Published in final edited form as:
Biomacromolecules. 2011 March 14; 12(3): 560–567. doi:10.1021/bm100980w.

Biodegradable and Biocompatible Synthetic Saccharide-Peptide Hydrogels for Three-Dimensional Stem Cell Culture

Kanika Chawla[†], **Ting-Bin Yu**[†], **Sophia W. Liao**[‡], and **Zhibin Guan**[*,†,‡]

[†]Department of Chemistry, University of California, 1102 Natural Sciences 2, Irvine, California 92697-2025

[‡]Department of Biomedical Engineering, University of California, 3120 Natural Sciences 2, Irvine, California 92697-2715

Abstract

Saccharide-peptide hydrogels have been developed in our laboratory as new synthetic extracellular matrices for regenerative medicine applications. In this work, we have expanded on our previously reported system and applied copolymerization of cysteine (Cys) and vinyl sulfone (VS)-functionalized saccharide-peptide polymers via Michael-type addition for encapsulation and three-dimensional (3D) culture of cells. Specifically, our aims were to (1) develop a novel hydrogel platform which could be applied for encapsulating and culturing mesenchymal stem cells (MSCs) in a 3D environment, (2) characterize the tunable properties of the hydrogel, specifically, degradation, mechanical, and gel network properties and (3) determine the biocompatibility of the saccharide-peptide hydrogel material with MSCs. Hydrogel mechanical properties were tunable by varying VS:Cys ratio (= 0.5, 1, or 2) as well as the pH (6, 7, or 8) of the cross-linking components. Stiffer gels were formed at VS:Cys = 1 and pH 6 or 7. Gels formed at pH 8 or with excess Cys (VS:Cys = 0.5) or VS (VS:Cys = 2) were significantly softer. Cross-linking pH and VS:Cys ratio also had an effect on the degradation behavior of the VS:Cys gels, with higher cross-linking pH resulting in an accelerated loss of mass. Based on Environmental Scanning Electron Microscopy (ESEM) analysis, all hydrogels appeared to be porous gel networks. MSCs cultured in monolayer and exposed to soluble Cys or VS copolymers (0.1–5 mg/ml) did not exhibit measurable cytotoxicity. In addition, MSCs were cultured in 3D for up to 14 days *in vitro* without deleterious effects on cell viability. In summary, we have established and characterized a tunable 3D saccharide-peptide hybrid copolymer hydrogel platform for culturing MSCs. Future studies will focus on utilizing the hydrogel system for controlling the differentiation of MSCs.

Keywords

hydrogel; saccharide-peptide copolymer; cell encapsulation; three-dimensional culture; mesenchymal stem cell

INTRODUCTION

Tissue engineering and regenerative medicine offer widespread opportunities for developing new therapies for treating disease. Adult bone-marrow derived mesenchymal stem cells (MSCs) may be obtained from a patient, isolated, and culture-expanded *in vitro*. Cells

[*] zguan@uci.edu .

Figures with essential color discrimination Certain figures in this article, particularly Figures 2, 3, and 5, are difficult to interpret in black and white. The full color images can be found in the online version at doi: XX.

cultured *in vitro* within artificial matrices in the form of extracellular mimicking hydrogels or scaffolds may be subjected to various stimuli (biochemical, biophysical) in order to coax a specific cell lineage. Eventually, these three-dimensional (3D) constructs may be transferred *in vivo* as implantable tissues for treatment of disease or injury.

Several cell sources, in conjunction with a variety of biomaterials, have been explored for tissue engineering and regenerative medicine applications. These include progenitor or stem cells from bone marrow[1], periosteum[2], and fat[3,4]. The regenerative potential of these cell types has been investigated with both natural materials, such as fibrin[5] and polysaccharides like hyaluronic acid[6] and alginate[7,8], as well as synthetic materials, such as polyethylene glycol (PEG)[9–12] and other polymer[13–16], protein-engineered[17], or peptide amphiphile based systems[18–20].

Culture of mammalian cells, including mesenchymal stem cells (MSCs), provides an opportunity for mechanistically identifying epigenetic factors associated with cell fate processes. In general, studies of cell culture conducted on 2D substrates, such as tissue culture plastic or biological substrates, have indicated the critical role of cellular microenvironment in influencing cell function[21,22]. Removing cells from their native 3D environment and culturing in 2D however, also has significant aberrant effects on cell migration[23], proliferation[23,24], and differentiation[24,25]. In addition, application of 3D cell culture has increased relevance to the *in vivo* environment[26,27]. Such 3D biomaterial environments could also be specifically tailored to generate “synthetic” ECMs which would provide specific biophysical and biochemical cues to encapsulated cells.

Trends have been moving toward synthesizing hybrid systems consisting of both natural and synthetic components. Ideally, the natural component will provide instructional cues to encapsulated cells, while the synthetic analog will provide desired mechanical properties. Saccharide- or peptide-based polymers are a common source for the natural component since they are naturally found in the body[6,28–30]. However, extracted proteins and polysaccharides from animals can be expensive and have batch-to-batch variations[8,31]. In addition, the complex structures of natural biopolymers have often limited their design versatility for fine structural modification to tailor specific functions.

We previously have reported the design and investigation of a novel sacchride-derived side-chain ether polymer as a protein-resistant material[32–34] and cationic saccharide–peptide hybrid copolymers as vectors for plasmid DNA transfection[35]. More recently, we have developed a family of hydrogels derived from anionic saccharide–peptide hybrid copolymer-based hydrogels derived from naturally occurring saccharides and amino acids[36]. In our design, we have chosen lysine and galactaric acid as the basis for our hybrid copolymer backbone due to their biocompatibility and physiologic relevance to various glycoproteins present *in vivo*[37 38 39]. Previously, we reported tunable mechanical properties of tyrosine functionalized saccharide-peptide hydrogels by controlling the time for cross-linking. While we were able to correlate certain cell functions with gel stiffness, application of this hydrogel system may be limited to 2D studies due to the need for hydrogen peroxide in the hydrogelation process[36].

In this work, we have expanded on our previously reported system and achieved mild hydrogelation for application of 3D cell culture, through copolymerization of cysteine and vinyl sulfone saccharide-peptide polymers employing Michael-type addition[14,40–44] (Fig. 1). While this type of chemistry has been applied previously to generate PEG-peptide[45,46], PEG-hyaluronic acid (HA)[47,48], or PEG-dextran gels[49], to our knowledge, this is the first study to apply Michael-type addition to form biodegradable synthetic hydrogels primarily composed of natural building blocks without a large synthetic component such as PEG. In

this study, our aims were to (1) develop a novel hydrogel platform which could be applied for encapsulating and culturing MSCs in 3D, (2) characterize the tunable properties of the hydrogel, specifically, degradation, mechanical, and gel network properties and (3) determine the biocompatibility of the saccharide-peptide hydrogel material with MSCs. Compared to other hydrogel systems, the saccharide–peptide hydrogels described here may offer the following advantages: (1) they are mainly composed of natural building blocks and will eventually degrade into natural, nontoxic, and bioabsorbable metabolites; (2) the hydrogels form under mild conditions by copolymerizing Cys and VS-functionalized saccharide-peptide polymers in culture medium; and (3) the hydrogels have the potential to be further functionalized in order to provide biophysical and biochemical cues to encapsulated cells and precisely control cell function and fates.

MATERIALS AND METHODS

Synthesis and characterizations of (4S,4′R,5R,5′S)–2,2,2′,2′–tetramethyl–4,4′–bi(1,3–dioxolane)–5,5′–dicarbonyl dichloride (1)

The synthesis is illustrated in Scheme 1. Compound **1** was synthesized by following our reported procedures[34].

Synthesis and characterizations of protected galactaric acid–lysine copolymer (2)

A solution of L–lysine–ethyl ester dihydrochloride (8.53 g, 34.5 mmol) and Na_2CO_3 (8.04 g, 75.9 mmole) in water solution (105 mL) was added dropwise to a solution of **1** (13.81 g, 34.5 mmol) in 690 mL of toluene cooled with an ice bath. This mixture was allowed to stir vigorously for 10 min. The solid was filtered and sequentially washed with water, sat. $NaHCO_3$, and finally hexane. The residue was dried in *vacuo* overnight to give 7.09 g of copolymer **5** (48%). ^{1}H NMR (600 MHz, DMSO–d_6) δ 8.27–8.00 (m, 2H), 4.54–4.19 (m, 5H), 4.41–4.02 (m, 2H), 3.16–3.03 (m, 2H), 1.74–1.73 (m, 2H), 1.49–1.04 (m, 16H). The number average molecular weight (M_n) of the polymer is 1.81×10^4 g/mol and weight number average molecular weight (M_w) is 2.69×10^4 g/mol based on GPC characterization using PEG as standards. The molecular weight of polymers was determined by gel permeation chromatography (GPC, Agilent 1100 Series, GPC–SEC Analysis System) using a mixed bed Plgel Mixed–C column from Polymer Labs. The eluent was DMF with 0.1% LiBr and a flow rate of 1.0 mL/min was used. The calibration was performed using poly(ethylene glycol) (PEG)–based molecular weight standards.

Hydrolysis of ester side chain for the copolymer (3)

To copolymer **2** (4.72 g) in (50 mL/ 2 mL) THF/Water solution, LiOH (4.0 eq, 1.85 g) was added. Then the reaction mixture was allowed to stir at room temperature overnight. The solution was concentrated in *vacuo* and added with hydrochloric acid (2 N) to acidify the solution. The resulting precipitate was filtered, washed with water thoroughly, and dried in *vacuo* providing 4.0 g of white solid polymer **6** (90%). ^{1}H NMR (600 MHz, DMSO–d_6) δ 8.06–7.72 (m, 2H), 4.54–4.19 (m, 5H), 3.13–2.99 (m, 2H), 1.73 (s, 2H), 1.41–1.25 (m, 16H).

Synthesis and characterizations of cysteine (Cys) functionalized saccharide–peptide copolymer (4)

To a solution of polymer **3** (12.89 mmole of carboxylic acid, 5.16 g) and Cysteine(Trt)-NH_2 (0.50 eq, 6.45 mmole, 2.336 g) in 35 mL of DMF, DIPEA (19.33 mmole, 3.37 mL) and HCTU (2-(6-Chloro-1Hbenzotriazole-1-yl)-1,1,3,3-tetramethylaminium hexafluorophosphate) (1.86 mmole, 0.710 g) were added. The reaction mixture was allowed to stir at room temperature for 16 h, at which time the DMF was removed in vacuo. 150 mL of 2N HCl was added into residue to generate a white suspension in aqueous solution. The

solid was filtered out and washed with 0.2N HCl to yield a white polymer. Then the protected polymer was further dissolved in a mixture of TFA (15.0 mL), H_2O (4.5 mL) and triisoproylsilane (0.5 mL) solution to remove protecting groups. After stirring the reaction mixture for 3 h, the solution was diluted with H_2O and purified by dialysis against 0.01N HCl for 3 days. Excess H_2O was then removed by lyophilization to give 3.29 g of pale brown solid polymer **4** (69%). 1H NMR integration of the polymer's cysteine β-protons revealed 1.27 mmole cysteine (thiol group) per gram of polymer. 1H NMR (600 MHz, D_2O) δ 4.69–4.38 (m, 3.5H), 4.05–4.04 (m, 2.0H), 3.32–3.28 (m, 2.0H), 3.03–2.90 (m, 0.94H, β-proton of cysteine), 1.97–1.86 (m, 2H), 1.60–1.44 (m, 4H).

Synthesis and characterizations of vinyl sulfone (VS) functionalized saccharide–peptide copolymer (6)

The protecting acetonide groups on copolymer **3** were first removed by TFA, leaving in their place free hydroxyl and carboxylic acid groups (polymer 3). To a solution of polymer 3 (6.24 mmole of carboxylic acid group, 2.00g) in PBS buffer (pH 8.0), divinyl sulfone (20eq, 124.8 mmole, 12.6 mL) and 10N NaOH (0.15eq, 0.0936 mL) were added. After stirring the reaction mixture at room temperature for 16 hours, the solution was afterwards diluted with H_2O, washed with Et_2O, and purified by dialysis against H_2O for 3 days. Excess H_2O was then removed by lyophilization to give 1.90 g of white solid polymer **6** (here after referred to as VS). 1H NMR integration of vinyl groups on the polymer revealed 1.54 mmole vinyl sulfone group per gram of polymer. For the purpose of viability studies (later described), the procedure above was repeated with 0.10eq of 10N NaOH to give 0.98 mmol/g VS copolymer. 1H NMR (600 MHz, D_2O) δ 7.00–6.95 (m, 0.68H, vinyl group), 6.54–6.36 (m, 1.36H, vinyl group), 4.48–3.18 (m, 12.4H), 1.89–1.78 (m, 2H), 1.60–1.41 (m, 4H).

Preparation of VS:Cys hydrogels

Hydrogels with VS:Cys molar ratios of 0.5, 1, or 2 were prepared by dissolving VS and Cys copolymers at a concentration of 100 mg/ml in Dulbecco's Modified Eagle's Medium (DMEM). Using a pH microprobe (Fisher Scientific, Pittsburgh, PA), the copolymer solutions were adjusted separately to pH 6, 7, or 8 by addition of the necessary amounts of HCl or NaOH prior to gelation. VS and Cys copolymer solutions were subsequently crosslinked through Michael-type conjugate addition (scheme 1), as previously reported[14,40, 43,44,45], in 6.5 mm diameter Transwell® inserts (Fisher).

Swellability of Hydrogels

Equilibrium swelling ratio (ESR), a measure of swellability of the hydrogels, was measured in DMEM at 37°C. Hydrogels were formed in triplicate and weighed wet (W_o). Dried hydrogels were weighed (W_d) after being frozen at −80°C and lyophilized. ESR was determined from the following equation:

$$ESR = \frac{(W_o - W_d)}{W_d}$$

Degradation Behavior of Hydrogels

The swollen ratio of the gels was tracked over 28 days *in vitro*. Gels, in triplicate, were incubated in DMEM with 10% FBS. The wet weight of the gel at time t (W_t) was normalized to the initial wet weight of the gel (W_o). Gels in Transwell® inserts were dabbed prior to weighing.

Measurement of Gelation Time of Hydrogels

In situ dynamic rheometry can be applied to determine the gelation point of hydrogel materials, by monitoring the crossover point of G' (storage modulus) and G" (loss modulus) during rheological measurements. In the present study, crosslinking and subsequent gelation of VS and Cys copolymer occurred very quickly, and in some cases, during the pre-equilibration portion of the rheological time sweep test. Consequently, we applied a previously described method for determining gelation time. Briefly, the two copolymers components (VS and Cys) were combined and pipette up and down until a sol-gel transition was completed and the solutions were unable to be pipetted up and down[50]. This is also similar to determination of gelation time by the vial inversion method[48,51]. Gelation times were determined in triplicate for each group.

Hydrogel Morphology Analysis

Microstructure of VS:Cys gels were examined by Environment Scanning Electron Microscopy (ESEM, Zeiss EVO LS 15 microscope). The environmental capability allowed for examination of the pore structure of the gels in a saturated water vapor environment which enabled imaging with minimal drying. Samples were observed at working distance of 8–10 mm and accelerating voltage of 20 kV with vacuum pressure levels in the range of 640–690 Pa at constant temperature of 1°C, in order to maintain relative humidity at 95%[52,53]. Some images were obtained at slightly lower pressures, leading to a relative humidity of 75%, in order to emphasize gel microstructure.

Pore network of hydrogels cross-linked at varying pH was also examined by fluorescence microscopy (Nikon Eclipse Ti, Melville, NY)[54]. Fluorescent hydrogels were prepared as previously described, by reacting 1 μ1 of 1% (w/v) fluorescein methacrylate[55] (Sigma) in dimethyl sulfoxide (DMSO, Sigma) with Cys copolymer and subsequently, VS copolymer. Samples were allowed to equilibrate in Tissue-Tek OCT compound (VWR, Radnor, PA) overnight, snap-frozen in liquid nitrogen-cooled isopentane, and then vertically cryosectioned to 50 μm using a cryostat (Microm HM550, Waltham, MA). Sections were placed in PBS, rinsed to remove OCT compound, and imaged by fluorescence microscopy to visualize the hydrogel pore network.

Gelation Kinetics by Oscillatory Rheology

Crosslinking kinetics of VS:Cys hydrogels was measured by oscillatory rheology with an ARG2 rheometer (TA Instruments, Newcastle, DE). Rheological behavior during crosslinking was determined with a plate-on-plate configuration (plate diameter, 20 mm) at 25°C over 1 hour (strain = 0.1%, frequency = 1 Hz)[56]. Both components were applied to the plate and mixed by pipetting prior to the onset of the time sweep, and values for storage modulus (G') were obtained. All analyses were done in triplicate.

Cytotoxicity of Soluble Hydrogel Components

Human MSCs (Lonza, Basel, Switzerland) were cultured in low-glucose DMEM containing 100 U/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin B and 10% fetal bovine serum (FBS). The extent of potential cytotoxic effects of soluble Cys or VS on MSCs plated in monolayer was evaluated. Briefly, MSCs were plated at 50,000 cells/cm^2. After 24h, DMEM was removed and replaced with DMEM containing 0, 0.1, 0.5, 1.0, or 5 mg/ml VS or Cys and 10% FBS. After another 24h, the MSC viability was assessed quantified by colorimetric 2-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay (Invitrogen, Carlsbad, CA) following manufacturer's instructions. Absorbance was measured λ=520 nm using a platereader (BioTek Synergy HT, Winooski, VT). A

standard curve for calibration of measured absorbances was obtained from cells seeded at known densities ranging between 2×10^3 and 7×10^6 cells/cm^2.

Cell Viability of MSCs in 3D Hydrogels

The feasibility of culturing MSCs in three-dimensional VS:Cys hydrogels was evaluated. Passage 4–6 MSCs were encapsulated in VS:Cys gels, at a cell density of 5 million cells/ml[57–59]. Briefly, MSCs were mixed with VS and Cys solutions (pH 7, VS:Cys ratio = 1, final concentration 100 mg/ml) and then 100 μl of cell suspension was transferred to Transwell inserts and allowed to complete gelation. Hydrogels were incubated at 37°C with 5% CO_2. Media was changed every two days and fed in slight excess of 1 ml/million cells/day.

Cell viability was qualitatively assessed with Live/Dead staining (Invitrogen). On days 1, 7, and 14 of culture, a separate quarter of the hydrogel disk was incubated in DMEM containing 2 μM calcein AM and 4 μM ethidium homodimer probes for 20 min. Portions of disks were rinsed with PBS and imaged by fluorescence microscopy (Nikon Eclipse Ti).

Statistical Analysis

Effects of VS:Cys ratio and pH on all endpoints were assessed by ANOVA and post-hoc Tukey test using SPSS 16.0 (SPSS, Chicago, IL). Effect of time on degradation behavior was assessed by repeated measured ANOVA.

RESULTS AND DISCUSSION

A series of saccharide-peptide copolymer based hydrogels was synthesized and investigated for 3D cell culture. Effects of pH and VS:Cys ratio on degradation, morphological, and mechanical properties, and gelation kinetics, of the saccharide-peptide hydrogels was determined. In addition, cytoxicity of soluble copolymers and viability of MSCs encapsulated in the hydrogel biomaterial was evaluated.

Synthesis and Characterization of Hydrogels

We have previously reported novel design of a new class of functional saccharide-peptide copolymer-based hydrogel[36]. While this biomaterial is highly functional and tunable, the need of H_2O_2 in effecting hydrogelation draws concerns for its application in 3D cell culture. To allow for hydrogel formation in the presence of cells, cross-linking ideally should be carried out in aqueous solution under mild conditions. Capitalizing on the versatility and high functionality of the saccharide-peptide copolymer, we have developed a mild hydrogelation via Michael-type conjugate addition between Cys and VS. Cys and VS conjugate addition has been extensively investigated in hydrogelation for synthetic ECM studies[14,40,43–45]. This Michael-type conjugate addition has been known as a “click” process which occurrs rapidly at mild physiological conditions without any additives[60]. Moreover, the particular Michael addition of Cys to VS yields a thiol-ether linkage which is hydrolytically stable and could avoid rapid degradation[43,61].

Accordingly, saccharide-peptide copolymer **1** was first synthesized. Approximately 47% of the free carboxylic acid groups on polymer **1** were coupled with cysteine(Trt)-NH_2 to give Cys–functionalized polymer via standard peptide coupling protocol using HCTU as the coupling reagent (Scheme 1). Global deprotection in TFA solution afforded the Cys-functionalized copolymer **2** containing 1.27 mmole Cys (thiol group) per gram of polymer. The calculation was based on the ^{1}H NMR integration of β-proton of cysteine on the polymer. The VS-functionalized saccharide–peptide copolymer **4** was synthesized from fully deprotected copolymer **3** with free hydroxyl and carboxylic acid group. Hydroxyl

group was then coupled with divinyl sulfone with catalytic amount of NaOH to afford vinyl VS copolymer **4** containing 1.54 mmole vinyl sulfone group per gram of polymer. The calculation of functionalization was based on the ^{1}H NMR integration of characteristic vinyl sulfone peaks at ~7.0 ppm on the polymer.

Swellability of Hydrogels

ESR of hydrogels was influenced by VS:Cys ratio and pH of the copolymer components at the time of cross-link. ESR was significantly affected by VS:Cys ratio ($p<0.001$) and pH of cross-linking components ($p<0.001$), as well as interactively ($p<0.01$) (Figure 2A). Gels which were formed with excess VS (VS:Cys = 2) generally resulted in higher ESR ($p<0.001$). This observation is most likely due to increased crosslinking between polymer chains at VS:Cys ratio = 1 when compared other ratios (VS:Cys ratio = 0.5 and 2). In addition, a trend of increasing ESR was observed with increasing pH. ESR was 1.4 and 1.3-fold lower, respectively at pH 6 and 7, than the ESR of equimolar gels formed at pH 8. Faster gelation kinetics at pH 8 appeared to contribute to increased ESR, suggesting the formation of polymer conformation which may not efficiently utilize all potential crosslinking sites available within the gel. Effects of pH on swelling properties of VS-Cys copolymerization has also been noted previously[45,49] indicating the potential for tuning hydrogel properties.

Degradation Behavior of Hydrogels

Degradation of saccharide-peptide hydrogels was dependent on the gelation pH and VS:Cys ratio, as well as the time in culture. Degradation of the VS:Cys gels, determined as a measure of wet weight over time (W_t), occurred fastest at pH 8 with less than 10% of the original mass present after 14 days *in vitro* at all VS:Cys ratios ($p<0.05$) (Figure 3). Hydrogels which were crosslinked at pH 6 or 7 appeared to be more stable, and maintained a swollen ratio close to 1 compared to gels cross-linked at pH 8 ($p<0.001$–$p<0.5$ for various VS:Cys ratios) after 21 days *in vitro*. In particular, an equimolar ratio of VS:Cys appeared to result in a longer lasting gel (Figure 3B) similar to degradable dextran hydrogels cross-linked by Michael addition[51]. Increased hydrolysis of hydrogels crosslinked at pH 8 and/or excess Cys (VS:Cys = 0.5, Figure 3A) and VS (VS:Cys = 2, Figure 3C) may be attributed, in part, to the previously reported contribution of secondary amines and hydroxyls to increased hydrolysis-based degradation of poly(glycoamidoamine)s[62]. Slower gelation kinetics at lower or physiologic pH may influence the efficiency of network formation, and consequently, degradation properties of the gel. These results suggest that conditions of pH 7 and equimolar ratios of VS and Cys components will be most relevant and advantageous to future longer term *in vitro* studies involving MSC culture and differentiation, in particular due to the time frame necessary (~ 2–3 weeks) to observe initial differentiation of MSCs[1].

Measurement of Gelation Time of Hydrogels

The time required to achieve gelation, determined as the time at which the two combined components (VS and Cys) completed a sol-gel transition and were unable to be pipetted up and down[50], was significantly affected by the pH of the two components. At slightly acidic pH (pH 6), equimolar gels required 12.3 min to form a gel, 3.8-fold and 9.0-fold higher than gels formed at pH 7 or pH 8, respectively ($p<0.001$) (Figure 4A). Gels which were formed at slightly basic pH (pH 8) required only 1.4 min to gel. Similarly, gels which were formed at physiologic pH formed in 3.2 min (Figure 4A). The aforementioned short duration required for complete hydrogelation of VS:Cys gels suggests that these gels could be applied *in situ* in a surgical setting.

Hydrogel Morphology Analysis

To examine gel microstructure, VS:Cys gels were examined by Environment Scanning Electron Microscopy (ESEM) and fluorescence microscopy. The environmental capability of the ESEM allowed for examination of the pore structure of the gels in a saturated water vapor environment which enabled imaging with minimal drying[52,53]. All hydrogels exhibited a macroporous gel network (Figure 4B–G) on the order of tens of microns when examined by both ESEM and fluorescence microscopy. Hydrogel pores in hydrogels cross-linked at pH 6 appeared smaller than pores in hydrogels cross-linked at pH 7 and pH 8 and were, comparatively, more challenging to resolve by ESEM and fluorescence microscopy. Slower gelation times associated with slightly acidic pH (Fig. 4A) could have contributed to a tighter gel network, and thus, smaller pores. Overall, pore sizes appeared to be slightly smaller in size when examined by ESEM, compared to examination by fluorescence microscopy. This could be attributed to some sample shrinkage when imaged at lower pressures and concomitant lower levels of relative humidity. Pores appeared to be continuous and allowed for diffusion of macromolecules such as dextran (MW ~ 40 kDa, unpublished results). The mechanisms underlying gel network formation in the VS-Cys system will be explored further in the future. It is likely that such hydrogels will provide suitable conditions for cell culture[63], allowing for oxygen diffusion and transport of nutrients while also providing mechanical support to cells during culture.”

Gelation Kinetics by Oscillatory Rheology

Rheological analysis of the crosslinking of saccharide-peptide hydrogels as a function of time (Figure 5) indicated an effect of both VS:Cys ratio and pH on the gelation profile and eventual formation of a stable gel (measured as a stabilization of storage modulus, G'). In general, gels that were formed with VS and Cys components at slightly acidic pH required longer time to gel and reach an equilibrium G' (Figure 5ABC). In contrast, gels that were formed with components at slightly basic pH formed gels relatively quickly. The effect of VS:Cys ratio was particularly evident when gels were formed with a VS:Cys ratio of 2 (Figure 5C). Excess VS appeared to diminish the overall G' of the resulting gel as function of time (Figure 4C, Figure 6), resulting in gels which were 26.8-fold softer (G' = 0.1 kPa) than those formed at VS:Cys = 1 (G' = 3.2 kPa) ($p<0.001$). In contrast, gels formed with excess Cys were more stiff (G' = 0.8 kPa) than those formed with excess VS, except for those formed at pH 6 which were similar (Figure 2B, Figure 5A). Overall, equivalent molar contributions of VS and Cys (VS:Cys = 1.0) resulted in the stiffest gels, likely due to more efficient crosslinking (Figure 2B, Figure 5B). Attainment of increased G' in off-stoichiometric gels with excess Cys (VS:Cys = 0.5) suggests the formation of disulfide bonds during crosslinking. In addition, higher pH conditions could further encourage disulfide bond formation, resulting in more robust gels. When gelation was attempted with Cys only (no VS), no gelation occurred implying the crosslinking mechanism to be primarily Michael-type addition. It is unlikely for electrostatic effects to play a significant role in gel crosslinking here since at pH 6, and higher, the free carboxyl groups on the saccharide-peptide polymer should be remain deprotonated. Moreover, if electrostatic effects were pronounced, repulsion would be greater at higher pH and lead to slower gelation or none at all, resulting in a trend opposite to that presented here. While relatively soft, the mechanical properties of VS:Cys gels were tunable by varying pH and VS:Cys ratios and also comparable to those of elastin-like polypeptide[64], hyaluronic acid[65], PEG-fibrinogen[66], and hydrolytically degradable PEG[67] hydrogels. In the future, it would be useful to incorporate amino acids of varying hydrophobicities into the polymer backbone in order to determine effects on mechanical properties of the hydrogel.

Cytotoxicity of Soluble Copolymer Components and Cell Viability in 3D Hydrogels

Incubation of MSCs with varying concentrations of each soluble copolymer components (VS or Cys) for 24h did not appear to have any significant deleterious effects on MSC viability ($p=0.1 – 0.9$) compared to MSCS which were incubated with no polymer. After 24h *in vitro*, MSCs were 88±2% viable when incubated with VS copolymer, and 101±5% viable when incubated with Cys copolymer. Viability of MSCs incubated with Cys polymer were statistically similar to control samples ($p>0.9$) (Figure 6A). Viability of MSCs encapsulated in hydrogels (VS:Cys =1, pH 7) was visualized qualitatively with Live/Dead staining, and appeared to be >90% after 1, 7, and 14 days *in vitro* (Figure 6B–D). Maintenance of MSC viability has been linked with differentiation potential and metabolic activity[68 69]. Our present study indicates the potential of VS:Cys hydrogels for culture of MSCs *in vitro* in 3D. Future studies will apply VS:Cys hydrogels for longer 3D cultures aimed at differentiation of MSCs *in vitro*.

CONCLUSIONS

We have developed and characterized a tunable, porous 3D saccharide-peptide hybrid copolymer hydrogel platform for culturing MSCs. Hydrogel mechanical properties were tunable by varying VS:Cys ratio as well as the pH of the cross-linking components. pH and VS:Cys ratio used for hydrogelation also had an effect on the degradation behavior of the VS:Cys gels, with basic pH (pH 8) resulting in accelerated loss of mass. We have applied Michael-type addition to generate biodegradable hydrogels composed of primarily natural components, with minimal inclusion of synthetic chemical groups (vinyl sulfone). In addition, we have established extended MSC viability in VS:Cys hydrogels without incorporation of cell adhesion peptides such as RGD, a usual requirement in bio-inert, PEG-containing hydrogels[70]. Overall, VS:Cys hydrogels did not have any deleterious effects on cell viability, and individual soluble components (VS or Cys) did not appear to be cytotoxic to MSCs. The combination of biodegradability, tunability of gel chemical and physical properties, non-cytotoxicity of soluble components, and good cell viability for 3D cell culture demonstrates the potential of our saccharide-peptide hydrogels as a novel family of synthetic ECM mimics for culture of MSCs in 3D. Our future studies will focus on utilizing the hydrogel system for controlling the differentiation of MSCs, as well as exploration of other biologically relevant saccharide-peptide motifs.

Acknowledgments

This work was generously supported by a grant from the NIH (R01 EB006797). We thank Dr. John Porter of the Carl Zeiss Center of Excellence for Electron Microscopy at the University of California, Irvine for assistance with ESEM. We thank Mr. Hanxiang Zeng for assistance with cell culture.

REFERENCES

(1). Mauck RL, Yuan X, Tuan RS. Osteoarthritis Cartilage 2006;14:179–89. [PubMed: 16257243]

(2). Emans PJ, Surtel DA, Frings EJ, Bulstra SK, Kuijer R. Tissue Eng 2005;11:369–77. [PubMed: 15869417]

(3). Wickham MQ, Erickson GR, Gimble JM, Vail TP, Guilak F. Clin Orthop Relat Res 2003:196–212. [PubMed: 12838072]

(4). Wang DW, Fermor B, Gimble JM, Awad HA, Guilak F. J Cell Physiol 2005;204:184–91. [PubMed: 15754341]

(5). Schense JC, Hubbell JA. Bioconjug Chem 1999;10:75–81. [PubMed: 9893967]

(6). Gerecht S, Burdick JA, Ferreira LS, Townsend SA, Langer R, Vunjak-Novakovic G. Proc Natl Acad Sci USA 2007;104:11298–303. [PubMed: 17581871]

(7). Heywood HK, Sembi PK, Lee DA, Bader DL. Tissue Eng 2004;10:1467–79. [PubMed: 15588406]

(8). Drury JL, Mooney DJ. Biomaterials 2003;24:4337–51. [PubMed: 12922147]

(9). Elisseeff J, Anseth K, Sims D, McIntosh W, Randolph M, Yaremchuk M, Langer R. Plast Reconstr Surg 1999;104:1014–22. [PubMed: 10654741]

(10). Burdick JA, Anseth KS. Biomaterials 2002;23:4315–23. [PubMed: 12219821]

(11). Bryant SJ, Anseth KS. J Biomed Mater Res 2002;59:63–72. [PubMed: 11745538]

(12). Hwang Y, Zhang C, Varghese S. J Mat Chem 2009;20:345–351.

(13). Nuttelman CR, Henry SM, Anseth KS. Biomaterials 2002;23:3617–26. [PubMed: 12109687]

(14). Lutolf MP, Lauer-Fields JL, Schmoekel HG, Metters AT, Weber FE, Fields GB, Hubbell JA. Proc Natl Acad Sci U S A 2003;100:5413–8. [PubMed: 12686696]

(15). Bonzani IC, Adhikari R, Houshyar S, Mayadunne R, Gunatillake P, Stevens MM. Biomaterials 2007;28:423–433. [PubMed: 16979756]

(16). Saha, K.; Keung, AJ.; Irwin, EF.; Li, Y.; Little, L.; Schaffer, DV.; Healy, KE. 2008. p. 4426-4438.

(17). Wong Po Foo CT, Lee JS, Mulyasasmita W, Parisi-Amon A, Heilshorn SC. Proc Natl Acad Sci U S A 2009;106:22067–72. [PubMed: 20007785]

(18). Ellis-Behnke RG, Liana Y-X, You S-W, Tay DKC, Zhang S, Kwok-Fai S, Schneider GE. Proc Natl Acad Sci 2006;103:5054–5059. [PubMed: 16549776]

(19). Silva GA, Czeisler C, Niece KL, Beniash E, Harrington DA, Kessler JA, Stupp SI. Science 2004;303:1352–1355. [PubMed: 14739465]

(20). Zhang S. Nat Biotech 2003;21:1171–1178.

(21). Engler AJ, Sen S, Sweeney HL, Discher DE. Cell 2006;126:677–689. [PubMed: 16923388]

(22). Chen CS, Mrksich M, Huang S, Whitesides GM, Ingber DE. Science 1997;276:1425–8. [PubMed: 9162012]

(23). Mann BK, West JL. J Biomed Mater Res 2002;60:86–93. [PubMed: 11835163]

(24). Petersen OW, Ronnov-Jessen L, Howlett AR, Bissell MJ. Proc Natl Acad Sci 1992;80:9064–9068. [PubMed: 1384042]

(25). Tanaka H, Murphy CL, Murphy C, Kimura M, Kawai S, Polak JM. J Cell Biochem 2004;93:454–462. [PubMed: 15372628]

(26). Place ES, Evans ND, Stevens MM. Nat Mater 2009;8:457–70. [PubMed: 19458646]

(27). Tibbitt MW, Anseth KS. Biotechnol Bioeng 2009;103:655–663. [PubMed: 19472329]

(28). Marolt D, Augst A, Freed LE, Vepari C, Fajardo R, Patel N, Gray M, Farley M, Kaplan D, Vunjak-Novakovic G. Biomaterials 2006;27:6138–6149. [PubMed: 16895736]

(29). Vercruysse KP, Marecak DM, Marecek JF, Prestwich GD. Bioconjugate Chemistry 1997;8:686–694. [PubMed: 9327132]

(30). Lee KY, Bouhadir KH, Mooney DJ. Macromolecultes 2000;33:97–101.

(31). Lee KY, Mooney DJ. Chem Rev 2001;101:1869–79. [PubMed: 11710233]

(32). Metzke M, Bai JZ, Guan Z. Journal of the American Chemical Society 2003;125:7760–7761. [PubMed: 12822968]

(33). Metzke M, Guan Z. Biomacromolecules 2007;9:208–215. [PubMed: 18078325]

(34). Metzke M, O'Connor N, Maiti S, Nelson E, Guan Z. Angew. Chem. Int. Ed 2005;44:6529–6533.

(35). Urakami H, Guan Z. Biomacromolecules 2008;9:592–597. [PubMed: 18220347]

(36). Liao S, Yu T, Guan Z. J Am Chem Soc 2009;131:17638–46. [PubMed: 19908839]

(37). Herzner H, Reipen T, Schultz M, Kunz H. Chem Rev 2000;100:4495–4538. [PubMed: 11749356]

(38). Gruner SAW, Locardi E, Lohof E, Kessler H. Chem Rev 2002;102:491–514. [PubMed: 11841252]

(39). Aoi K, Tsutsumiuchi K, Okada M. Macromolecules 1994;27:875–877.

(40). Kraehenbuehl TP, Ferreira LS, Zammaretti P, Hubbell JA, Langer R. Biomaterials 2009;30:4318–24. [PubMed: 19500842]

(41). Lutolf MP, Hubbell JA. Biomacromolecultes 2003;4:713–722.

(42). Lutolf MP, Weber FE, Schmoekel HG, Schense JC, Kohler T, Muller R, Hubbell JA. Nat Biotechnol 2003;21:513–8. [PubMed: 12704396]

(43). Mather BD, Viswanathan K, Miller KM, Long TE. Progress in Polymer Science 2006;31:487–531.

(44). Pratt AB, Weber FE, Schmoekel HG, Muller R, Hubbell JA. Biotechnol Bioeng 2004;86:27–36. [PubMed: 15007838]

(45). Lutolf MP, Hubbell JA. Biomacromolecules 2003;4:713–22. [PubMed: 12741789]

(46). Rizzi SC, Hubbell JA. Biomacromolecules 2005;6:1226–1238. [PubMed: 15877337]

(47). Jin R, Moreira Teixeira LS, Krouwels A, Dijkstra PJ, van Blitterswijk CA, Karperien M, Feijen J. Acta Biomaterialia 2010;6:1968–1977. [PubMed: 20025999]

(48). Shu XZ, Ahmad S, Liu Y, Prestwich GD. Acta Biomaterialia 2006;79A:902–912.

(49). Hiemstra C, van der Aa LJ, Zhong Z, Dijkstra PJ, Feijen J. Macromolecules 2007;40:1165–1173.

(50). Strehin I, Nahas Z, Arora K, Nguyen T, Elisseef J. Biomaterials 2010;31:2788–2797. [PubMed: 20047758]

(51). Hiemstra C, van der Aa LJ, Zhong Z, Dikjkstra PJ, Feijen J. Biomacromolecules 2007;8:1548–1556. [PubMed: 17425366]

(52). Gagnon M-A, Lafleur M. J Phys Chem B 2009;113:9084–9091. [PubMed: 19522479]

(53). Motta A, Migliaresi C, Faccioni F, Torricelli P, Fini M, Giardino R. J Biomater Sci Polym Ed 2004;15:851–864. [PubMed: 15318796]

(54). Bodugoz-Senturk H, Macias CE, Kung JH, Muratoglu OK. Biomaterials 2009;30:589–596. [PubMed: 18996584]

(55). da Silva J, Lautenschlager F, Sivaniah E, Guck JR. Biomaterials 2010;31:2201–2208. [PubMed: 20015545]

(56). Hu BH, Su JS, Messersmith PB. Biomacromolecules 2009;10:2194–2200. [PubMed: 19601644]

(57). Kloxin AM, Kasko AM, Salinas CN, Anseth KS. Science 2009;324:59–63. [PubMed: 19342581]

(58). Alhadlaq A, Mao JJ. J Dental Res 2003;82:951–956.

(59). Buxton AN, Bahney CS, Yoo JY, Johnstone B. Tissue Eng A. 2010 epub.

(60). van Dijk M, Rijkers DTS, Liskamp RMJ, van Nostrum CF, Hennink WE. Bioconj Chem 2009;20:2001–2016.

(61). Morpurgo M, Veronese FM, Kachensky D, Harris JM. Bioconj Chem 1996;7:363–368.

(62). Liu Y, Reineke TM. Biomacromolecules 2010;11:316–325. [PubMed: 20058913]

(63). Peppas N, Hilt JZ, Khademhosseini A, Langer R. Adv Mater 2006;18:1–17.

(64). Lim DW, Nettles DL, Setton LA, Chilkoti A. Biomacromolecules 2007;8:1463–1470. [PubMed: 17411091]

(65). Seidlits SK, Khaing ZZ, Petersen RR, Nickels JD, Vanscoy JE, Shear JB, Schmidt CE. Biomaterials 2010;31

(66). Almany L, Seliktar D. Biomaterials 2005;26:2467–2477. [PubMed: 15585249]

(67). Zustiak SP, Leach JB. Biomacromolecules. 2010 ASAP.

(68). Salinas CN, Cole BB, Kasko AM, Anseth KS. Tissue Eng 2007;13:1025–1034. [PubMed: 17417949]

(69). Nuttelman CR, Tripodi MC, Anseth KS. Matrix Biol 2005;24:208–218. [PubMed: 15896949]

(70). Salinas CN, Anseth KS. J of Tissue Eng and Reg Med 2008;2:296–304.

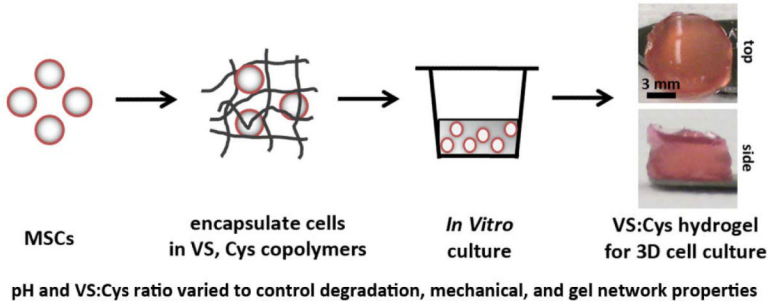


Figure 1.
Schematic of mesenchymal stem cell (MSC) encapsulation and culture in tunable VS:Cys 3D hydrogels.

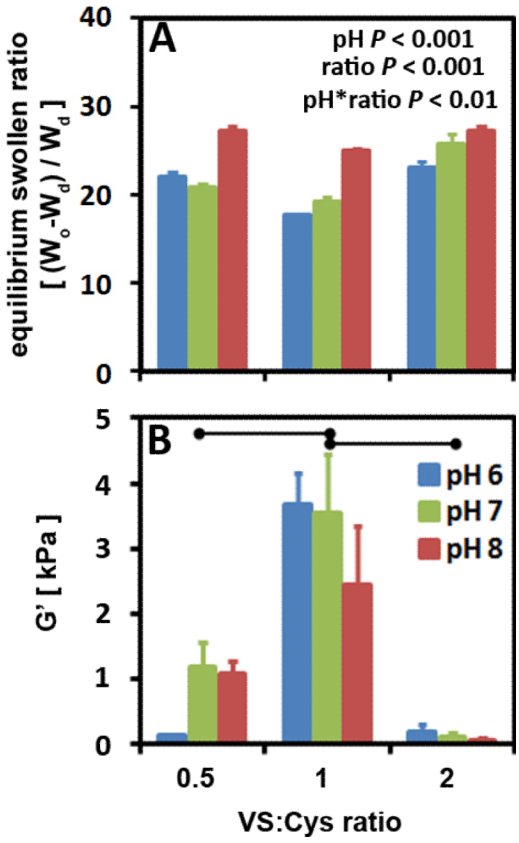


Figure 2.

Effects of VS:Cys ratio and pH on hydrogel swelling, cross-linking, and mechanical properties. **(A)** Equilibrium swelling ratio and **(B)** storage modulus, G', as a function of VS:Cys ratio and pH. pH 6, 7, and 8 are depicted in blue, green, and red, respectively. Mean ±SEM, n=3. (•) p<0.001.

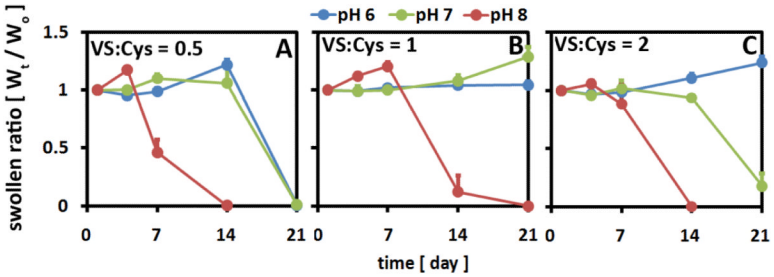


Figure 3.

Effects of VS:Cys ratio and pH on degradation behavior. Gels composed at VS:Cys ratios of **(A)** 0.5, **(B)** 1, and **(C)** 2 at pH 6 (●), 7 (●), and 8 (●) were observed over 21 days *in vitro*. Mean±SEM, n=3.

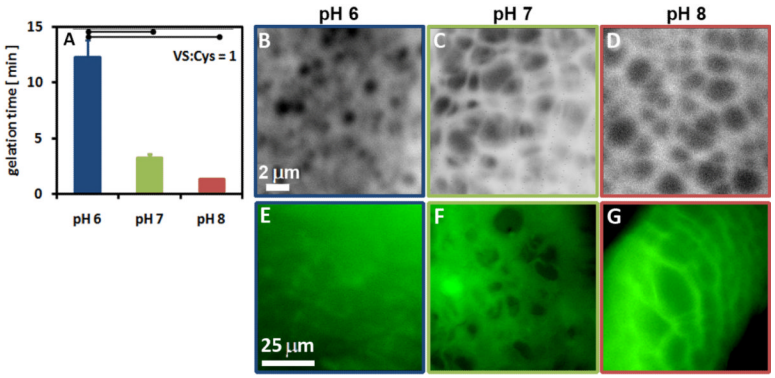


Figure 4.

Effect of pH on **(A)** gelation time and pore network of VS:Cys gels, examined by (B–D) Environmental Scanning Electron Microscopy (ESEM) and (E–G) fluorescence microscopy. Gelation time was evaluated as the time required for complete sol-gel transition. Mean ±SEM, n=3. (•) $P < 0.001$.

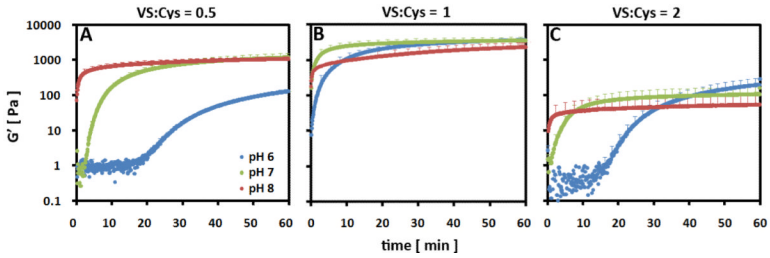


Figure 5.

Rheological characterization of VS:Cys gels as a function of VS:Cys ratio: **(A)** 0.5, **(B)** 1, and **(C)** 2; and pH: 6 (●), 7 (●), and 8 (●). Time sweeps were conducted with an oscillation frequency = 1 Hz and strain = 0.1%. Mean±SEM, n=3.

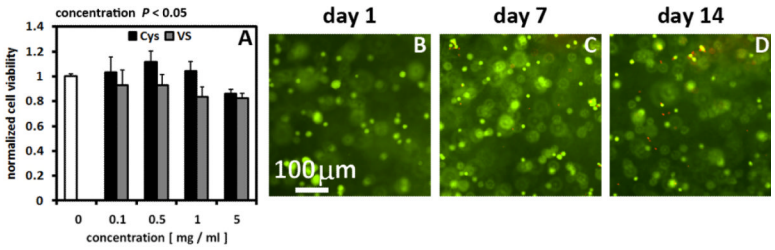
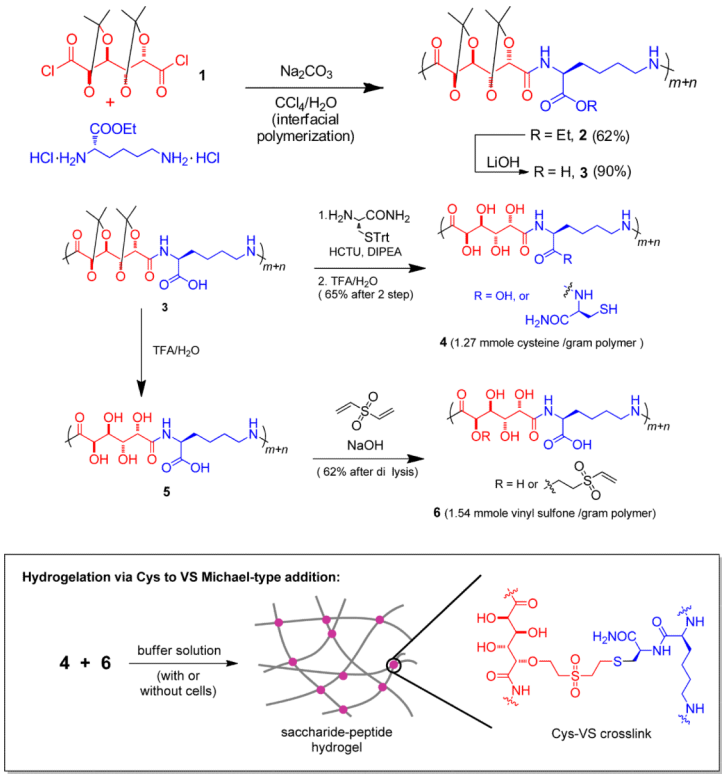


Figure 6.

Effects of polymer components and hydrogel encapsulation on cytotoxicity and viability of MSCS. **(A)** Cytotoxicity of soluble copolymer components (Cys, black; VS, gray). Data are normalized to the number of viable cells present in samples which were not incubated with either copolymer. Mesenchymal stem cells were exposed to copolymer components for 24 h and then analyzed for viability by MTT assay. Viability of MSCs assessed by Live/Dead stain on days **(B)** 1, **(C)** 7, and **(D)** 14. Live cells are stained with calcein AM (green) and dead cells with ethidium homodimer-1 (red). Mean±SEM, n=3.



Scheme 1.
Hydrogel synthesis through vinyl sulfone-thiol conjugated addition.