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Sulfated Alginate Hydrogels Prolong the Therapeutic Potential of MSC Spheroids by Sequestering the Secretome

Marissa Gionet-Gonzales,

Department of Biomedical Engineering, University of California, Davis, Davis, CA 95616

Alena Casella,

Department of Biomedical Engineering, University of California, Davis, Davis, CA 95616

Daphne Diloretto,

Department of Biomedical Engineering, University of California, Davis, Davis, CA 95616

Clara Ginnell,

Department of Biomedical Engineering, University of California, Davis, Davis, CA 95616

Katherine H. Griffin,

School of Veterinary Medicine, University of California, Davis, CA, 95616, USA

Department of Orthopaedic Surgery, UC Davis Health, Sacramento, CA 95817

Anne Bigot,

Universite de Paris, Institut de Myologie, Paris, France 75013

J. Kent Leach*

Department of Biomedical Engineering, University of California, Davis, Davis, CA 95616

Department of Orthopaedic Surgery, UC Davis Health, Sacramento, CA 95817

Abstract

Cell-based approaches to tissue repair suffer from rapid cell death upon implantation, limiting the window for therapeutic intervention. Despite robust lineage-specific differentiation potential *in vitro*, the function of transplanted mesenchymal stromal cells (MSCs) *in vivo* is largely attributed to their potent secretome comprised of a variety of growth factors (GFs). Furthermore, GF secretion is markedly increased when MSCs are formed into spheroids. Native GFs are sequestered within the extracellular matrix (ECM) *via* sulfated glycosaminoglycans, increasing the potency of GF signaling compared to their unbound form. To address the critical need to prolong the efficacy of transplanted cells, we modified alginate hydrogels with sulfate groups to sequester endogenous heparin-binding GFs secreted by MSC spheroids. We assessed the influence of crosslinking method and alginate modification on mechanical properties, degradation rate, and degree of sulfate modification. Sulfated alginate hydrogels sequestered a mixture of MSC-secreted endogenous biomolecules, thereby prolonging the therapeutic effect of MSC spheroids for tissue

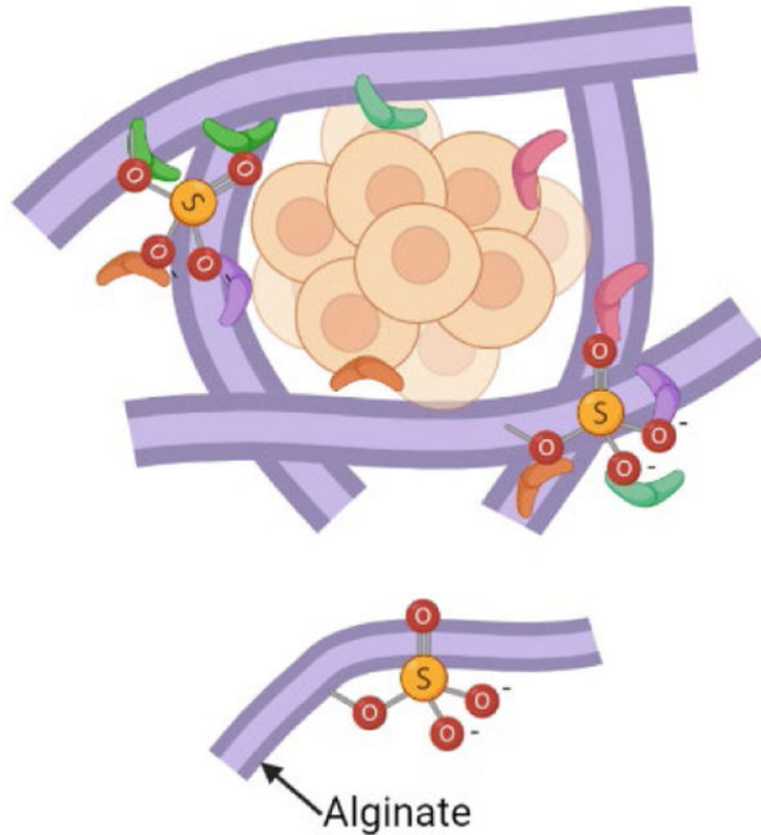
*Corresponding author: J. Kent Leach, Ph.D., University of California, Davis, Department of Orthopaedic Surgery, 4860 Y Street, Suite 3800, Sacramento, CA 95817, jkleach@ucdavis.edu.

7. Disclosure Statement

The authors have nothing to disclose.

regeneration. GFs were sequestered for longer durations within sulfated hydrogels and retained their bioactivity to regulate endothelial cell tubulogenesis and myoblast infiltration. This platform has the potential to prolong the therapeutic benefit of the MSC secretome and serve as a valuable tool for investigating GF sequestration.

Graphical Abstract



Spheroids of mesenchymal stromal cells (MSCs) secrete high levels of relevant growth factors (GFs) for tissue regeneration. Sulfated alginate can sequester these GFs through ionic interactions, allowing increased therapeutic potency to invading cells. This paper investigates how MSC spheroid-secreted GFs are retained in sulfated alginate and their ability to influence cell function *in vitro*.

Keywords

mesenchymal stromal cells; secretome; heparin-binding; spheroids; sulfate

1. Introduction

Cells are the building blocks of new and replacement tissues that offer a therapeutic benefit often unmatched by cell-free approaches. Consequently, a multitude of tissue engineering strategies have employed the delivery of cells to wound sites for improved regeneration.[1]

Mesenchymal stromal cells (MSCs) are commonly used for this application due to their ease of harvest from adult patients, trilineage potential, and their therapeutic secretome that recruits host cells to the tissue site, promotes angiogenesis, and modulates the local inflammatory microenvironment.[2] The MSC secretome is composed of a complex mixture of tissue-reparative cytokines and endogenous growth factors (GFs) including VEGFA, IGF-1, and IL-8. Despite their robust differentiation potential *in vitro*, it is widely assumed that MSCs primarily contribute to tissue regeneration and repair *via* this potent secretome. [3]

Cell-based tissue engineering approaches are limited by rapid cell death upon implantation into a harsh microenvironment, thereby reducing the duration and overall potential of this strategy.[4] In contrast to monodisperse cells, the aggregation of MSCs into spheroids results in prolonged cell viability and enhanced secretion of endogenous GFs.[5, 6] The behavior of MSC spheroids can be further influenced by the biophysical properties of carriers used to transplant cells. For example, hydrogel stiffness and adhesivity can be tuned to upregulate GF production by MSC spheroids.[6, 7] However, the therapeutic effects of MSC spheroids are limited by their survival and rapid diffusion of endogenous GFs from many cell carriers and delivery vehicles.

To address the challenge of short-term cellular contributions to cell-based therapies, there is a critical need for the development of advanced biomaterials that prolong or retain the bioactivity of endogenous GFs or other bioactive moieties.[8, 9] Beyond tuning the morphological and mechanical properties of biomaterials, one strategy is designed to mimic a key role of the native extracellular matrix (ECM): retention and presentation of endogenous GFs secreted by neighboring cells. The presentation and release of GFs from biomaterials can be regulated by controlling pore density and gel composition, covalently tethering GFs to materials, and sequestering GFs *via* charged polymers.[10–12] Heparan sulfate (HS), a negatively charged glycosaminoglycan that reversibly binds positively charged GFs, is a key GF-binding constituent of the native ECM. HS function has been mimicked using naturally occurring sulfated polymers (*e.g.*, heparin) or modifying polymers such as polyethylene glycol (PEG) and alginate with functional sulfate groups.[13, 14] Sulfated alginate has been used as a GF delivery vehicle for applications in hind limb ischemia and myocardial infarction, demonstrating a 3-fold greater retention of entrapped GFs compared to unmodified alginate.[15] However, this platform was utilized to deliver a single GF for a therapeutic application, which severely limits the potential of endogenous GFs to be retained in the platform. MSC spheroids are under investigation for the utility of their secretome,[16] a complex mixture of endogenous GFs, and cells are commonly transplanted using engineered hydrogels.[17, 18] Thus, it is imperative to develop and characterize the ability of biomaterial platforms to sequester the endogenous secretome produced by entrapped MSC spheroids.

We hypothesized that alginate hydrogels could be modified to support survival of entrapped MSC spheroids and sequester components of the endogenous secretome as a means to prolong their therapeutic potential. We sought to independently control initial moduli, degradation rate, and degree of sulfation of these modified hydrogels to tune the production and retention of endogenous GFs. We then investigated the capacity of sulfated alginate

hydrogels to capture the bioactive MSC spheroid secretome and enhance cell infiltration to demonstrate the utility of this platform.

2. Materials and Methods

2.1 Modification of alginate with functional groups

PRONOVA UP VLVG alginate (<75,000 g/mol; NovaMatrix Sandvika, Norway) was modified with sulfate groups on the polymer backbone as described.[19] Briefly, dried VLVG was dissolved in formamide at 2.5% (w/v). Chlorosulfonic acid (HSO_3Cl ; Sigma Chemical, St. Louis, MO) at 0–2% (v/v) was added to the solution and incubated at 60°C for 2.5 hrs while stirring. Alginate was then precipitated out of solution in cold acetone and dissolved in ultrapure water overnight. Sodium hydroxide was added dropwise to the alginate solution until each sample reached a neutral pH (~7). The solutions were pipetted into 3500 Da molecular weight cut off (MWCO) dialysis tubing (Spectrum Laboratories, New Brunswick, NJ). The tubes were maintained in 2 L of 100 mM sodium chloride solution for 12 hrs and then ultrapure water the next 2.5 days, with water changes every 6–12 hrs. The alginate was then recovered from the dialysis tubing, filtered through a 0.22 μm pore filter, and lyophilized for up to 7 days until dry.

PRONOVA UP MVG (>200,000 g/mol; NovaMatrix Sandvika) was oxidized as previously described.[20] Alginate was dissolved overnight in ultrapure water at a 1% (w/v) solution and reacted with 1 mM of sodium periodate for 1% oxidation or 5 mM for 5% oxidation. The reaction was quenched after 17 hrs in darkness with stirring using an equimolar amount of ethylene glycol. The alginate was then dialyzed in ultrapure water, filtered, and lyophilized.

PRONOVA UP VLVG and oxidized PRONOVA UP MVG were modified with Arg-Gly-Asp (RGD) through standard carbodiimide chemistry.[21] Alginate was first dissolved in a 1% (w/v) solution in MES buffer (0.1 M MES, 0.3 M NaCl pH 6.5) overnight. The next day, N-(3-Dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC) and N-Hydroxysulfosuccinimide sodium salt (Sulfo-NHS) were added to the reaction at a ratio of 2:1 per gram of alginate. The peptide G4RGDSP (Commonwealth Biotechnologies, Richmond, VA) was then added to the reaction to achieve a degree of substitution (DS) of 2. The resulting RGD-alginate was put in a dialysis tube (6–8 kDa MWCO, Spectrum Laboratories) in a water bath for three days. The solution was sterile filtered and lyophilized for 4 days.

2.2 Detection of sulfate groups on the alginate polymer

Sulfate modification of alginate was confirmed *via* ^1H NMR as described.[21] Briefly, lyophilized alginate samples were dissolved in D_2O at 3.33% (w/v) concentration and recorded using an 800 MHz Bruker Avance III NMR spectrometer. Alginate sulfate modification was further verified through 1,9-Dimethyl-Methylene Blue zinc chloride double salt (DMMB) assay.[22] The DMMB solution pH was reduced to 1.5 to eliminate false readings from the negatively charged alginate polymer. Sulfated alginate was compared *via* DMMB assay with a sample of decellularized ECM derived from human MSCs and

commercially available heparin sodium salt (Fisher Scientific, Hampton, NH). ECM was decellularized as previously described, including treatment with a detergent solution and deoxyribonuclease.[23]

2.3 Sulfated hydrogel synthesis

All alginate was dissolved at a concentration of 25 mg/mL in PBS. Alginate hydrogels were produced by combining sulfated alginate with non-sulfated alginate in a ratio of 1:7 (sulfated:non-sulfated). The sulfated alginate consisted of VLVG alginate reacted with 0, 1 or 2% chlorosulfonic acid. The non-sulfated alginate was composed of 1 part RGD-modified VLVG alginate and 1 part oxidized (either 1% or 5%) RGD-modified MVG, as described in Table 1. A visual representation of the gel composition can be seen in Figure 2A. After mixing *via* gentle tube rotation for 10 min, the alginate mixture was pipetted into 8 mm diameter circular silicone molds sandwiched between two dialysis membranes (6–8 kDa MWCO, Spectrum Laboratories). A solution of either 6 mM BaCl₂ and 200 mM CaCl₂ (high elastic modulus) or 3 mM BaCl₂ and 100 mM CaCl₂ (low elastic modulus) was pipetted onto the top dialysis membrane for 5 min. The gels were then flipped, and the ionic solution was pipetted onto the other dialysis membrane for an additional 5 min. Both dialysis membranes were removed, and the gels were placed in a bath of the same ionic solution for an additional 10 min. Gels were then removed from the mold and used immediately.

2.4 Mechanical testing and degradation

We tested the shear storage moduli of 8 mm diameter gels using a Discovery HR2 Hybrid Rheometer (TA Instruments, New Castle, DE) with a stainless steel, cross hatched, 8 mm plate geometry. We performed an oscillatory strain sweep ranging from 0.004% to 4% strain on each gel to obtain the linear viscoelastic region (LVR) before failure.[21] At least 5 data points were collected for the LVR and averaged to obtain gel shear storage modulus. Gels were measured after 1, 14, or 21 days in serum-free α MEM at standard sterile culture conditions (37°C, 21% O₂, 5% CO₂). After testing, gels were frozen and lyophilized for 24 hrs or until dry. The dry mass of the gels was determined using a Mettler Toledo XPR2 Microbalance (Mettler-Toledo, Columbus, OH).

2.5 Cell culture and MSC spheroid formation

Human MSCs (RoosterBio, Frederick, MD) were cultured in α MEM (Invitrogen, Carlsbad, CA) with 10% fetal bovine serum (FBS) (Biotechne, Minneapolis, MN) and 1% penicillin-streptomycin (pen/strep) (Gemini Bio Products, West Sacramento, CA) in standard conditions. MSCs were used at passage 3–5 for all experiments. TdTomato-expressing human hTERT/cdk4 immortalized myoblasts (Institute of Myology, Paris, France) were cultured in 1:4 ratio of Media 199 (Thermo Fisher, Chicago, IL) and DMEM (Invitrogen) supplemented with 20% FBS, 1% pen/strep, 25 μ g/mL fetuin, 5 ng/mL of hEGF, 0.5 ng/mL of bFGF, 5 μ g/mL of insulin, and 0.2 μ g/mL dexamethasone (all from Millipore Sigma, Burlington, MA) under the same conditions.[24] Myoblasts were cultured in flasks until 80% confluence or lower to discourage cell fusion and myotube formation. Human dermal microvascular endothelial cells (HDMECs, Lot #437592; Lonza, Basel, Switzerland) were cultured in complete endothelial cell growth media (EGM2-MV). HDMECs were cultured

for 6 days under standard conditions or until 75–80% confluent before use, and cells were used between passage 4–5 for all experiments.

MSC spheroids were produced using the forced aggregation technique as we described.[21, 25] Once cultured to confluence, MSCs were trypsinized and centrifuged down into 2,000 μm pores made out of 1.5% agarose to form 40,000-cell spheroids. After 48 hrs in static conditions to enable spheroid formation, MSC spheroids were pipetted into the alginate solutions at 800,000 cells per gel and crosslinked into hydrogels as described above.

2.6 Retention of proteins in modified alginate gels

Hydrogels were loaded with 1 μg HGF (PeproTech, Rocky Hill, NJ) as a model protein to verify that sulfate groups bind heparin-binding GFs. As a control experiment, another set of hydrogels was loaded with 1 μg IGF-1 (PeproTech), a non-heparin binding GF, to verify the specificity of GF retention. All gels were cultured in serum-free media collected at various time points for GF quantification. HGF and IGF detection was quantified using Human Quantikine ELISA Kits specific for each GF (R&D Systems, Minneapolis, MN) and assessed on a Synergy HTX Multi-Mode Reader (BioTek, Winooski, VT) at 450 nm. To quantify hydrogel-retained cytokines, MSC spheroids were cultured in sulfated or non-sulfated hydrogels for 3 days, then collected in 1 mg/mL alginate lyase (Millipore Sigma) in a 1.4 M NaCl solution. MSC spheroid-secreted GFs were quantified and identified using the IsoLight System (IsoPlexis, Branford, CT) with the Human Innate Immune cytokine panel.

2.7 Endothelial cell tubulogenesis assay

MSC spheroids were entrapped in alginate sulfated with either 0, 1, or 2% HSO_3Cl and cultured for 21 days. The media was refreshed every 2–3 days and subsequently stored at -20°C for later use. Before use, the conditioned media was thawed on ice, vortexed briefly, then centrifuged at $700\times g$ for 8 min to pellet any cell debris or ECM. Immediately before use, the media was warmed to 37°C , and 200 μL of supernatant was used for experimentation.

Matrigel (Corning, Corning, NY) was pipetted into a 48-well plate and incubated at 37°C for 1 hr to ensure gelation. HDMECs were trypsinized and resuspended at a concentration of 30,000 cells per well in either complete EGM2 as the positive control, GF-deficient media as the negative control, or conditioned media for the experimental groups. Tubule formation proceeded for 6 hrs. The media was then removed, a 2 mM calcein AM solution was added to each well, and plates were incubated for an additional 30 min. Each well was imaged in 3 different locations; network length and branch number were quantified using the ImageJ Angiogenesis Analyzer plugin. Network length was quantified as the sum of all segments in the network, and branches were defined as segments linked to one junction, a bifurcation point, and one extremity, a segment with a free end.[26]

2.8 Myoblast infiltration into hydrogels

MSC spheroids were loaded into sulfated alginate hydrogels at 800,000 cells per gel (20 spheroids per gel; 40,000 cells per spheroid). The gels were cultured in complete αMEM for 4 days to allow for accumulation of the secretome within the hydrogel. To test the potential

of GFs retained after cell death, gels were frozen at -80°C for 2 weeks, while live spheroid hydrogels were used immediately after the 4-day culture. Hydrogels were then placed in non-tissue culture treated 24-well plates and seeded with 1×10^6 tdTomato myoblasts in a 50:50 α MEM:myoblast media mixture. Hydrogels were moved to new plates after 24 hrs and media was refreshed every 2–3 days to prevent GFs secreted from non-adherent myoblasts to confound results. After 8 days, myoblast infiltration was characterized *via* confocal imaging. Z stacks of 100–300 μm depth were analyzed using Imaris software to quantify myoblast infiltration depth and sphericity.

2.9 Macrophage migration into hydrogels and subsequent polarization

Conditioned media was collected from 1×10^6 rat MSCs formed into 40k spheroids cultured in 100 μL of media for 3 days. Alginate was then dissolved into the collected media and formed into hydrogels. IC-21 macrophages (ATCC, Manassas, VA) were seeded in non-tissue culture-treated wells containing the hydrogels at 500,000 cells per well immediately following gel formation. Gels were maintained in RPMI 1640 (ATCC) supplemented with 10% FBS. After 24 hrs, gels were moved to a new plate with fresh media, maintained in culture for another 24 hrs, and then collected.

Cells were recovered from alginate *via* digestion at 37°C in a 200 mM sodium citrate and 100 mM EDTA solution in PBS supplemented with 3% FBS, pH 6.8, at a 10:1 v:v ratio with alginate. Gels were digested for 45 min until alginate appeared dissolved. Solutions were filtered through a 100 μm filter to create a single cell suspension, and macrophage polarization was characterized using flow cytometry (Attune NxT, Life Tech). Following Fc γ receptor blocking (1:40, TruStain FcX, BioLegend, San Diego, CA), cells were stained with antibodies against CD86 (1:160, eBioscience, Hatfield, United Kingdom, #47-0862-82) and CD206 (1:40, eBioscience #48-2061-82). Cellular viability was evaluated with fixable Zombie Aqua (1:250, Life Tech, Carlsbad, CA). Cells were then fixed with 2% PFA, permeabilized with 0.1% Triton-X, and stained for intracellular markers, iNOS (1:500, eBioscience #12-5920-82) and Arginase-1 (1:500, eBioscience #53-3697-82), overnight at 4°C with gentle agitation.

Macrophages with an M1 phenotype were characterized by CD86+iNOS+ populations and M2 phenotypes by CD86-CD206+ARG1+ populations. The frequency of each type of macrophage was quantified as a function of sulfation. Polarization controls (*data not shown*) consisted of IC-21s seeded on TC wells in monolayer treated with basal media (M0), 200 ng/mL LPS (M1), and 20 ng/mL IL-4 (M2) for 24 hrs. Cells were lifted with cold 2.5 mM EDTA solution in PBS and cell scraping. Cells were filtered and stained as described.

2.10 Statistical analysis

Data are presented as means \pm standard deviation. All experiments represent at least three independent experiments unless otherwise noted. Statistical analysis was performed using a one-way or two-way analysis of variance with Bonferroni correction for multiple comparisons in Prism 9 software (GraphPad, San Diego, CA); p-values less than 0.05 were considered statistically significant. Significance is denoted by alphabetical letterings; groups

with no significance are linked by the same letters, while groups with significance do not share the same letters.

3. Results

3.1 Addition of sulfate groups to alginate increases retention of growth factors in hydrogels

Alginate was reacted with chlorosulfonic acid (HSO_3Cl), resulting in the substitution of hydroxyl groups with sulfate groups (Fig. 1A). The success of this reaction was evaluated using ^1H NMR analysis to verify peak shifts. Protons on the 1 and 5 positions on the G block of alginate (G1, G5) and the proton in the 1 position on the M block (M1) were identified and labeled. We observed increased substitution of sulfate groups on the alginate backbone with increasing concentration of HSO_3Cl . All peaks exhibited a shift upfield with increasing sulfate modification, indicative of shielding or increase in electron density (Fig. 1B). This verifies that increasing the concentration of HSO_3Cl in the reaction results in alginate possessing higher negative charges due to the addition of sulfate groups. Subsequently, three concentrations were explored (0%, 1%, and 2% HSO_3Cl) and identified as non-sulfated, low-sulfated, and high-sulfated alginate, respectively.

Sulfate modification was further confirmed using a DMMB assay, a protocol used to determine the GAG content in physiological samples. In agreement with ^1H NMR analysis, increases in the HSO_3Cl correlated with greater sulfate modification of the alginate. Differences among the hydrogels were apparent with as low as 0.5% HSO_3Cl , indicating the efficiency of this reaction (Fig. 1C). To further validate this sulfate modification method, we evaluated batch-to-batch variation of modification with 1% HSO_3Cl using the DMMB assay. Sulfation was similar among three batches, confirming the reproducibility of this modification method (Fig. 1D). Crosslinked hydrogels were stained with DMMB to qualitatively observe the spatial distribution of sulfate in a 3D hydrogel. DMMB exhibits a blue color until it is bound to sulfate and turns pink. Gels exhibited relatively homogenous staining, with sulfate-bound pink staining most apparent in the 2% HSO_3Cl gels, 0% HSO_3Cl gels exhibiting minimal blue staining, and 1% HSO_3Cl exhibiting a combination of both blue and pink staining (Fig. 1E). The sulfate concentration detected on the alginate was also compared to heparin and decellularized ECM *via* DMMB assay (Supplemental Fig. 1). All sulfated alginate groups exhibited lower sulfate modification than heparin but higher sulfate concentrations than decellularized ECM secreted by human MSCs. Collectively, these data demonstrate the efficiency, reproducibility, and tunability of this method to modify alginate with sulfate groups.

Upon verification of sulfate modification of the alginate, we sought to demonstrate that the sulfate groups were functional and could sequester GFs. We loaded all hydrogels with known quantities of HGF and monitored its release over 7 days. Compared to the non-sulfated control, HGF was retained within sulfated alginate longer, exhibiting slower release curves over 7 days compared to non-sulfated ($p < 0.0001$) and 1% ($p = 0.046$) hydrogels (Fig. 1F,G). We confirmed the functionality of sulfated alginate to retain GFs by studying the encapsulation and release of a non-heparin binding GF, IGF-1. These curves revealed no differences in release rate, indicating sulfation had no effect on the retention of non-heparin

binding GFs (Supplemental Fig. 2). These data demonstrate that sulfate-modified alginate can effectively retain heparin binding growth factors, while non-heparin binding growth factors readily diffuse from the gel into the surrounding environment.

3.2 Sulfate modification, degradation and initial moduli can be decoupled

Hydrogels were prepared from a mixture of sulfated VLVG, non-sulfated VLVG, and oxidized non-sulfated MVG alginates (Fig. 2A). 1% oxidized MVG gels were dual-crosslinked using either 6 mM BaCl₂ and 200 mM CaCl₂ to achieve a high initial modulus or 3 mM BaCl₂ and 100 mM CaCl₂ for a low initial modulus (Table 1). Since sulfate groups can inhibit the formation of ionic crosslinks, BaCl₂ was used to strengthen hydrogel formation, as it is able to crosslink both guluronic (G) and mannuronic (M) acid blocks in alginate, while CaCl₂ primarily crosslinks only G blocks.[27] A higher concentration of CaCl₂ was supplemented with BaCl₂ to further increase crosslinking since higher concentrations of BaCl₂ can be cytotoxic.[28] On day 1, we observed significant differences in initial elastic moduli between the high moduli (14.5 ± 3.2 kPa) and low moduli groups (7.5 ± 2.4 kPa; p<0.0001) regardless of the level of sulfation (Fig. 2B). Next, we assessed hydrogel degradation by quantifying changes in moduli at day 14 (Fig. 2C,D) and measuring dry mass over time (Supplemental Fig. 3). Both groups exhibited significant reductions in moduli compared to initial elastic modulus by day 14, with the low moduli group decreasing to 1.8 ± 0.9 kPa and the high moduli decreasing to 3.3 ± 0.9 kPa (p<0.0001 for both groups). We did not detect differences in modulus as a function of sulfate modification at either time point. These data confirm that the degree of sulfate modification on the backbone of alginate can be controlled without sacrificing the ability to tune initial moduli.

In order to decouple the interplay between sulfate modification and degradation, we incorporated our sulfated alginate into a faster degrading hydrogel composed of 5% oxidized MVG (Table 1). The initial moduli for these hydrogels were 13.2 ± 3.7 kPa for the high moduli group and 7.1 ± 2.4 kPa for the low moduli group, similar to moduli for the slower degrading 1% oxidized gels. As expected, the moduli of the 5% oxidized high group decreased more by 14 days to 1 ± 0.5 kPa (Fig. 2E,F). These changes were further confirmed by measuring the dry mass degradation of the high initial moduli group (Supplemental Fig. 3A,B). The dry mass decreased significantly for all gels from day 1 to 14 in the low moduli group except those modified with 1% HSO₃Cl (Supplemental Figure 3B). To verify that degradation did not significantly influence the availability of sulfate groups, we performed a DMMB assay on gels after 14 days to determine if degrees of sulfate modification could be detected. All formulations exhibited clear differences in sulfate modification between 0% and 2% HSO₃Cl groups, with all except the high moduli 1% oxidation group exhibiting statistical differences between all groups (Supplemental Fig. 4). We also measured swelling ratio and mesh size of sulfated hydrogels. Sulfation did not appreciably influence these parameters, confirming that hydrogel physical properties are independent of modification with sulfate groups (Supplemental Fig. 5A,B). To investigate how hydrogel properties change over longer periods of time, we measured the moduli and mesh size at day 21. As predicted, the moduli decreased, and mesh size increased after 21 days, however there were no differences between sulfated and non-sulfated hydrogels. These data verify that the

degree of sulfate modification, degradation rate, and initial modulus can be independently tuned.

3.3 MSC spheroids entrapped in sulfated alginate are viable and secreted GFs are retained

All subsequent experiments were carried out with 1% oxidized hydrogels crosslinked with 6 mM BaCl₂ and 200 mM CaCl₂, as this composition exhibited the most durable hydrogels at later time points. To verify that our dual ionic crosslinking method did not adversely affect cell viability, we entrapped human MSC spheroids within our hydrogels and evaluated viability after 8 and 21 days (Fig. 3A). At both time points, we observed high viability of the MSC spheroids and no observable differences between sulfated and non-sulfated groups when stained with LIVE/DEAD stain, indicating that neither the exposed sulfate groups nor ionic crosslinking method were detrimental toward cell viability (Fig. 3B–C). We detected comparable metabolic activity *via* alamarBlue between groups at day 21 (Fig. 3D).

We then sought to determine if sulfated alginate could retain cell-secreted GFs. After 24 hours, the retained MSC-secreted VEGF in all gels was equivalent. However, the amount of VEGF retained in the 1% and 2% gels increased significantly from day 1 to day 4 ($p < 0.0001$ and $p = 0.0002$), whereas the 0% group exhibited no change in VEGF retention (Fig. 3E). These data confirm the functionality of sulfated hydrogels to sequester both recombinant GFs as well as endogenous, cell-secreted VEGF at higher levels than non-sulfated hydrogels. We examined the diversity of GFs and cytokines within sulfated gels to assess the efficiency of MSC secretome retention. A variety of factors were retained at higher levels in sulfated alginate compared to unmodified gels. The greatest cytokine signals were derived from IL-8, IL-6, and VEGF, with sulfated gels exhibiting significantly higher levels of VEGF compared to their non-sulfated counterparts. Cytokines detected at lower concentrations exhibited similar trends, with heparin binding factors such as interferon gamma (IFN- γ) present in significantly increased concentrations in sulfated gels (Fig. 3F–G). However, non-heparin binding GFs such as epidermal growth factor (EGF) exhibited no increase in retention. These data indicate that sulfated alginate hydrogels can retain an array of cytokines secreted by MSC spheroids.

3.4 MSC spheroid-secreted GFs are effectively retained in sulfated hydrogels

We indirectly assessed the bioactivity of sequestered factors within sulfated alginate gels by collecting conditioned media from sulfated alginate hydrogels with entrapped MSC spheroids at 4 and 21 days. We stimulated HDMECs seeded on Matrigel with this media, and tubule formation was quantified after 6 hours (Fig. 4A). We hypothesized that sulfated alginate hydrogels would sequester more GFs, resulting in fewer GFs in the conditioned media and thus, inferior tubule formation. Tubule formation was most visibly affected when stimulated by 21-day conditioned media. Tubule formation was greatest using conditioned media from unmodified alginate, followed by 1% sulfated gels and virtually no tubule formation was observed using media from 2% sulfated hydrogels (Fig. 4B). Day 4 conditioned media elicited no statistical differences in tubule length among conditioned media from the different gels, though HDMECs stimulated by media from 2% sulfated gels trended lower. The day 21 conditioned media showed clearer results, with statistically

greater tubule length for the unmodified gels compared to 2% sulfated alginate ($p < 0.001$) (Fig. 4C). Tubule branch number exhibited similar trends to the tubule length data, although no significant differences were observed among alginates (Fig. 4D). To further verify growth factors from the FBS in the media were not contributing to these results, we analyzed a control group exposed to media incubated with acellular hydrogels and found this spurred limited tubule formation (*data not shown*). These data indicate that GFs are retained for longer durations in more sulfated alginate gels, while non- or lower-sulfated hydrogels elute factors that enhance HDMEC tubule formation. As previously stated, mesh size and moduli were comparable for all groups at day 21, supporting the fact that the observed differences are not due to variations in hydrogel degradation, but rather sequestration of the GFs by sulfate groups.

3.5 MSC spheroids entrapped in sulfated alginate stimulate myoblast infiltration

Having established differences in the bioactivity of GFs eluted from sulfated hydrogels, we investigated the bioactivity of GFs retained within the hydrogel. We cultured MSC spheroids for 4 days to enable accumulation of the MSC spheroid secretome within the sulfated hydrogels. We then assessed the invasion of tdTomato-expressing human myoblasts into the gels over 8 days *via* confocal microscopy (Fig. 5A). Confocal z-stack images confirmed greater numbers of myoblasts within 2% sulfated hydrogels that also exhibited a more elongated morphology. In contrast, myoblasts in unmodified and 1% hydrogels were sparse, rounded, and often remained at the periphery of the hydrogel (Fig. 5B). Image quantification of myoblast invasion confirmed visual observations, with significantly higher fluorescence, corresponding to higher myoblast infiltration, detected in 2% sulfated hydrogels compared to 0% ($p = 0.001$) and 1% ($p = 0.001$) groups (Fig. 5C). We observed significant increases in the depth of myoblast invasion as a function of sulfate modification (Fig. 5D). Myoblasts that invaded the 2% sulfated alginate gels exhibited significantly lower sphericity compared to lower sulfated and non-sulfated groups ($p < 0.0001$ for both), in agreement with representative images of increased spreading and elongation in 2% gels (Fig. 5E).

To further verify that the effect of infiltration was due to GFs sequestered by the sulfated alginate and not the MSC spheroids themselves, we repeated the experiment by killing the MSC spheroids *via* flash freezing after 4 days in culture. Hydrogels were thawed, and myoblasts were introduced in the same manner as the previous experiment (Fig. 5F). After 8 days, we observed similar infiltration in gels containing dead MSC spheroids, with myoblast infiltration and elongation corresponding to the degree of sulfate modification (Fig. 5G). Quantification of these images indicated increased myoblast infiltration in higher sulfated hydrogels, with trends mirroring those studies using viable MSC spheroids and significant increases in the 2% sulfated hydrogels compared to non-sulfated alginate ($p = 0.001$) (Fig. 5H). Z-position and sphericity were in agreement with data using live cells, evidenced by significant increases in infiltration depth and myoblast elongation within 2% sulfated hydrogels ($p < 0.0001$ for all) (Fig. 5I–J). We performed a control experiment using acellular sulfated hydrogels to investigate if myoblast infiltration was influenced by the sulfate groups themselves in the absence of MSC spheroids (Supplemental Fig. 6). These data indicate that sulfation alone is sufficient to induce myoblast migration, even in the absence of cells.

Indeed, penetration depth of the myoblasts increased in acellular hydrogels, suggesting an increase in migration of cells. However, pixel sum for the 2% sulfated hydrogels decreased in acellular conditions. Thus, acellular sulfated hydrogels enable enhanced migration but lower overall engraftment of myoblasts. These data support the hypothesis that sulfated alginate sequesters endogenous, cell-secreted GFs, and these bound GFs retain their bioactivity.

3.6 Macrophage migration and differentiation are not influenced by alginate sulfation

Given the first-response nature of immune cells to sites of injury and the capability of immune cells to secrete pro-inflammatory cytokines, we evaluated the response of macrophages in the presence of sulfated alginate hydrogels containing MSC spheroid-conditioned media. Live macrophages and their polarization were quantified using flow cytometry (Fig. 6A). We observed a nonsignificant trend in the number of macrophages recovered from alginate gels with increasing degrees of sulfation (Fig. 6B). We characterized the polarization of macrophages seeded on sulfated alginate gels and found no statistical differences in the number of M1 or M2 macrophages as a function of sulfation (Fig. 6C,D). Furthermore, the frequency of macrophages with a well-defined M1 phenotype after interaction with our materials is significantly lower than the M1-polarized control group (*data not shown for control group*). This may indicate either a limited pro-inflammatory response by macrophages to sulfated alginate groups with retained growth factors or may demonstrate the anti-inflammatory action of the growth factors retained by the material. Distinguishing between these mechanisms would require further investigation, but ultimately these data demonstrate that the immunomodulatory signaling provided by retained growth factors is not abrogated by the local pro-inflammatory response of infiltrating macrophages.

4. Discussion

Poor cell viability upon transplantation remains a critical challenge for the success of cell-based approaches to tissue repair and regeneration. MSCs are widely acknowledged to support tissue repair upon implantation through their potent secretome, yet the utility of MSCs persists only while cells remain viable. We developed a biomaterial platform that supports the viability of entrapped MSC spheroids while sequestering a variety of endogenous secreted GFs for prolonged presentation. To our knowledge, this is the first study to explore utilizing GF sequestering moieties to retain and prolong the therapeutic benefit of the MSC spheroid secretome. We designed a unique crosslinking method that facilitates the tunable regulation of initial elastic moduli, degradation, and degree of sulfate modification within alginate hydrogels. Our results demonstrate that increasing the sulfate modification of alginate improves the retention of MSC spheroid-secreted GFs. Importantly, sulfated hydrogels enhanced myoblast infiltration even when MSC spheroids were no longer viable. These findings highlight how our platform could aid in solving challenges related to rapid cell death upon transplantation in cellular therapies.

The addition of GF sequestering moieties to biomaterials has been reported by several groups.[29, 30] Heparin is a well-characterized anticoagulant that has been thoroughly investigated in the biomedical field as a GF delivery agent. Foundational studies of

heparin immobilization were initially motivated by increasing blood compatibility on the surface of biomaterials. These methods leveraged both the localized, sustained release of heparin[31] and direct covalent binding to the material surface[32], though the latter was more advantageous for long-term results. These studies ultimately led to the successful design of adhesion methods for bioactive heparin that have been implemented commercially in polytetrafluoroethylene (PTFE) vascular grafts.[33]

Heparin has been used for GF delivery due to its ability to bind bioactive factors strongly and reversibly. Heparin has been incorporated into various hydrogel polymers, including polyethylene glycol (PEG)[34], fibrin[35], poly(lactic-co-glycolic) acid (PLGA)[36] and alginate[37] for effective delivery of therapeutic GFs for tissue engineering applications. Notably, heparin was co-polymerized with PEG to form hydrogels that delivered basic fibroblast growth factor (bFGF) to MSCs for up to five weeks, resulting in increased cellular proliferation, adhesion, and osteogenic differentiation.[38] Although heparin incorporation is an effective method for GF sequestration, alternative approaches have been evaluated to avoid the complexity, cost, and antithrombotic effects of using the full heparin molecule. For example, the modification of alginate macromer with sulfate groups derived from sulfonic acid treatment capitalizes on the polymer's high degree of material tunability.[13, 19] Like heparin, sulfated alginate can be used for controlled GF delivery. The formulation has been successfully used to sequester and release BMP-2[39] and FGF-2[40], resulting in increased cell differentiation and response compared to GFs released from unmodified alginate.

GF delivery alone is often insufficient for tissue regeneration due to unintended side effects when using GFs at supraphysiological concentrations, prohibitive cost of these GFs, and poor replication of endogenous signaling that occurs with a multitude of GFs presented to neighboring cells.[41] MSCs produce a complex secretome composed of multiple GFs at lower concentrations than routinely used for therapeutic applications.[42] Although the incorporation of sulfate groups onto polymers such as alginate has been reported, previous studies have focused on delivering a single recombinant GF, while neglecting to capitalize on the complex and physiologically tuned MSC secretome.[19, 39] The innovation of our work is in sequestering a diverse array of factors secreted by MSC spheroids as opposed to pre-loading individual recombinant GFs for delivery.

To investigate this aspect further, we characterized MSC spheroid-secreted factors retained in sulfated and non-sulfated hydrogels. We found increased retention of an array of heparin binding growth factors and cytokines in sulfated hydrogels, verifying that this platform is capable of retaining several therapeutic biomacromolecules. These included high levels of heparin binding cytokines IL-8, IL-6, and VEGF, which are in agreement with other reports describing the composition of the MSC spheroid secretome.[43, 44] We also detected increased retention of INF- γ in sulfated hydrogels, corresponding with previous work indicating that INF- γ has high heparin affinity.[45] This is especially promising considering INF- γ sequestered by heparin and collagen has enhanced ability to increase MSC cytokine secretion and integrin binding.[46] When evaluating the capacity for extracellular matrix proteins to be sequestered, we did not detect the retention of collagen within sulfated alginate gels (*data not shown*). Identification of potent cytokines within this platform indicates MSC

spheroid loaded sulfated alginate has high therapeutic potential, which we further evaluated through a series of *in vitro* studies.

Upon accumulation of endogenous factors of the MSC secretome, we observed increased adherence, spreading, and invasion of myoblasts into sulfated alginate compared to unmodified hydrogels. It is important to note that the sulfate-modified alginate is likely retaining MSC spheroid-secreted GFs as well as myokines that may further contribute to myoblast infiltration. Indeed, we observed myoblast infiltration from the acellular sulfated hydrogel control groups, indicating myokine and serum GF retention is likely contributing to our results. These results are in agreement with previous observations of the bioactivity of sequestered GF within hydrogels.[38] A possible limitation to this platform is that the sulfated alginate will retain proinflammatory cytokines produced by the injured tissue when implanted in a wound site. To investigate this possibility and any immunological repercussions, we assessed macrophage invasion and polarization on sulfated and non-sulfated hydrogels loaded with MSC spheroid conditioned media. We found no significant increase in adherence of macrophages to sulfated hydrogels, nor did our data indicate that sulfated hydrogels induced a greater M1 or M2 phenotype. The high secretion of chemokines such as IL-8 and IP-10 by MSC spheroids and their subsequent retention by more sulfated materials may be involved in the homing of immune cells to 2% sulfated alginate hydrogels. However, this hypothesis warrants further investigation and is beyond the scope of this work. Overall, these data confirm that sulfated groups do not enhance pro-inflammatory responses from macrophages and therefore are likely not adhering excessive pro-inflammatory cytokines. Future studies are required to evaluate the efficacy of sulfated alginate *in vivo* and determine if preloading hydrogels with MSC secretome is necessary so endogenous GFs produced by the tissue do not dominate the hydrogel.

When formed into spheroids, MSCs exhibit enhanced viability, differentiation, and increased secretion of immunomodulatory and reparative GFs compared to monodisperse MSCs.[44, 47–49] The MSC secretome can also be influenced by the biophysical properties of the surrounding ECM. Fibrin hydrogels were designed to increase the concentration of VEGF or PGE₂ within the MSC spheroid secretome [16], and degree of alginate RGD modification influences MSC spheroid migration, GF secretion, and differentiation.[21] These findings highlight the importance of the mechanical and chemical tunability of sulfated alginate, as these properties can be leveraged to manipulate secretome and cell function for different applications that may be further amplified through GF sequestration.

We used a dual ionic crosslinking technique to independently modulate the physical and chemical properties of sulfated alginate hydrogels. Few studies have directly measured and established this tunability using an ionically crosslinked sulfated alginate hydrogel, likely because the negatively charged sulfate groups can interfere with ionic crosslinking, influencing hydrogel degradation and moduli. By crosslinking with both CaCl₂ and BaCl₂, we produced a divalent cation combination strong enough to overcome these issues. CaCl₂ and BaCl₂ have been successfully utilized for bio-printing applications of alginate[50], although not with this reported simultaneous crosslinking technique. One possible limitation of using BaCl₂ as a crosslinker is that it can injure myofibers, and injection of the chemical has been well established as a muscle injury model.[51] Our crosslinker concentration is

well below that of the concentration used for muscle injury, yet we have not tested our hydrogel *in vivo* to verify the BaCl₂ will not induce adverse effects. Using this method, we demonstrated that sulfate modification can be modulated independently of initial elastic moduli and degradation. Although not explicitly explored in this paper, modification with different adhesion sites and densities could further be pursued as another independent factor, as adhesion peptide degree of substitution does not influence alginate mechanical properties. [21, 52]

These studies establish an exciting platform of study, facilitating the independent regulation of mechanical properties, adhesive moiety type and concentration, and sulfate modification within alginate hydrogels using simple and well-established chemical modifications. This platform can be used to further interrogate the effect of higher sulfate modification levels, as previous studies indicate sulfate concentration on the alginate backbone increases when using up to 3.5% HSO₃Cl.[53] Sulfated alginate also exhibits promising translational potential, as alginate is FDA approved for wound healing and as a food additive. The sulfated alginate described herein requires significantly lower concentrations of sulfate than that contained in heparin (Supplemental Fig. 1) while still inducing significant biological differences. This is advantageous, as heparin levels that exceed the effective threshold for VEGF delivery correlated with reductions in network formation by endothelial cells.[54] These data emphasize the importance of striking a balance between sequestration and delivery of GF.

5. Conclusion

We established and characterized a sulfated alginate hydrogel platform with independent tunability of sulfate modification, initial elastic moduli, and degradation rate. We determined that sulfated alginate could bind both recombinant and cell-secreted GFs. Sulfated alginate sequestered a multitude of bioactive GFs from the secretome of entrapped MSC spheroids. These studies indicate that sulfate groups aid in retaining and increasing the therapeutic effect of the MSC spheroid secretome within the hydrogel, potentially prolonging the therapeutic effect of cells transplanted for their secretome.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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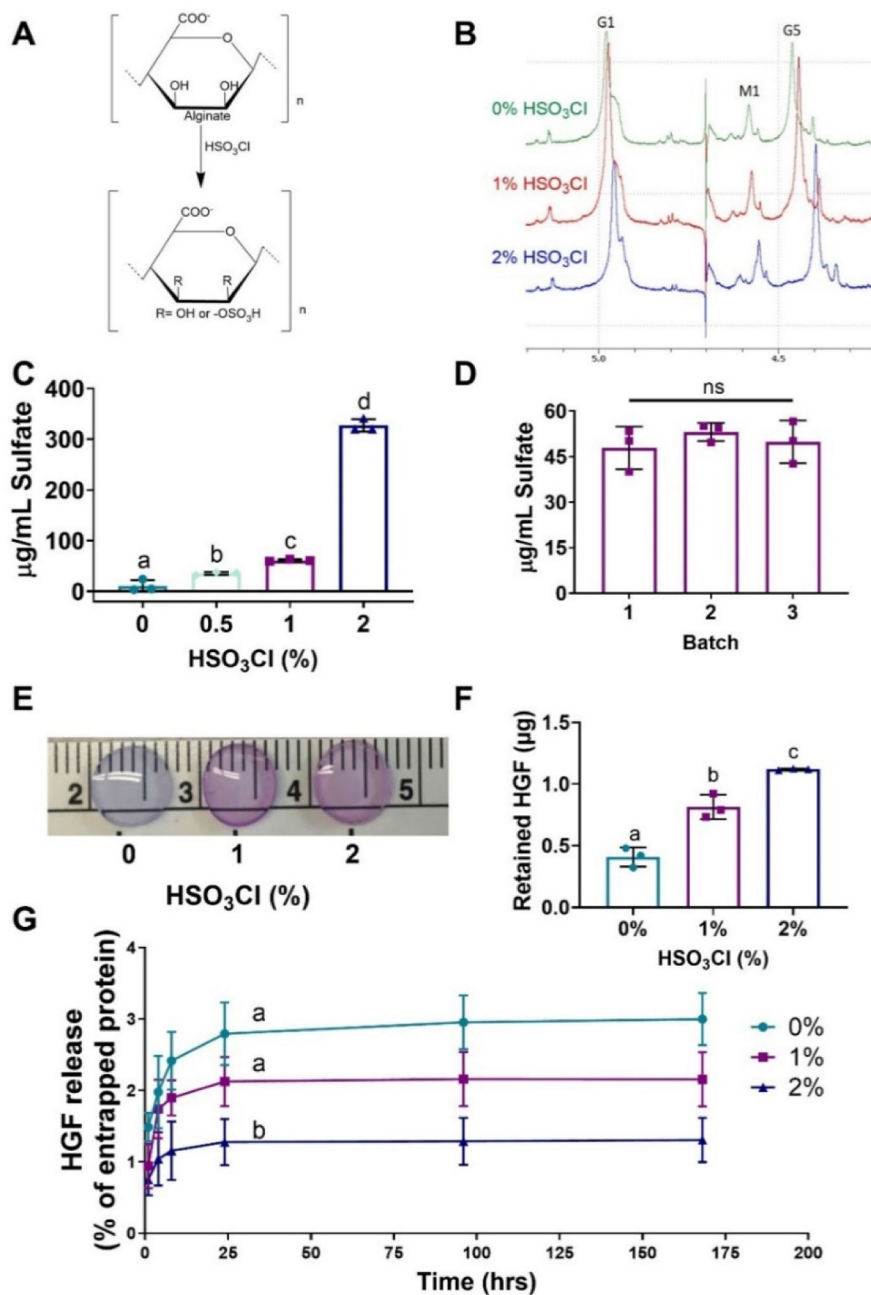


Figure 1. Quantification of the amount and functionality of sulfate-modified alginate. (A) The reaction of alginate with chlorosulfonic acid yields sulfated alginate. (B) Sulfate modification of alginate was verified by ^1H NMR. (C, D) Sulfate modification was quantified at different chlorosulfonic acid concentrations and between different batches *via* DMMB assay. (E) DMMB staining of crosslinked hydrogels exhibited uniform staining throughout the gel. (F) Recombinant HGF was entrapped in sulfated alginate hydrogels and the amount retained after 7 days was quantified. (G) Quantification of HGF release confirms that sulfate groups are functional and retain this heparin binding GF. Data are mean \pm SD

(n=3–4). Groups with statistically significant differences do not share the same letters; ns denotes no significance among all groups.

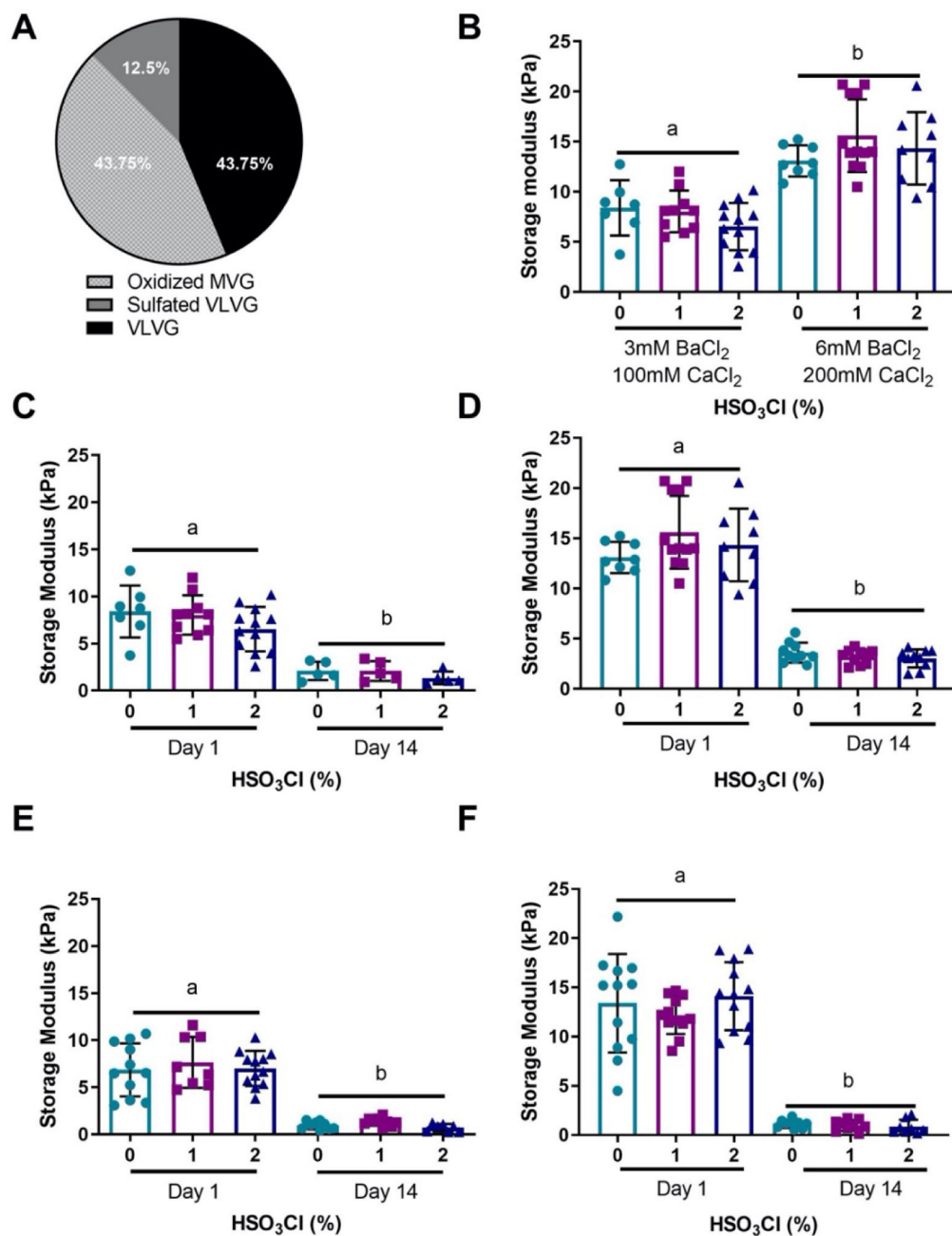


Figure 2. Hydrogel formulation and modulation of mechanical and degradation properties.

(a) Composition of sulfated alginate hydrogels. (b) Initial moduli of sulfated alginate can be regulated by varying the concentration of ionic crosslinkers. A 3 mM BaCl₂ and 100 mM CaCl₂ concentration produced a hydrogel with low initial modulus, and 6 mM BaCl₂ and 200 mM CaCl₂ produced a high initial modulus. The moduli for 1% oxidized (c) low initial moduli, and (d) high initial moduli decreased to similar levels by day 14. Increasing the oxidation percentage of the MVG alginate from 1% to 5% caused the day 14 moduli to decrease further for (e) low and (f) high initial moduli groups without affecting initial

moduli. Data are mean \pm SD (n=7–12). Groups with statistically significant differences do not share the same letters.

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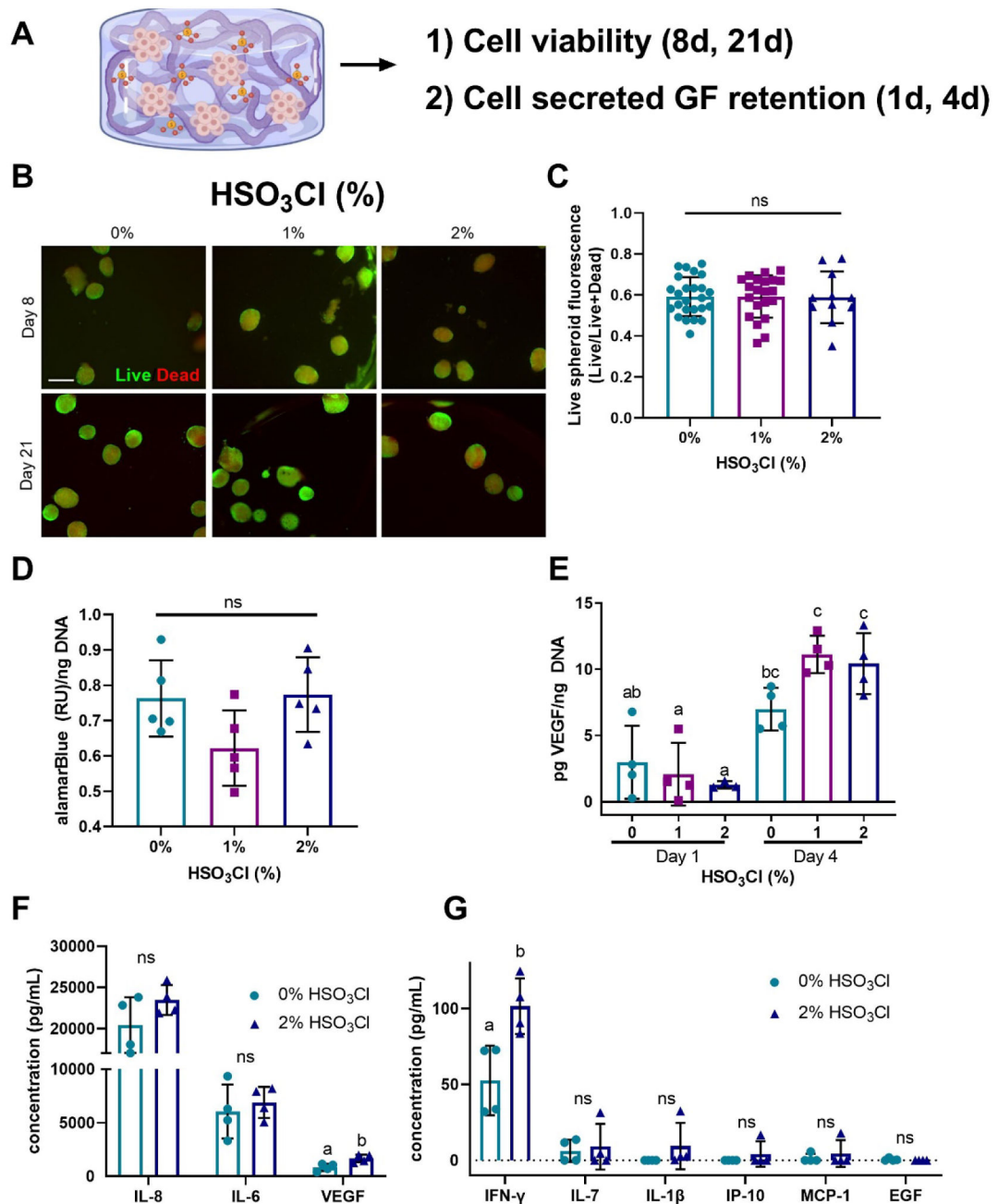


Figure 3. Spheroid-produced GFs are retained within sulfated alginate hydrogels.

(a) MSC spheroids were entrapped in sulfated alginate hydrogels. (b) No differences were observed in cell viability by LIVE/DEAD stain of MSC spheroids entrapped in sulfated and non-sulfated alginate on day 8 (scale bar = 500µm). Live cells are green, dead cells are red. (c) Ratio of live cell fluorescence to total cell fluorescence revealed no differences. (d) Metabolic activity is comparable for MSC spheroids entrapped in sulfated and non-sulfated alginate on day 8. (e) MSC-secreted VEGF is retained in sulfated alginate hydrogels on days 1 and 4. (f-g) Retained MSC spheroid cytokines in sulfated and non-sulfated hydrogels. Data

are mean \pm SD (n=4–5). Groups with statistically significant differences do not share the same letters; ns denotes no significance among all groups.

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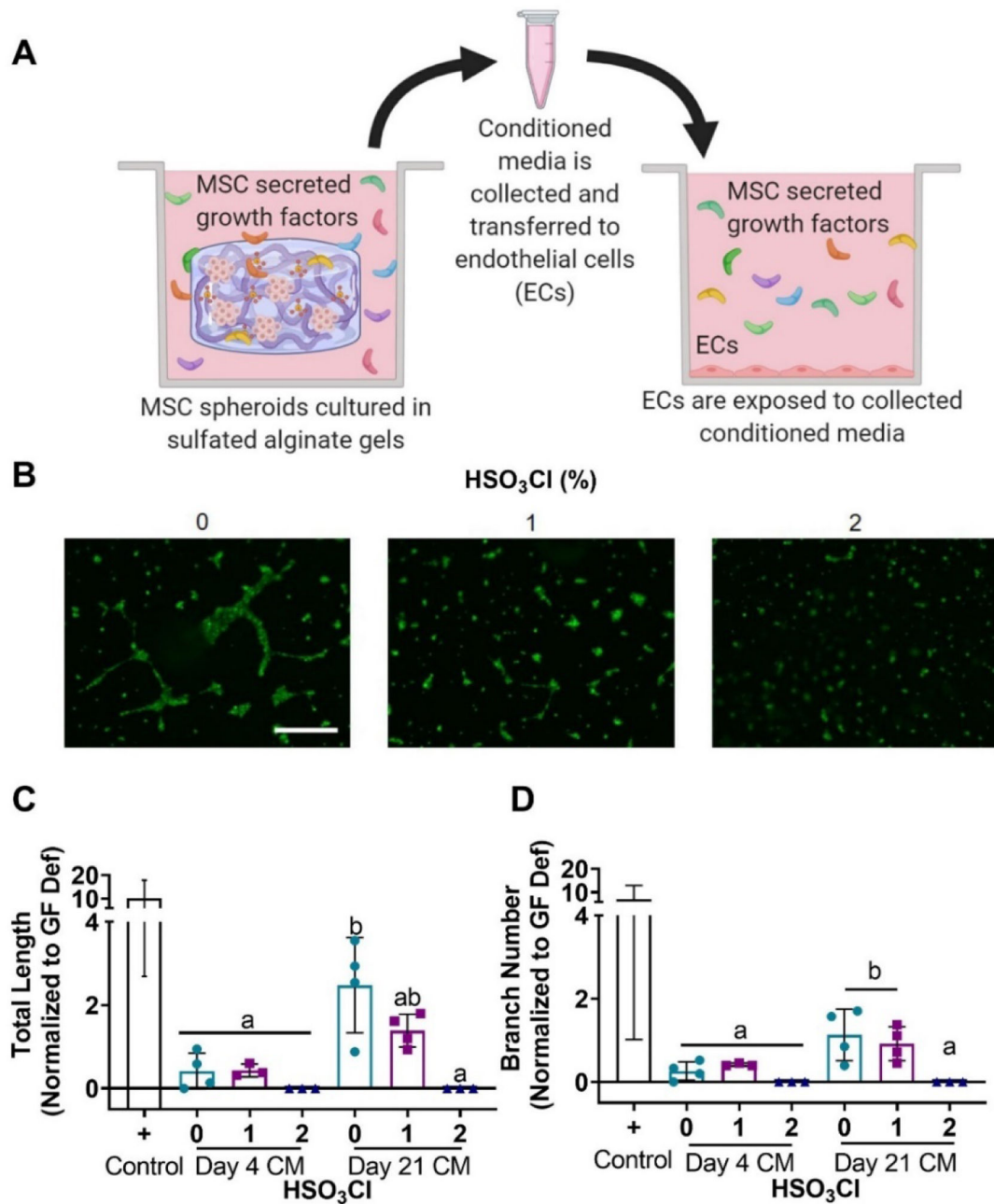


Figure 4. GFs eluting from sulfated alginate are functional and stimulate tubule formation.

(a) Conditioned media was collected from sulfated hydrogels loaded with MSC spheroids and pipetted on HDMECs to establish the retention of function GFs indirectly by determining tubule formation. (b) Calcein AM stain of HDMECs exposed to day 21 conditioned media (scale bar = 500 μ m). Quantification of (c) total tubule length and (d) branch number from HDMEC images confirm that more GFs are eluted from non- and low-sulfated alginate hydrogels. Positive control is tubule formation in complete endothelial cell growth media. All values were normalized to the negative control that was performed in growth factor deficient media. Data are mean \pm SD (n=3–4). Groups with statistically

significant differences do not share the same letters; ns denotes no significance among all groups.

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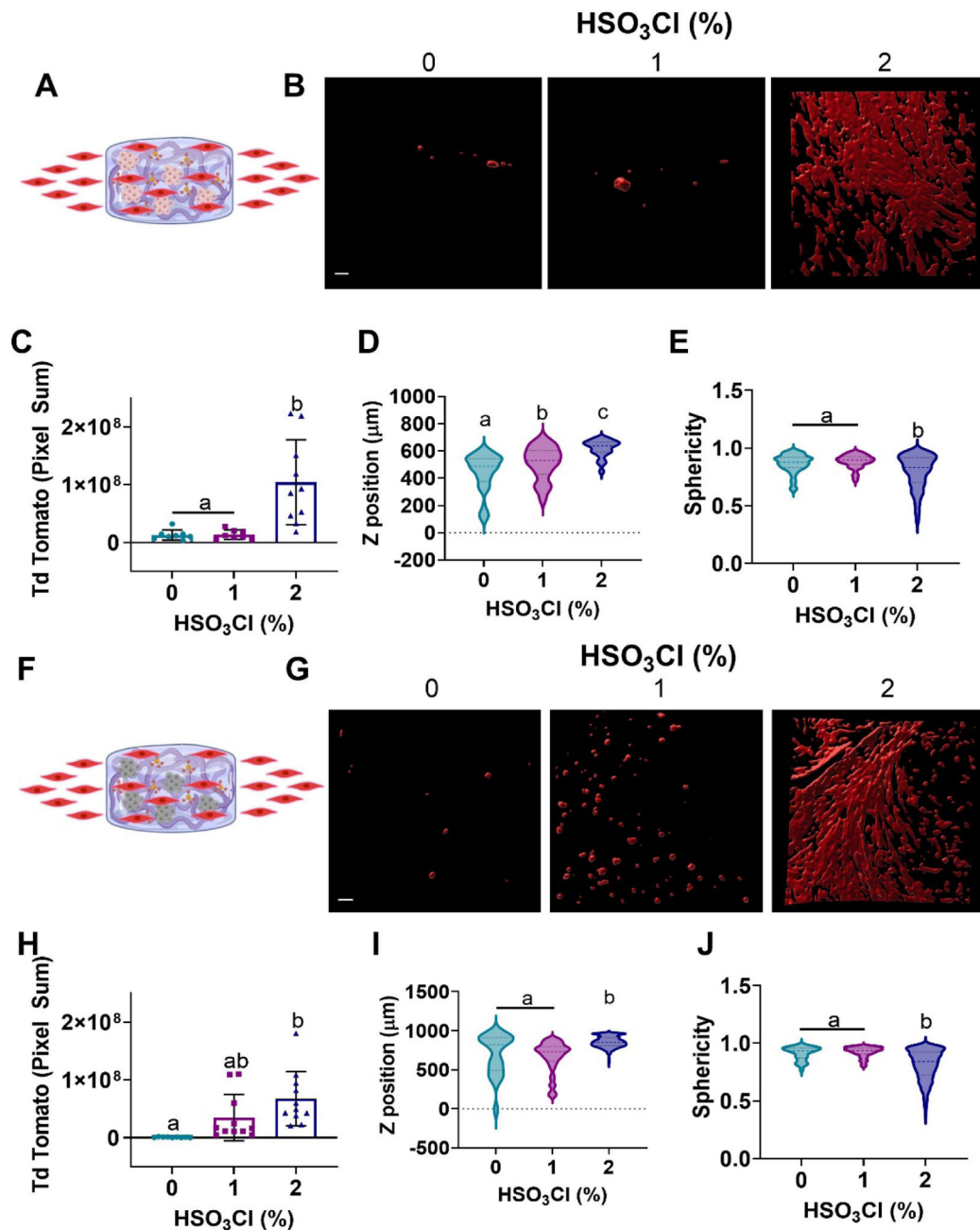


Figure 5. MSC spheroids entrapped in sulfate-modified alginate hydrogels enhance myoblast infiltration.

(a) MSC spheroids were entrapped and cultured in alginate hydrogels for 4 days, then tdTomato-expressing myoblasts were introduced to the media and cultured for an additional 8 days. (b) Representative z stack 3D reconstruction images of tdTomato myoblasts within MSC spheroid gels on day 8 (scale bar = 100 μm). (c) Pixel sum of tdTomato fluorescence, (d) depth of penetration and (e) sphericity of the infiltrating myoblasts were quantified from the z stacks, confirming greater myoblast invasion and elongation in 2% sulfated gels. (f-j) The experiment was repeated with spheroids that were killed after 4 days of culture to confirm that GF retention induced myoblast infiltration. Data are mean ± SD (n=9–1145).

Groups with statistically significant differences do not share the same letters; ns denotes no significance among all groups.

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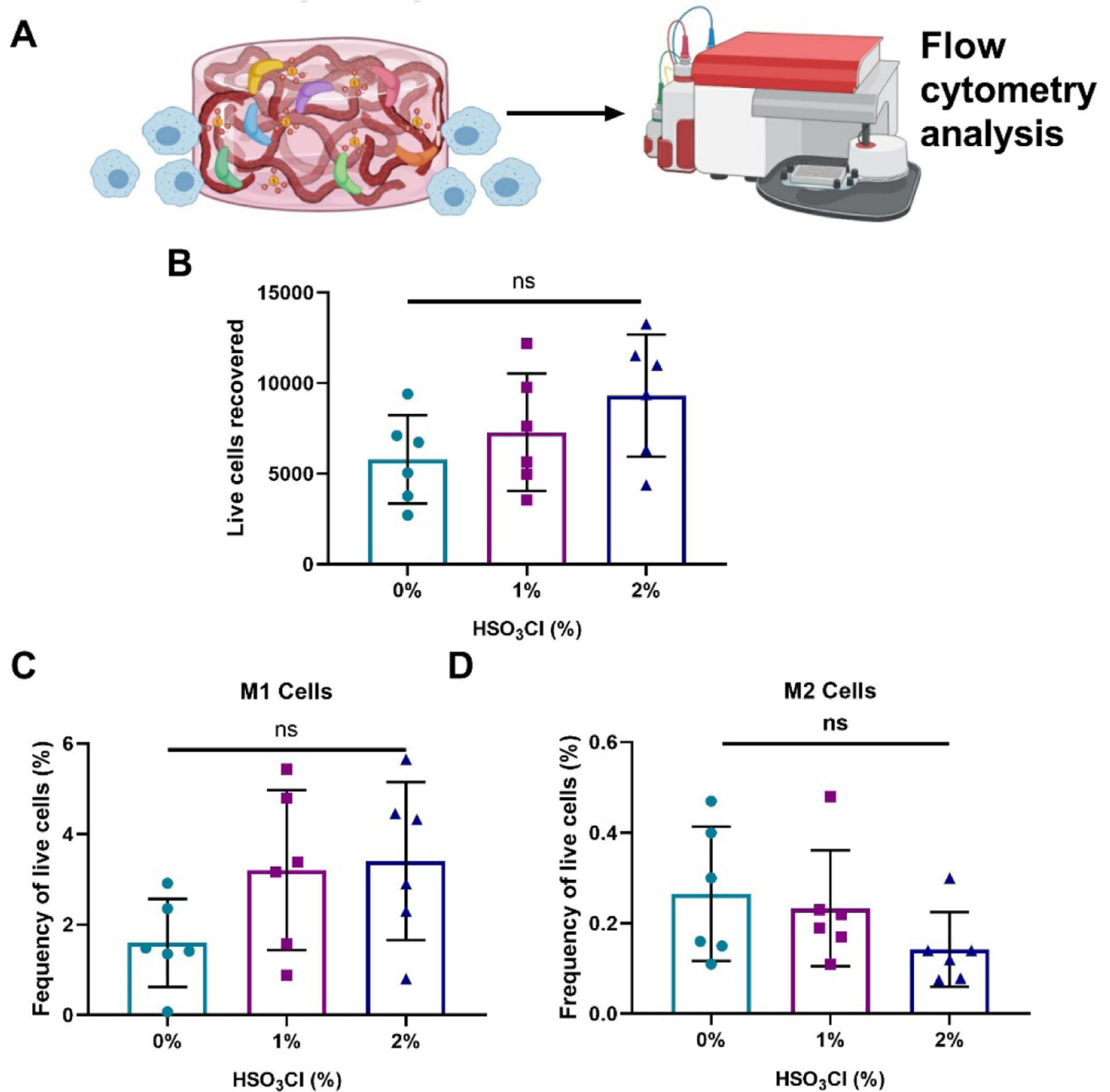


Figure 6. MSC spheroid conditioned media entrapped in sulfate-modified alginate hydrogels exhibited no significant increases in macrophage invasion.

(a) MSC spheroid conditioned media was entrapped in sulfated and non-sulfated hydrogels, and IC-21 macrophages were then seeded on the hydrogels. (b) Total macrophages recovered from the hydrogel. (c) M1 polarized macrophages (CD86+, iNOS+) and (d) M2 polarized macrophages (CD86-, CD206+, ARG1+) were quantified *via* flow cytometry. Data are mean \pm SD (n=6), ns denotes no significance among all groups.

Table 1.

Composition of the different hydrogel groups.

| Hydrogel Group | Hydrogel component (percent volume in gel) | | | |
|------------------------------------|--|---------------|-----------------------|---|
| | MVG (43.75%) | VLVG (43.75%) | Sulfated VLVG (12.5%) | Crosslinker |
| <i>High moduli, slow degrading</i> | 1% oxidized RGD modified | RGD modified | Sulfate modified | 6 mM BaCl ₂ 200 mM CaCl ₂ |
| <i>Low moduli, slow degrading</i> | 1% oxidized RGD modified | RGD modified | Sulfate modified | 3 mM BaCl ₂ 100 mM CaCl ₂ |
| <i>High moduli, fast degrading</i> | 5% oxidized RGD modified | RGD modified | Sulfate modified | 6 mM BaCl ₂ 200 mM CaCl ₂ |
| <i>Low moduli, fast degrading</i> | 5% oxidized RGD modified | RGD modified | Sulfate modified | 3 mM BaCl ₂ 100 mM CaCl ₂ |

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