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MODULATION OF CHONDROCYTE BEHAVIOR THROUGH TAILORING FUNCTIONAL SYNTHETIC SACCHARIDE-PEPTIDE HYDROGELS

Kanika Chawla^{1,2}, Ting-bin Yu^{1,3}, Lisa Stutts¹, Max Yen^{1,4}, and Zhibin Guan^{1,+}

¹Department of Chemistry, University of California-Irvine, Irvine, CA

Abstract

Tailoring three-dimensional (3D) biomaterial environments to provide specific cues in order to modulate function of encapsulated cells could potentially eliminate the need for addition of exogenous cues in cartilage tissue engineering. We recently developed saccharide-peptide copolymer hydrogels for cell culture and tissue engineering applications. In this study, we aim to tailor our saccharide-peptide hydrogel for encapsulating and culturing chondrocytes in 3D and examine the effects of changing single amino acid moieties differing in hydrophobicity/hydrophilicity (valine (V), cysteine (C), tyrosine (Y)) on modulation of chondrocyte function. Encapsulated chondrocytes remained viable over 21 days *in vitro*. Glycosaminoglycan and collagen content was significantly higher in Y-functionalized hydrogels compared to V-functionalized hydrogels. Extensive matrix accumulation and concomitant increase in mechanical properties was evident over time, particularly with the presence of Y amino acid. After 21 days *in vitro*, Y-functionalized hydrogels attained a modulus of 193±46 kPa, compared to 44±21 kPa for V-functionalized hydrogels. Remarkably, mechanical and biochemical properties of chondrocyte-laden hydrogels were modulated by change in a single amino acid moiety. This unique property, combined with the versatility and biocompatibility, makes our saccharide-peptide hydrogels promising candidates for further investigation of combinatorial effects of multiple functional groups on controlling chondrocyte and other cellular function and behavior.

Keywords

tissue engineering; chondrocyte; cartilage; sacchride-peptide; amino acid; functional group

1. INTRODUCTION

Articular cartilage is a connective tissue that normally provides a load-bearing, low friction, wear-resistant tissue located at the ends of long bones through its depth-varying cell and matrix properties. Upon damage due to disease, trauma, or as a consequence of aging, articular cartilage possesses limited capacity to self-repair[1, 2]. Several existing surgical

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⁺Corresponding Author: Dr. Zhibin Guan, Department of Chemistry, 1102 Natural Sciences II, Irvine, CA 92606, TEL: 949-824-5172, FAX: 949-824-2210, zguan@uci.edu.

²current location: Cellerant Therapeutics, San Carlos, CA

³current location: DuNing Inc., Irvine, CA

⁴current location: Hewlett-Packard, Rancho Bernardo, CA

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repair strategies, such as microfracture[3, 4], allograft transplantation[5], mosaicplasty[6], and total knee arthroplasty[7], result in inferior tissue formation, site morbidity, and/or exhibit limited lifetime and function. Engineering cartilaginous tissues *in vitro*, by incorporating various cells, scaffolds or materials, as well as biophysical and biochemical cues, in combination or alone, holds promise as an alternative treatment for repair of focal cartilage defects.

A variety of biomaterials have been explored for cartilage tissue engineering applications. Chondrocytes have been encapsulated in three-dimensional (3D) matrices composed of natural materials, such as agarose[8, 9], alginate[10, 11], chitosan[12, 13], and hyaluronic acid[14], as well as synthetic materials such as poly(ethylene) glycol[15], poly(glycolic acid) or poly(lactic) acid[16, 17], thermal gelling polypeptide [18, 19], and self-assembling peptide amphiphile[20] based systems. Although the aforementioned materials have been shown to support viable chondrocytes, little is known regarding the ability of such three-dimensional (3D) matrices to modulate chondrocyte function, and resulting cell-material properties.

To date, 3D matrices have been applied in combination with growth factors and mechanical stimulation in order to modulate chondrocyte function (proliferation, matrix accumulation) as well as resulting mechanical properties of chondrocyte-laden biomaterials. Previous studies have applied a variety of growth factors (TGF- β 1[21], BMP-2[22, 23], BMP-7[24], and IGF-I[25, 26]) with and without dynamic stimulation[21, 27, 28], in order to enhance matrix deposition and regulate chondrocyte growth. Application of low oxygen tension has also indicated promise in promoting cartilaginous tissue formation[29]. Although resulting tissues indicated increased matrix content, reported mechanical properties were inferior to native cartilage. Tailoring 3D biomaterial environments would provide specific biophysical and biochemical cues in order to modulate function of encapsulated cells; potentially without the need of the aforementioned exogenous cues.

Several studies have investigated the influence of microenvironment, in the form of hydrophobicity/hydrophilicity, on chondrocyte behavior; largely on two-dimensional (2D) surfaces[30–33]. Chondrocytes cultured on hydrophilic 2D surfaces[30], or, in one case, encapsulated in 3D poly(ethylene) glycol (PEG)-based hydrogels[34], exhibited greater growth than on hydrophobic surfaces. Given the potential of regulating function of mesenchymal stem cells encapsulated in a hydrogel material simply by providing cues in the form of small functional groups[35], it is likely that modulation of chondrocyte behavior is also possible.

Our laboratory has recently developed a family of 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]. Our previous studies have shown that saccharide-peptide copolymers are non-toxic, non-immunogenic, and biodegradable [36, 38, 39]. Mild and quick hydrogelation, beneficial for application of 3D cell culture, was achieved by Michael addition reaction [40–45] [38] of cysteine and vinyl sulfone functionalized saccharide-peptide polymers (referred to as polymer-C and polymer-VS, respectively, in latter discussion). While this type of chemistry has been applied previously to generate PEG-peptide [41, 46], PEG-hyaluronic acid (HA)[47], or PEG-dextran gels[48], to our knowledge, our design is the first to apply Michael-type addition to form biodegradable synthetic hydrogels composed of natural building blocks without a large synthetic component such as PEG. In addition, the modular copolymer design, along with free carboxylic acid groups along the backbone, allows for convenient derivatization with small

chemical functionalities, such as amino acids. In this study, our aims were to determine the utility of the **VS:C** hydrogel for encapsulating and culturing chondrocytes in 3D and examine the effects of changing single amino acid moieties differing in hydrophobicity/hydrophilicity[49] on modulation of chondrocyte function.

2. MATERIALS AND METHODS

2.1. Materials

Materials for cartilage explant, chondrocyte isolation, monolayer culture, hydrogel culture, biochemical procedures, and immunohistochemical procedures were obtained as described previously [50]. Mouse monoclonal anti-collagen type I antibody, and testicular hyaluronidase were from Sigma (St. Louis, MO). Mouse monoclonal anti-collagen type II antibody cocktail was from Chemicon (Temecula, CA). Non-specific mouse monoclonal IgG antibody was from Pierce (Rockford, IL).

2.2. Experimental Design

To determine the effects of cysteine, valine, and tyrosine amino acid functionalities on chondrocyte-laden hydrogel properties and matrix accumulation by encapsulated chondrocytes, cells were encapsulated in *de novo* synthesized saccharide-peptide hydrogels. The polymer backbone was functionalized with cysteine (designated as **C**), cysteine and valine (designated as **V**), or cysteine and tyrosine (designated **Y**) and used to generate **VS:C**, **VS:CV**, or **VS:CY** hydrogels for culture *in vitro*.

2.3. Polymer Synthesis and Characterization

2.3.1 Synthesis and characterizations of copolymer backbone—The galactaric acid-lysine copolymer backbone (referred to as polymer **1**) was synthesized by following previously reported procedures[36].

2.3.2 Synthesis and characterizations of cysteine functionalized saccharide-peptide copolymer (here after referred to as polymer-C)—To a solution of polymer **1** (12.89 mmole of carboxylic acid, 5.16 g) and cysteine(Trt)-NH₂ (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-1H-benzotriazole-1-yl)-1,1,3,3-tetramethylaminium hexafluorophosphate) (8.38 mmole, 3.15 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 generating a white suspension in aqueous solution. The solid was filtered out and washed with 0.2N HCl to yield a white polymer. The protected polymer was further dissolved in a mixture of TFA (15.0 mL), water (4.5 mL) and triisopropylsilane (0.5 mL) solution to remove protecting groups. After stirring the reaction mixture for 3 h, the solution was diluted with water and purified by dialysis against 0.01N HCl for 3 days. Excess water was removed by lyophilization to give 3.29 g of pale brown solid polymer functionalized with cysteine (**C**) with a yield of 65%. The cysteine thiol groups on polymer-C was employed in latter hydrogelation via Michael-type addition with **VS** polymer. ¹H NMR integration of the polymer's cysteine β-protons revealed 1.27 mmole cysteine (thiol group) per gram of polymer, which is equivalent to ~50 mol% of the free carboxylate groups on the α-proton of lysine and cysteine, proton of backbone galactaric acid), 4.05–4.04 (m, 2.0H, proton of backbone galactaric acid), 3.32–3.28 (m, 2.0H, ε-proton of backbone lysine), 3.03–2.90 (m, 0.9H, β-proton of cysteine, corresponding to 0.47 cysteine unit per repeating unit), 1.97–1.86 (m, 2.0H, β-proton of backbone lysine), 1.60–1.44 (m, 4.0H, γ, σ-proton of backbone lysine).

2.3.3 Synthesis and characterizations of cysteine and tyrosine (~15 mol% to the total carboxylic acid groups) functionalized saccharide-peptide

copolymer (here after referred to as polymer-CY)—To a solution of polymer **1** (3.75 mmole of carboxylic acid, 1.5 g), tyrosine(*t*Bu)-O*t*Bu (0.15eq, 0.56 mmole, 0.176g) and cysteine(Trt)-NH₂ (0.50 eq, 1.87 mmole, 0.679 g) in 15 mL of DMF, DIPEA (7.49 mmole, 0.88 mL) and HCTU (2-(6-chloro-1H-benzotriazole-1-yl)-1,1,3,3-tetramethylammonium hexafluorophosphate) (3.75 mmole, 1.550 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. The protected polymer was further dissolved in a mixture of TFA (15.0 mL), water (4.5 mL) and triisopropylsilane (0.5 mL) solution to remove protecting groups. After stirring the reaction mixture for 3 h, the solution was diluted with water and purified by dialysis against 0.01N HCl for 3 days. Excess water was then removed by lyophilization to give 0.78 g of white solid polymer functionalized with cysteine and tyrosine (**CY**) with a yield of 51%. ¹H NMR integration of the polymer's cysteine β-protons reveals 1.22 mmole cysteine (thiol group) per gram of polymer, equivalent to ~48 mol% of the free carboxylic acid groups being functionalized by cysteine, comparable to the cysteine functionalization level on polymer-**C**. Similarly, the cysteine thiol groups on the polymer-**CY** will be employed in latter hydrogelation via Michael-type addition with **VS** polymer. ¹H NMR integration of the polymer's tyrosine β-protons indicates ~15 mol% of the free carboxylic acid groups being functionalized by **Y**. ¹H NMR (600 MHz, D₂O) δ 7.15 (s, 0.3H, aromatic proton of tyrosine), 6.86 (s, 0.3H, aromatic proton of tyrosine), 4.71–4.05 (m, 4.0H, proton of backbone galactaric acid), 3.76–3.75 (m, 0.4H), 3.43–3.28 (m, 2.0H, ε-proton of backbone lysine), 3.04–2.93 (m, 1.0H, β-proton of cysteine, corresponding to 0.5 cysteine per repeating unit), 1.94–1.74 (m, 2.0H, β-proton of backbone lysine), 1.60–1.25 (m, 4.0H, γ, σ-proton of backbone lysine).

2.3.4 Synthesis and characterizations of cysteine and valine (~15 mol%, to the total carboxylic acid groups) functionalized saccharide-peptide copolymer (here after referred to as polymer-CV)

—To a solution of polymer **1** (3.75 mmole of carboxylic acid, 1.5 g), valine-OMe (0.15eq, 0.56 mmole, 0.094g) and cysteine(Trt)-NH₂ (0.50 eq, 1.87 mmole, 0.679 g) in 15 mL of DMF, DIPEA (7.49 mmole, 0.88 mL) and HCTU (2-(6-chloro-1H-benzotriazole-1-yl)-1,1,3,3-tetramethylammonium hexafluorophosphate) (3.75 mmole, 1.550 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), water (4.5 mL) and triisopropylsilane (0.5 mL) solution to remove protecting groups. After stirring the reaction mixture for 3 h, the solution was diluted with water and purified by dialysis against 0.01N HCl for 3 days. Excess water was then removed by lyophilization to give 0.811 g of white solid polymer functionalized with cysteine and valine (**CV**) with a yield of 53%. ¹H NMR integration of the polymer's cysteine β-protons revealed 1.22 mmole cysteine (thiol group) per gram of polymer, equivalent to ~48 mol% of the free carboxylic acid groups being functionalized by cysteine, comparable to the cysteine functionalization level on polymer-**C**. Again, the cysteine thiol groups on the polymer-**CV** will be employed in latter hydrogelation via Michael-type addition with **VS** polymer. ¹H NMR integration of the polymer's valine β-protons revealed ~15 mol% of the free carboxylic acid groups being functionalized by **V**. ¹H NMR (600 MHz, D₂O) δ 4.70–4.33 (m, 3.8H, α-proton of cysteine and lysine, proton of backbone galactaric acid), 4.12–4.06 (m, 2.0H, proton of backbone galactaric acid), 3.89–3.80 (m, 1.0H), 3.33–3.30 (m, 2.0H, ε-proton of backbone lysine), 3.03–2.91 (m, 1.0H, β-

proton of cysteine, corresponding to 0.5 cysteine per repeating unit), 2.27–2.23 (m, 0.15H, β -proton of vproto, corresponding to 0.15 valine per repeating unit), 1.93–1.87 (m, 2.0H, β -proton of backbone lysine), 1.61–1.43 (m, 4.0H, γ , σ -proton of backbone lysine), 1.00–0.97 (m, 1.0H, γ -proton of valine).

2.3.5 Synthesis and characterizations of vinyl sulfone functionalized saccharide–peptide copolymer (here after referred as polymer-VS)—To a solution of polymer-C as described in section 2.3.2 (4.90 mmole of cysteine group, 2.00g) in 100 mL PBS buffer (pH 6.0), excess divinyl sulfone (30eq, 147.0 mmole, 14.8 mL) was added. After stirring the reaction mixture at room temperature for 48 hours, the solution was diluted with water, washed with diethyl ether, and purified by dialysis against water for 3 days. Excess water was then removed by lyophilization to give 1.90 g of white solid polymer VS (here after referred to as polymer-VS). ^1H NMR integration of vinyl groups on the polymer revealed 0.90 mmole vinyl sulfone group per gram of polymer. ^1H NMR (600 MHz, D_2O) δ 6.95–6.91 (m, 0.4H, vinyl group), 6.53–6.43 (m, 0.8H, vinyl group), 4.69–4.06 (m, 5.0H), 3.58–3.56 (m, 0.9H, α -proton of backbone lysine), 3.29–2.94 (m, 4.0H), 1.91–1.80 (m, 2.0H, β -proton of backbone lysine), 1.60–1.41 (m, 4.0H, γ , σ -proton of backbone lysine).

2.4. Preparation and Characterization of Hydrogels

All hydrogels (VS:C, VS:CV, and VS:CY) were prepared with a molar ratio of vinyl sulfone to cysteine of 1. Hydrogels were prepared by dissolving polymer-VS and one of the C functionalized copolymers at a total polymer concentration of 100 mg/ml in Dulbecco's Modified Eagle's Medium (DMEM) [38]. Using a pH microprobe (Fisher Scientific, Pittsburgh, PA), the copolymer solutions were adjusted separately to pH 7 by addition of the necessary amounts of HCl or NaOH prior to gelation. Polymer-VS and one of the C functionalized copolymers (polymer-C, polymer-CV, or polymer-CY) solutions were subsequently crosslinked through Michael-type conjugate addition (Figure 1), as previously reported [38], in 6.5 mm diameter Transwell® inserts (Fisher).

2.4.1. Swellability and Degradation 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:

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

The swollen ratio of the gels was tracked over 21 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.

2.4.2. Gelation Kinetics by Oscillatory Rheology—Crosslinking kinetics of VS:C, VS:CV, and VS:CY 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) [51]. 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.

2.5. Primary Culture and Formation of Hydrogel Constructs

Chondrocytes were isolated from articular cartilage slices (up to 1 mm thickness) obtained from the patellofemoral groove and femoral condyles of six immature (1–3 wk old) bovine calf knees (Research 87, Boylston, MA) and digested in medium (Dulbecco's Modified Eagle Medium [DMEM], 10 mM HEPES, 0.1 mM nonessential amino acids, 0.4 mM L-proline, 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, and 0.25 µg/ml amphotericin B) with 0.2% pronase for 1 hour, and 0.02% collagenase-P for 16 hours [50].

Primary chondrocytes were encapsulated in **VS:C**, **VS:CV**, or **VS:CY** gels, at a cell density of 20 million cells/ml. Briefly, chondrocytes were resuspended in polymer-**VS** and one of the **C** functionalized copolymers (polymer-**C**, polymer-**CV**, or polymer-**CY**) solutions and then 80 µl of the cell suspension was transferred to 6.5 mm diameter Transwell® inserts (Fisher) where hydrogelation was allowed to complete [38]. Hydrogels were incubated at 37°C with 5% CO₂. Media (DMEM with 10% FBS) was changed every two days and fed in slight excess of 1 ml/million cells/day for up to 21 days *in vitro*.

2.6. Chondrocyte-Laden Hydrogel Analyses

2.6.1. Chondrocyte Viability in Hydrogels—Cell viability was qualitatively assessed with Live/Dead staining (Invitrogen). On day 21 of culture, a quarter of the chondrocyte-laden hydrogel disk was incubated in DMEM containing 2 µM calcein AM and 4 µM ethidium homodimer probes for 20 min [38]. Portions of disks were rinsed with PBS and imaged by fluorescence microscopy (Nikon Eclipse Ti, Melville, NY).

2.6.2. Morphology of Chondrocytes Encapsulated in Hydrogels—Morphology of chondrocytes encapsulated in hydrogels was documented by phase contrast microscopy using a Nikon Eclipse Ti with Digital Sight camera (Melville, NY) on days 1, 10, and 21 of culture.

2.6.3. Biochemical Analysis—Portions of *in vitro* generated chondrocyte-laden hydrogels were biochemically analyzed at specified time points in culture (days 1, 10, and 21). Half of the 6.5 mm diameter hydrogel was solubilized in proteinase K overnight [52]. Portions of the digest were analyzed for DNA using PicoGreen [53], sulfated glycosaminoglycans (GAG) by 1,9 dimethylmethylene blue (DMMB) [54], and collagen by hydroxyproline. DNA content was converted to cell number using a conversion constant of 7.7 pg of DNA per cell. Hydroxyproline content was converted to collagen (COL) content by assuming a mass ratio of collagen to hydroxyproline of 7.14 [55].

2.6.4. Biomechanical Analysis—Bulk mechanical (compression) properties of chondrocyte-laden hydrogels (**VS:C**, **VS:CV**, **VS:CY**) over time in culture (days 1, 10, and 21) were measured using an MTS Synergie 100 (MTS Systems, Eden Prairie, MN) with a 10 N load cell. [56]. Hydrogel samples were subjected to uniaxial confined compression at a constant strain rate of 1%/s from 0 to 20% strain. The compressive modulus was calculated as the slope of the linear portion of the stress-strain curve, determined at low strain regions (0–4% strain).

2.6.5. Histology and Immunohistochemistry—Portions of *in vitro* generated hydrogels were histologically and immunohistochemically analyzed on day 21 of culture. Samples were pre-treated in a solution of 50 mg/ml 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) and lysine (K) for 30 min, in order to introduce cross-linking sites in hydrogel samples for subsequent fixation. After EDC/K treatment, samples were copiously rinsed and fixed in 10% formalin overnight. Samples were then embedded in paraffin, and sectioned vertically to 5 µm thick. Sections were deparaffinized, digested with testicular

hyaluronidase (5000 U/ml), and probed with non-specific monoclonal mouse IgG, anti-collagen I, or anti-collagen II antibody cocktail using the R.T.U. Vectastain University Elite ABC kit and Vector VIP peroxidase substrate kit. Serial sections were stained with 0.5% Alcian Blue in 0.4 M MgCl₂ and 0.025 M sodium acetate (pH 5.6) to stain GAG [57, 58]. Results were documented by brightfield microscopy using a Nikon Eclipse Ti with Digital Sight camera (Melville, NY).

2.7. Statistical Analysis

Effects of hydrogel type and time in culture on all endpoints were assessed by ANOVA with Tukey post-hoc tests. Data are expressed as mean±SEM.

3. RESULTS

3.1. Polymer Synthesis and Characterization

The aims of the current study were to determine the utility of the **VS:C** based hydrogel for encapsulating and culturing chondrocytes in 3D and examine the effects of small functional groups on modulation of chondrocyte function. For this purpose, cysteine and vinyl sulfone functionalized copolymers, polymer-**C** and polymer-**VS** respectively, were first synthesized and characterized following our reported procedure[38]. To probe the influence of small functional groups on chondrocyte behavior, copolymers containing other amino acids differing in hydrophobicity/hydrophilicity were prepared. Specifically, tyrosine or valine was co-functionalized with cysteine to the galactaric acid-lysine copolymer backbone (Figure 1). While **Y** or **V** functionalization modulated the hydrophilicity/hydrophobicity of the hydrogels, the **C** groups were needed in Michael-addition crosslinking during the hydrogelation. Following dialysis purification, ¹H NMR integration was used to estimate the polymer's cysteine, tyrosine and valine functionalization level. Polymers co-functionalized with cysteine/tyrosine or cysteine/valine were capable of crosslinking with polymer-**VS** to afford more hydrophilic or hydrophobic hydrogels compared to **VS:C** hydrogel, respectively. Importantly, surface functionalization was achieved without varying crosslinking density or the basic chemical structure of the hydrogels, allowing investigation of the direct influence of changes in small functional groups on chondrocyte behavior.

3.2. Preparation and Characterization of Hydrogels

3.2.1. Swellability and Degradation of Hydrogels—All hydrogels (**VS:C**, **VS:CV**, **VS:CY**) had similar swellability at the time of cross-linking ($p=0.6$) (Figure 2A). Similarly, the swollen ratio or extent of degradation, determined as a measure of wet weight over time (W_t), of all hydrogels over three weeks *in vitro* was also similar ($p=0.4$) for all the hydrogels used in this study (Figure 2B). Hydrogel degradation was dependent on time in culture, with gels at day 21 representing 97% of the initial hydrogel mass on day 1 ($p<0.05$).

3.2.2. Gelation Kinetics by Oscillatory Rheology—Rheological analysis of the crosslinking of saccharide-peptide hydrogels as a function of time (Figure 2C) indicated similarity among all hydrogels ($p=0.3$). Gels reached an equilibrium storage modulus (G') of 1.5 kPa.

3.3. Chondrocyte-Laden Hydrogel Analyses

3.3.1. Chondrocyte Viability in Hydrogels—Viability of chondrocytes encapsulated in hydrogels (**VS:CV**, **VS:C**, **VS:CY**) was visualized qualitatively with Live/Dead staining, and appeared to be >95% after 21 days *in vitro* (Figure 3A–C).

3.3.2. Morphology of Chondrocytes Encapsulated in Hydrogels—Morphology of chondrocytes encapsulated in hydrogels was observed by phase contrast microscopy during *in vitro* culture. After 1 day *in vitro*, chondrocytes encapsulated in **VS:CV**, **VS:C**, or **VS:CY** hydrogels were round in appearance and did not possess pericellular matrix (Figure 4A–C). By day 10 *in vitro*, differences in pericellular matrix accumulation were evident. Halos of pericellular matrix were evident around chondrocytes encapsulated in **VS:C** and **VS:CY** hydrogels (Figure 4EF). Chondrocytes in **VS:CV** hydrogels did not, qualitatively, appear to accumulate much matrix (Figure 4D). After 21 days *in vitro*, extensive matrix accumulation around encapsulated chondrocytes was evident in **VS:CY** (Figure 4I) hydrogels, followed by chondrocytes encapsulated in **VS:C** (Figure 4H) and **VS:CV** (Figure 4G) hydrogels, which appeared more similar.

3.3.3. Biochemical Analysis—Some biochemical content varied among hydrogels based on the amino acid incorporated into the hydrogel side chain, as well as the time in culture. DNA content of hydrogels were similar regardless of the amino acid incorporated ($p=0.4$), but was significantly lower at day 1 compared to DNA content at day 10 ($p<0.01$) or 21 ($p<0.001$) (Figure 5A). DNA content increased 1.6-fold by day 10 and 2.0-fold by day 21 in culture. GAG content of hydrogels differed significantly based on the type of hydrogel ($p<0.05$) and the time in culture ($p<0.001$), however, interaction effects were not significant ($p=0.6$) (Figure 5B). GAG content of **VS:CY** hydrogels was 2.0-fold higher than **VS:CV** hydrogels ($p<0.05$), and tended to be significantly higher than GAG content of **VS:C** hydrogels ($p=0.1$). GAG content of **VS:CV** and **VS:C** hydrogels were similar ($p=0.7$). GAG content of all hydrogels increased significantly over time in culture. GAG content at day 21 and day 10 were 8.7-fold ($p<0.001$) and 6.5-fold ($p<0.001$) higher than GAG content on day 1 of culture. Total COL content also varied significantly based on the type of hydrogel ($p<0.01$) and time in culture ($p<0.001$), without significant interaction effects ($p=0.4$) (Figure 5C). Total COL content was 1.7-fold higher in **VS:CY** hydrogels compared to **VS:CV** hydrogels, and similar to **VS:C** hydrogels ($p=0.9$). Total COL content of **VS:C** hydrogels was also 1.7-fold higher than **VS:CV** hydrogels ($p<0.05$). In addition, total COL content increased over time in all hydrogels. Total COL content at day 21 and day 10 were 17.2-fold ($p<0.001$) and 9.2-fold ($p<0.001$) higher, respectively, than content at day 1 of culture.

3.3.4. Biomechanical Analysis—Compressive biomechanical properties of chondrocyte-laden hydrogels differed significantly based on the type of hydrogel ($p<0.001$), day in culture ($p<0.001$), and interactive effects of the two aforementioned factors ($p<0.01$) (Figure 6). Overall, both **VS:CY** ($p<0.001$) and **VS:C** ($p<0.05$) hydrogels were significantly stiffer than **VS:CV** hydrogels. Compressive properties of hydrogels significantly increased over time in culture, particularly between days 1 and 10 of culture ($p<0.05$). Increases in compressive modulus between day 10 and day 21 were noted ($p=0.1$) but were less than increases observed earlier in culture. By day 21, the compressive modulus of chondrocyte-laden **VS:CY** hydrogels was significantly higher than **VS:C** (**VS:CY** 2.5-fold higher than **VS:C**, $p<0.05$) and **VS:CV** (**VS:CY** 4.4-fold higher than **VS:CV**, $p<0.05$) hydrogels. **VS:CY** hydrogels had a modulus of 193 ± 46 kPa, compared to 78 ± 18 kPa for **VS:C** and 44 ± 21 kPa for **VS:CV** hydrogels. Correlation analysis (Figure 7) indicated a significant positive correlation between GAG accumulation and compressive modulus ($p<0.01$).

3.3.5. Histology and Immunohistochemistry—Histological and immunohistochemical analyses indicated differences in GAG and collagen staining based on hydrogel type. GAG accumulation was evident in all hydrogel types on day 21 (Figure 8ABC), although staining appeared more intense for **VS:CY** hydrogels (Figure 8C) compared to **VS:CV** (Figure 8A) or **VS:C** hydrogels (Figure 8B). Staining for type I

collagen appeared negative for all hydrogel types (Figure 8DEF). Presence of type II collagen was evident in all hydrogel types but was increased in **VS:C** and **VS:CY** hydrogels, with intense pericellular staining (Figure 8FI) compared to **VS:CV** hydrogels which had comparatively little type II collagen present (Figure 8C).

4. DISCUSSION

In this study, we have tailored our functional saccharide-peptide hydrogel material for 3D chondrocyte encapsulation. Furthermore, we have investigated the effects of amino acid moieties differing in hydrophobicity/hydrophilicity on modulation of chondrocytes three-dimensionally encapsulated in **VS:C** hydrogels. Cysteine or vinyl sulfone functionalized saccharide-peptide copolymers were synthesized for hydrogel preparation[38]. To investigate the effects of amino acid moieties on chondrocyte culture, the saccharide-peptide polymers were co-functionalized with either valine/cysteine (**CV**) or tyrosine/cysteine (**CY**). Whereas the cysteine moiety serves as the cross-linking site for subsequent hydrogelation, amino acids **V** or **Y** were introduced to vary the surface properties of the resulting hydrogels. We chose amino acids for our functionalization in order to maintain the biocompatibility/biodegradability of our hydrogels. While **V** was chosen as a representative hydrophobic amino acid, **Y** was selected as a hydrophilic amino acid.

When cysteine (**C**) and vinyl sulfone (**VS**) functionalized copolymers were combined in stoichiometry, hydrogelation occurred under mild conditions by Michael addition (Figure 1), generating **VS:CV**, **VS:C**, and **VS:CY** hydrogels, respectively. The biocompatibility of our saccharide-peptide hydrogels, combined with mild hydrogelation conditions resulted in high viability of encapsulated chondrocytes in 3D hydrogels up to 21 days *in vitro* (Figure 3). In contrast to many other synthetic hydrogels in which changing one property is often linked with other physical/chemical changes, all the hydrogels used in this study had identical crosslinking density, due to the same amount of cysteine and vinyl sulfone present on the polymer backbone (Figure 2). All the hydrogels had similar swelling ratios with subtle changes in amino acid moieties likely generating cellular microenvironments which differ in hydrophobicity/hydrophilicity. In addition, the synthesis procedure detailed here reproducibly produces cysteine and vinyl sulfone copolymers, resulting in reproducible gelation kinetics. This allows us to probe the effects of small functional groups on cellular behavior. Interestingly, matrix production and accumulation by chondrocytes was significantly enhanced in more hydrophilic hydrogels (**VS:CY**) compared to more hydrophobic hydrogels (**VS:CV**) (Figures 4, 5B, 8). GAG content was significantly increased in **VS:CY** hydrogels compared to **VS:C** or **VS:CV** hydrogels, which correlates well with increased compressive modulus of chondrocyte-laden hydrogels after 21 days of *in vitro* culture (Figures 6, 7). Histological differences in **VS:CY** hydrogels compared to **VS:C** or **VS:CV** hydrogels were evident with increased GAG and type II collagen staining in **VS:CY** hydrogels.

Biomaterial surface functionality and other microenvironmental cues have previously been shown to affect biological properties of cell-laden matrices. Increased collagen deposition was obtained on hydrophilic porous high density polyethylene (HDPE) matrices compared to hydrophobic HDPE matrices[59]. The presence of negatively charged, hydrophilic glycosaminoglycans, such as chondroitin sulfate, within a hydrogel matrix, were shown to support chondrocyte viability and deposition of collagen and proteoglycan production [60, 61]. Finally, encapsulation and culture of chondrocytes in a self-assembling peptide hydrogel, with alternating hydrophilic and hydrophobic moieties, indicated increased matrix deposition and stiffness over time in culture.[62] In the current study, functionalization of a single amino acid moiety, varying in side groups, to three-dimensional hydrogel matrices has resulted in significant changes in chondrocyte behavior over a relatively short period of

time (21 days *in vitro*). In agreement with previous observations[62–65], we observed a significant increase in matrix production for **VS:CY** hydrogel functionalized with a hydrophilic **Y** moiety [49, 63–67] as compared to **VS:CV** hydrogel functionalized with a hydrophilic **V** amino acid. In addition, the mechanical properties achieved here for chondrocyte-laden **VS:C** hydrogels are significantly higher than those obtained previously under comparable cell culture conditions [9] or extended culture durations [68].

The mechanism underlying the observed influence of amino acid moieties on chondrocyte function warrants further investigation. Although the effect of matrix stiffness on cellular function is well known[69], in this study, the stiffness of all acellular matrices was similar, allowing us to isolate the effect of amino acid moieties on chondrocyte function. Here, the relatively low level of functionalization of amino acid moiety did not have a statistically significant effect on equilibrium swollen ratio. Presumably, a more subtle effect is present in influencing chondrocyte behavior and matrix accumulation. In native cartilage, hydrophilic proteoglycans[70] make up a significant portion of cartilage content. Hydrogels which mimic the chondrocyte microenvironment, particularly the presence of hydrophilic polysaccharides[71], have shown promise for cartilage tissue engineering. It is possible that, similarly, hydrophilic amino acids (such as **Y**) could have more beneficial influence on chondrocyte behavior than more hydrophobic amino acids (such as **V**).

In future studies, longer durations for cell culture *in vitro*, and individual or combinatorial exploration of other amino acids would be useful in further optimizing the hydrogel function as well as gaining mechanistic understanding of modulating chondrocyte function and related implications for cartilage repair. In addition, even though significant mechanical properties of chondrocyte-laden **VS:C** hydrogels were achieved here, the influence of dynamic loading during *in vitro* culture of these hydrogels has not been established and could potentially further enhance mechanical properties[9, 28]. Finally, the highly functional and versatile saccharide-peptide copolymer employed here could be modified with other biologically relevant components to further enhance the performance.

5. CONCLUSIONS

In this study, we have tailored a saccharide-peptide hydrogel material to impart specific microenvironmental cues, via amino acid functionalities, to encapsulated chondrocytes. Cysteine and vinyl sulfone-functionalized saccharide-peptide polymers were further functionalized with amino acid moieties differing in side groups (valine, cysteine, tyrosine). Mechanical and biochemical properties of chondrocyte-laden hydrogels were controlled by inclusion of hydrophobic/hydrophilic amino acid functional groups. Extensive matrix accumulation and concomitant increase in mechanical properties was evident over time in culture, particularly with the presence of **Y** amino acid. Overall, this study indicates the promise of subtly altering the cellular microenvironment with single amino acid moieties, resulting in modulation of chondrocyte-laden hydrogel properties. In the future, studies will focus on applying combinatorial effects of functional groups on cellular behavior via the **VS:C** hydrogel platform.

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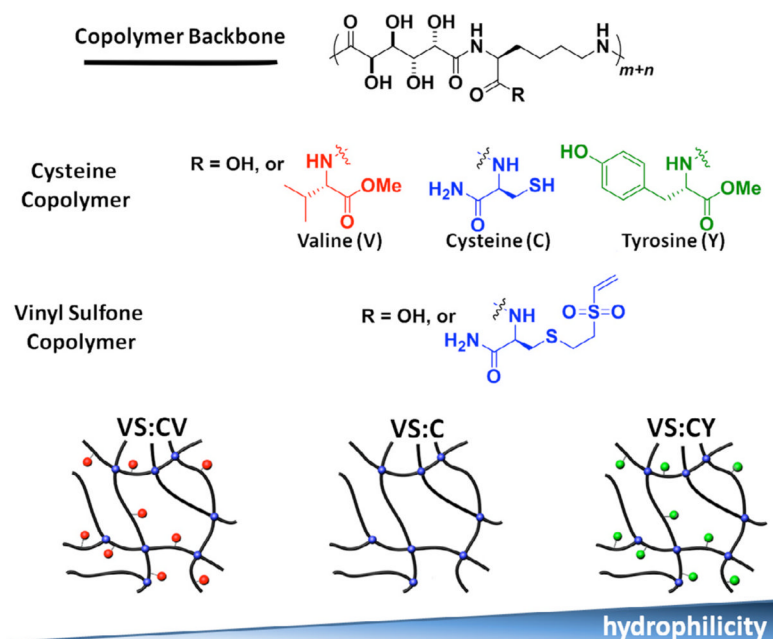


Figure 1. Schematic of methods used to generate **VS:CV**, **VS:C**, **VS:CY** hydrogels, varying in hydrophobicity/hydrophilicity. Two types of saccharide-peptide copolymers were generated. Cysteine (**C**) functionalized copolymers were further functionalized with either valine (**V**) or tyrosine (**Y**) amino acids. Separately, vinyl sulfone (**VS**) functionalized copolymers were also generated. Hydrogelation occurred by Michael addition, when cysteine and vinyl sulfone functionalized copolymers were combined at equimolar ratio. This resulted in three types of hydrogels, varying in hydrophobicity/hydrophilicity: **VS:CV**, **VS:C**, **VS:CY**.

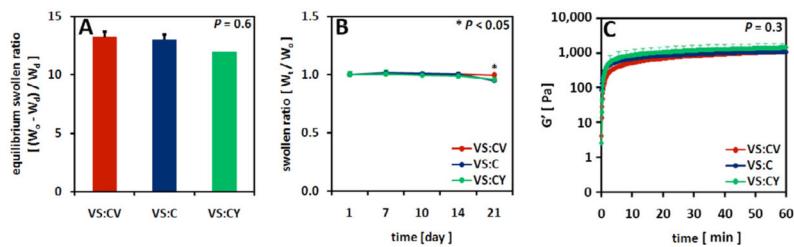


Figure 2. Effects of hydrogel type on hydrogel swelling properties, degradation, and evolution of rheological storage modulus (G'). VS:CV, VS:C, VS:CY hydrogels were analyzed for (A) equilibrium swelling ratio, (B) monitored for degradation over 21 days *in vitro* culture, and (C) rheologically characterized to assess evolution of G' over time. Mean \pm SEM, n=3.

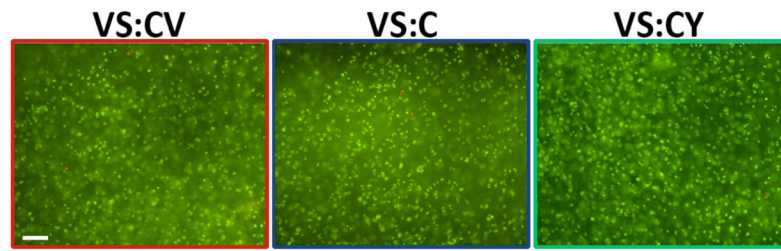


Figure 3. Viability of chondrocytes encapsulated in **VS:CV**, **VS:C**, **VS:CY** hydrogels, assessed by Live/Dead stain on day 21 of *in vitro* culture. Live cells are stained with calcein AM (green) and dead cells with ethidium homodimer-1 (red). Bar = 100 μm .

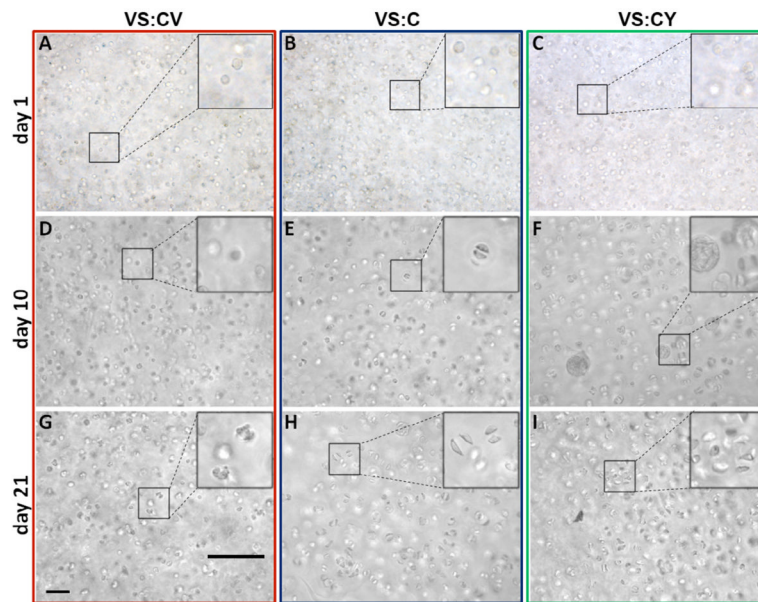


Figure 4. Matrix production and accumulation by chondrocytes encapsulated in **VS:CV**, **VS:C**, **VS:CY** hydrogels on (A–C) days 1, (D–F) 10, and (G–I) 21 *in vitro*. Matrix production and accumulation was documented by phase microscopy. Bar= 100 μ m.

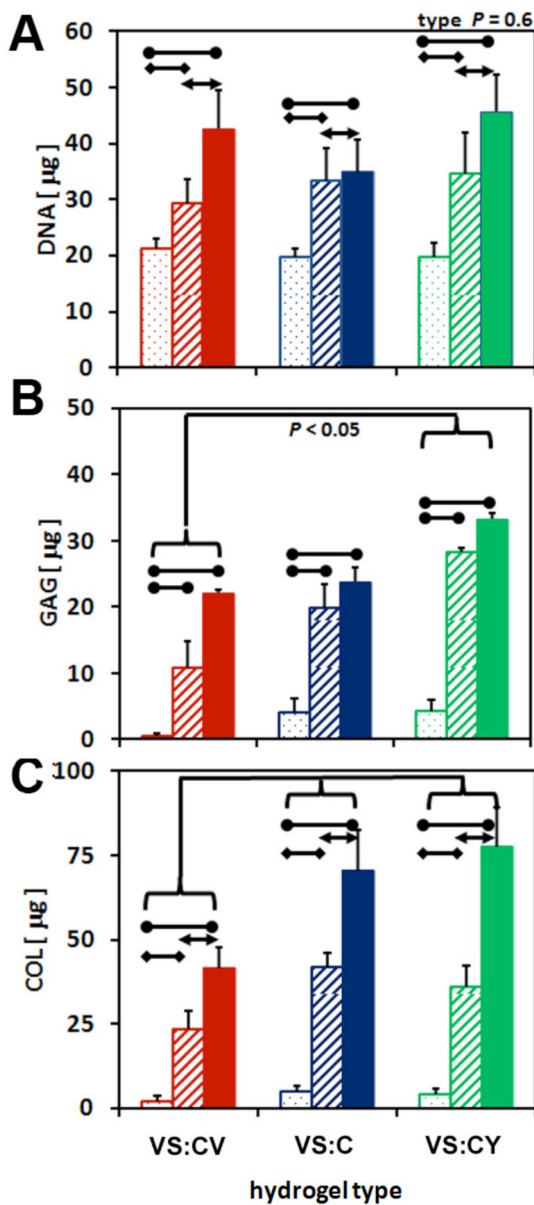


Figure 5. Effects of hydrogel type (VS:CV, VS:C, VS:CY) on biochemical properties of hydrogels. (A) DNA, (B) glycosaminoglycans (GAG), and (C) collagen (COL) content of hydrogels were determined on days 1 (dot), 10 (stripe), and 21 (solid) of *in vitro* culture. Data are mean \pm SEM, $n = 5-10$.

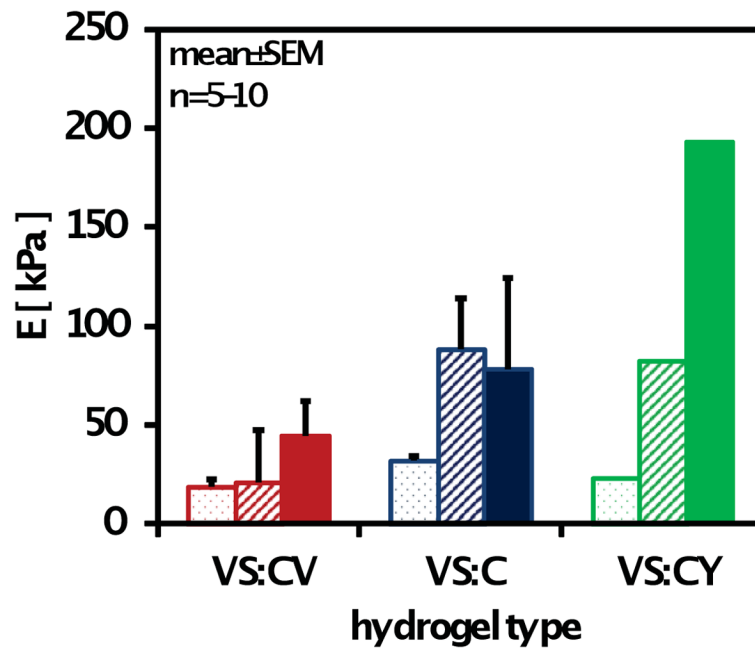


Figure 6. Effects of hydrogel type (VS:CV, VS:C, VS:CY) on compressive properties of hydrogels. Modulus of hydrogels were determined on days 1 (dot), 10 (stripe), and 21 (solid) of *in vitro* culture. Data are mean \pm SEM, $n = 5-10$.

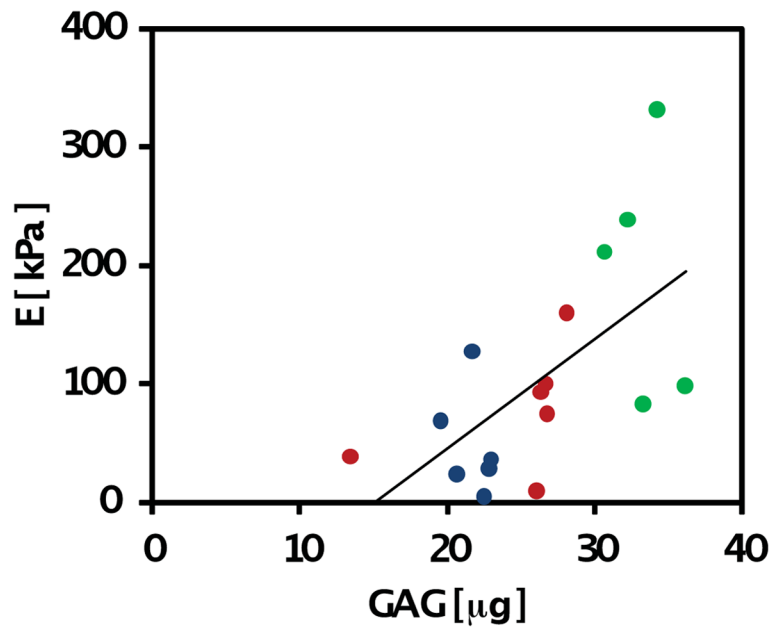


Figure 7. Correlation analysis of GAG content of VS:CV, VS:C, VS:CY and compressive modulus of hydrogels on day 21 of *in vitro* culture. Linear regression resulted in significant correlation ($P < 0.01$).

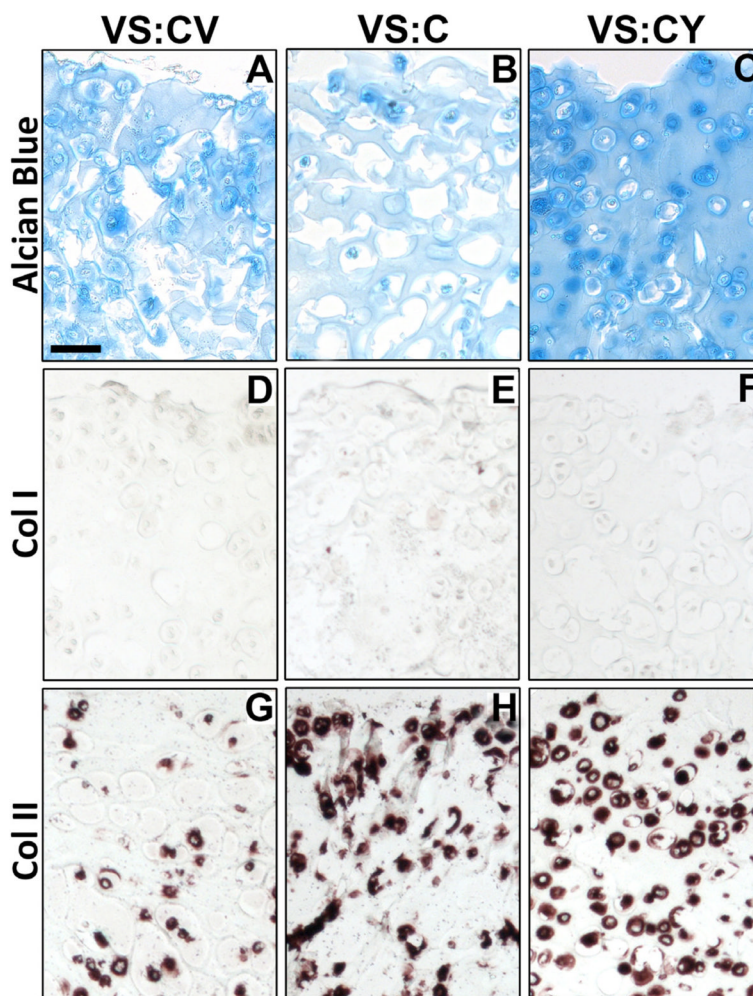


Figure 8. Effects of VS:CV, VS:C, VS:CY hydrogels on localization of glycosaminoglycans (GAG), type I collagen, and type II collagen by encapsulated chondrocytes on day 21 of *in vitro* culture. Sections of chondrocyte-laden hydrogels were stained with (A–C) Alcian blue, or antibodies for (D–F) type I collagen (Col I), or (G–I) type II collagen. Bar = 50 μm .