroitin sulfate has been reported to interact with a variety of growth factors such as FGF [70], hepatocyte growth factor (HGF) [71] and BMP-4 [20] and thus to be involved in various cellular processes, such as cell proliferation, differentiation, morphogenesis and mineralization of bone. To investigate the binding capability of CS components in CS-PEG hybrid networks we utilized BMP-2 as a model growth factor and its binding to CS chains was demonstrated via assessing its release profile using ELISA. Our data demonstrates that CS-PEG hybrid hydrogels can be tailored towards their binding capacity for BMP-2 by varying the molar ratio of CS and PEG hydrogel precursors (Fig. 7b).

Since CS-PEG hybrid hydrogels can mimic growth factor binding properties of naturally occurring ECMs we aimed at showing their contribution to controlling the sustained delivery of BMP-2 for osteogenic cell differentiation. By forming hydrogels in presence of BMP-2 and BM-MSCs and culturing them under standard culture conditions we demonstrate that CS-bound BMP-2 is made available to the encapsulated cells and promotes their osteogenic differentiation (Fig. 7c). It should be noted that differentiation efficiency for CS containing hydrogels seems to be inversely related to BMP-2 binding affinity and hydrogel cross-link density. Since hydrogels are only partially degraded during this short time of culture it can be assumed that, dependent on the amount of remaining matrix-immobilized CS, BMP-2 is retained within the hydrogel and thus not made available to the encapsulated cells. These findings are consistent with our previous study where the availability of biotinylated BMP-2 in streptavidin modified PEG hydrogels was dependent on BM-MSC mediated hydrogel degradation [36].

Earlier GAG mimicking biomaterials have mostly taken advantage of heparin sulfate (HS) or hyaluronic acid (HA) precursors, which were cross-linked by chemical means or by affinity interaction or combinations thereof. While HA due to its specific binding to CD44 and its great flexibility for engineering was an ideal starting material, the lack of sulfate groups made its combination with HS necessary to generate hydrogels with growth factor binding properties [70]. Despite its low molecular weight and consequently restricted possibility for modifications, HS has been successfully used to generate growth factor releasing hybrid hydrogels with PEG, which could be modified towards the binding of growth factors by controlling the degree sulfation [71]. By uniting the major advantages of HA and

HS, such as the large number of repeating units and the inherent sulfate group mediated growth factor binding properties, CS provides an ideal starting point for the tailoring of materials towards specific application by adapting their architectural and chemical properties. Thus the generation of CSs serving as substrates for FXIIIa mediated cross-linking combines several advantages: i) building blocks with great variability of architecture and chemical properties can be combined ii) available engineered building blocks derived from PEG hydrogels can be employed iii) hydrogel components can be modularly assembled and enzymatically cross-linked under mild physiological conditions which are highly compatible with biological systems.

Conclusions

We describe an enzymatically formed, modular designed nano-porous hybrid materials platform, which mimics both GAG and protein based properties of the naturally occurring ECM by integrating CS and PEG based hydrogel precursors. We show that the degree of functionalization, the molecular ratio of hydrogel precursors and initial precursor concentrations can be employed to tune the hydrogel network properties. Additionally the formulation with different concentrations of CS can be employed to tune growth factor binding and release in order to generate cell instructive matrices which can be specifically tailored to promote tissue healing and regeneration.

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