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Development of Nano- and Micro-Scale Chondroitin Sulfate Particles for Controlled Growth Factor Delivery

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Abstract

Size scale plays an important role in the release properties and cellular presentation of drug delivery vehicles. Because negatively charged chondroitin sulfate (CS) is capable of electrostatically sequestering positively charged growth factors, CS-derived nanoscale micelles and microscale spheroids were synthesized as potential growth factor carriers to enhance differentiation of stem cells. Particles were characterized for morphology, size distribution, surface charge, cytocompatibility, as well as release of transforming growth factor- β 1 (TGF- β 1) and tumor necrosis factor- α (TNF- α). CS micelles were spherical and negatively charged with a bimodal distribution of 324.1 ± 8.5 nm and 73.2 ± 4.4 nm diameters, and CS microspheres possessed a rounded morphology and a diameter of 4.3 ± 0.93 μ m. Positively charged TGF- β demonstrated minimal release after loading in CS microspheres, while negatively charged TNF- α exhibited substantial release over the first 15 hours, suggesting TGF- β 1 electrostatically complexed with CS. The micelles and microparticles were found to be cytocompatible at moderate concentrations with marrow stromal cell (MSC) monolayers and within embryonic stem cell (ESC) embryoid bodies. These synthesis techniques, which allow the formation of CS-based carriers over a variety of nano- and microscale sizes, offer versatility for tailored release of positively charged growth factors and controlled CS presentation for a variety of stem cell-based applications in tissue engineering and regenerative medicine.

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Keywords

chondroitin sulfate; micelles; microparticles; drug delivery; tissue engineering

1. INTRODUCTION

Chondroitin sulfate (CS) is a sulfated glycosaminoglycan (GAG) that is covalently bound to a variety of protein cores to form proteoglycans that are found throughout the human body [1]. During development, one of the key roles of GAGs is to establish morphogen gradients that pattern tissue morphogenesis by sequestering secreted growth factors at the cell membrane [2]. Negatively charged GAGs, including heparin, heparan sulfate, and CS, are capable of electrostatically interacting with positively charged growth factors, including basic fibroblast growth factor (bFGF), insulin-like growth factor (IGF), vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), and transforming growth factor- β (TGF- β), to stabilize and prevent degradation of the growth factors in solution [3–5], indicating that CS biomaterials may be a promising vehicle for delivery of cationic growth factors, particularly to direct differentiation of stem cells.

Size scale plays an important role in the release kinetics of a given delivery vehicle, including the diffusion rate of molecules out of the material and the degradation properties of the material [6]; therefore, control of size enables one to tailor the release profile for a variety of applications. Nanospheres and microspheres possess a high surface area-to-volume ratio, accelerating the diffusion of molecules from the particles and potentially enhancing the hydrolytic or enzymatic degradation of the carrier and further accelerating release [6–7]. The small size of nano- and microspheres, relative to cells and tissues, make them especially advantageous carriers for delivery and sustained release within dense tissues, high-density cell pellets, or tissue engineering scaffolds with numerous barriers to diffusion [8], because these small particle carriers can be incorporated or locally injected within a tissue for controlled release of specific molecules to achieve a localized or more homogenous cell response [7].

Additionally, nano-sized particles (< 500 nm diameter) are capable of being internalized by cells, permitting efficient transport across the cell membrane [7]. Generally, smaller particles are endocytosed more effectively than larger particles, though an optimal size range commonly exists around 100–200 nm [9]. Larger micro-scale delivery vehicles, however, may be advantageous for preventing cell endocytosis if extracellular release is desired, and their larger size scales also possess potential for greater molecule loading and prolonged release due to their larger volumes.

In this study, we explore the means to fabricate both micron-scale particles and nano-scale micelles from CS. Micelles are nanoscale, self-assembling particles composed of amphiphilic molecules. In an aqueous environment, micelles self-assemble into sphere-like structures with a hydrophilic shell surrounding a hydrophobic core, making them largely stable in the aqueous environment of the body [10]. Due to its negative charge density and hydrophilic nature, CS can be utilized as a hydrophilic segment of a polymeric micelle. CS has been previously reported as the hydrophilic component with poly(L-lactic acid) (PLLA) and poly(lactic-co-glycolic acid) (PLGA) copolymers to form amphiphilic microspheres for use as drug and protein delivery carriers [10–12]. With its nanoscale size and high negative charge density, CS-derived micelles have promising potential for many drug and protein delivery applications.

The long term goal of this research is to examine CS interactions with stem cells, particularly as a means to control growth factor release in order to direct differentiation. Therefore, the first set of experiments in this study were designed to develop facile means to control the size of CS-based particles over a broad range of size scales (at least two orders of magnitude) and characterize the resulting materials. Subsequently, the ability of these CS-based biomaterials to interact electrostatically with positively charged growth factors, as well as the cytocompatibility of these materials with both embryonic and mesenchymal stem cells was demonstrated, confirming the potential for these materials as naturally-derived carriers for growth factor delivery for a variety of stem cell-based tissue engineering and regenerative medicine applications.

2. MATERIALS AND METHODS

2.1. Modification of Chondroitin Sulfate

2.1.1. Chondroitin Sulfate Modification for Micelle and Hydrogel Fabrication—

To examine the effects of reaction stoichiometry on the resulting modification of CS, N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide (EDC) chemistry, which forms an amide bond between a primary amine and a carboxylic acid, was used to synthesize chondroitin sulfate methacrylamide (CSMAm; Figure 1a). N-(3-aminopropyl)methacrylamide (APMAm) was conjugated to chondroitin sulfate A, using EDC chemistry adapted from [13]. 2.5 mM CS (~48.7 kDa; Sigma-Aldrich, St. Louis, MO) [14] based on CS disaccharide molecular weight was reacted with APMAm (Polysciences, Warrington, PA), EDC (Sigma-Aldrich), and N-hydroxysulfosuccinimide (sulfo-NHS; Sigma-Aldrich) at various molar ratios depicted in Table 1. Since CS possesses 1 carboxyl moiety per disaccharide, this represents the molar ratio of reagent to each reactive carboxyl group. The reaction mixture was incubated at room temperature and pH 5.0 for 2 hours with stirring.

Sulfo-NHS stabilizes the EDC reaction and was used to control the degree of substitution of the reaction; however, to reduce the degree of modification for micelle formation, the reaction with the highest molar ratio of 4:1 was repeated in the absence of sulfo-NHS. Initially a 2:1 ratio of APMAm and EDC to CS was used and allowed to react for 2 hours, and then a second round of APMAm and EDC was added to the solution for a total 4:1 molar ratio to CS. The reaction without sulfo-NHS was allowed to proceed for 2 more hours at pH 5.0. All reaction mixtures were dialyzed in 1,000 Da molecular weight cutoff (MWCO) dialysis membrane (Spectrum Laboratories, Rancho Dominguez, CA) against distilled water (dH₂O) for 3 days, and the product was then lyophilized (Labconco FreeZone 4.5, Kansas City, MO) for 4 days and stored at 4°C until use.

2.1.2. Chondroitin Sulfate Modification for Microsphere Fabrication—

For microsphere formation, CS was reacted with methacrylic anhydride (Sigma-Aldrich) to conjugate methacrylate groups to the existing hydroxyl groups in CS (Figure 1b). Methacrylation of CS was carried out per established protocols [14]. After complete dissolution, 60 ml methacrylic anhydride was added dropwise into 60 ml of a 25% w/v chondroitin sulfate A solution (20.6:1 molar ratio of methacrylic anhydride to reactive hydroxyl) under basic conditions. The reaction solution was stirred at 60°C for 24 hours, then precipitated and washed in cold methanol:isopropanol (10:1). The resulting chondroitin sulfate methacrylate (CSMA) precursor was subsequently dried under vacuum at room temperature. The resulting product was dialyzed (1,000 Da MWCO) against dH₂O for 3 days, followed by lyophilization for 4 days.

2.2. Characterization of CS Nano- and Microspheres

2.2.1. Proton Nuclear Magnetic Resonance Spectroscopy—Proton nuclear magnetic resonance (^1H NMR) was utilized to determine the degree of the conjugation of the APMAM groups to the CS chains. The initial components (CS and APMAM) and resulting modified CS products (CSMAM and CSMA) were solubilized in deuterated water (D_2O ; Sigma-Aldrich), and ^1H NMR was measured on a Bruker AMX-400 spectrometer (Billerica, MA) at 400 MHz. The resulting spectra were analyzed by calibrating the region from 3.61–3.88 ppm according to the known number of protons in the chondroitin sulfate backbone. This provided an internal standard which allowed monitoring of the amount of modification attached to the different modification reactions.

2.2.2. Formation and Characterization of Bulk CS Hydrogels—Solutions of CSMAM or CSMA at 90% water content in phosphate buffered saline (PBS) were crosslinked between 2 glass slides at 1 mm thickness under 15 mW/cm^2 , 365 nm UV light (UVP, Upland, CA) with 0.2 wt% Irgacure 2959 photoinitiator (D2959, Ciba, Ludwigshafen, Germany) for 12.5 minutes, and 6 mm diameter discs were punched out using a cork borer. After 1 day swelling in PBS, the wet weight of crosslinked hydrogels was recorded, and following lyophilization overnight, dry weight was recorded. Swelling ratio was calculated as wet weight/dry weight ($n=4$).

2.2.3. Dynamic Light Scattering and Zeta Potential Measurements of CS Micelles—Dynamic light scattering (DLS) measurements were taken to determine the average diameter of CSMAM micelles in distilled, deionized water (ddH_2O), and zeta potential measurements were also recorded as a measure of surface charge and colloidal stability in ddH_2O . DLS and zeta potential measurements were taken on a NICOMP 380ZLS (Particle Sizing Systems, Santa Barbara, CA) at 23°C . A helium-neon laser at 632.8 nm wavelength was used at a detector angle of 90° . Samples of 1.0 mg/ml CSMAM were run in ddH_2O , while samples of unmodified CS at the same concentration served as controls ($n=4$). Prior to measurement, the solutions were filtered through a $5 \mu\text{m}$ pore size cellulose acetate filter (Sartorius, Goettingen, Germany), and the sample was briefly centrifuged (14,000 RPM) to remove any dust particles from the solution. Hydrodynamic diameter was calculated using the inverse Laplace transform of the correlation function [15].

Zeta potential measures colloid mobility of charged particles in solution under an electric field. Zeta potential measurements were taken on 0.1 mg/ml CSMAM solutions in an electric field of 4 V/cm (1.6 V, 0.4 cm electrode distance; $n=3$). Zeta potential was calculated according to the Smoluchowski limit [16].

2.2.4. CS Microsphere Fabrication and Characterization—CS microspheres were fabricated using a water-in-oil, single-emulsion technique similar to a previously described protocol [17]. CSMA (55.6 mg) was dissolved in PBS (440 μl) and combined with ammonium persulfate (30 μl , 0.3 M) (Sigma-Aldrich) and tetramethylethylenediamine (30 μl , 0.3 M) (Sigma-Aldrich) free radical initiators on ice. This mixture was added drop-wise into corn oil at 4°C and homogenized at 3,800 RPM for 5 minutes. The temperature was then raised to 50°C with stirring for 30 minutes to promote crosslinking. The resulting solution was centrifuged at 300 RCF, and the corn oil was removed. Microspheres were then washed with dH_2O and stored in dH_2O at 4°C . The diameter of the microsphere population was analyzed using a Z2 Coulter Particle Counter (Beckman Coulter, Fullerton, CA) equipped with a 100 μm aperture. Microspheres were suspended in Isoton II diluent (Beckman Coulter) prior to counting.

For imaging, microspheres were treated with a solution of 1,9-dimethylmethylene blue (DMMB; Sigma-Aldrich) for 30 minutes to stain CS purple, washed with ddH₂O, and imaged using an inverted microscope (Nikon Eclipse TE2000-U, Melville, NY).

2.2.5. Complexation and Release from CS Microspheres—To examine the ability of negatively charged CS microspheres to electrostatically bind positively charged TGF- β 1, approximately 2 mg of lyophilized microspheres were suspended in 500 μ l of 50 ng/ml human recombinant TGF- β 1 (Peprotech, Inc., Rocky Hill, NJ) and 1% bovine serum albumin (BSA) in PBS. Microspheres were incubated with TGF- β 1 overnight at 4°C with shaking (n=3).

To examine the release kinetics of positively charged TGF- β 1 and negatively charged tumor necrosis factor- α (TNF- α), approximately 2 mg of lyophilized CS microspheres were loaded with 5 μ l of 5 μ g/ml human recombinant TGF- β 1 or TNF- α (R&D Systems, Minneapolis, MN) per mg of microspheres overnight at 4°C. Microspheres were then suspended in 750 μ l of solution containing 1% BSA in PBS (time 0) and incubated at 37°C with gentle shaking for 5 days.

At various time points, samples were centrifuged for 90 seconds at 4,000 RPM, and the supernatant was sampled and analyzed for factor content using the corresponding enzyme-linked immunosorbent assay (ELISA; R&D Systems) kit specific to human TGF- β 1 or TNF- α (n=3).

2.3. Cytocompatibility of CS Nano- and Microspheres

2.3.1. In Vitro Cytotoxicity of CS Micelles and Microspheres on 2D MSC Monolayers—For CSMAM micelle cytotoxicity studies, bovine marrow stromal cells (MSCs) were isolated in a modification of the procedure outlined in [18]. Briefly, bone marrow was harvested from the tibia and femur of an immature calf (Research 87, Marlborough, MA). Cells were cultured in media composed of low-glucose DMEM (Mediatech, Manassas, VA) supplemented with 10% fetal bovine serum (FBS; Thermo Scientific Hyclone, Logan, Utah), 1% antibiotic/antimycotic, 0.1% Fungizone antimycotic (Invitrogen, Carlsbad, CA), and 1 ng/ml bFGF (Peprotech). The adherent MSCs were expanded until confluence and were then detached with 0.05% trypsin/EDTA (Mediatech), frozen in media containing 20% FBS and 10% dimethyl sulfoxide (DMSO; Sigma-Aldrich), and cryopreserved in liquid nitrogen until use (P1).

Prior to use, MSCs were thawed and plated for at least 3 days to eliminate transitory effects from the thawing process. After this time, cells were trypsinized (P2) and plated at 10,000 cells/cm² in a tissue culture-treated 96-well plate. After 24 hours to allow cell attachment, culture media was replaced with 100 μ l of media containing CSMAM micelles or CSMA microparticles equivalent to 1, 10, 100, 320 mg per 10⁶ cells (0.064, 0.64, 6.4, and 20.5 mg/ml, respectively). After 24 hours of exposure to the CSMAM- or CSMA-containing media, cells were stained (30 minutes) with LIVE/DEAD stain (Invitrogen). Calcein stains live cells to fluoresce green (excitation/emission: 494/517 nm), and ethidium homodimer-1 stains dead cells to fluoresce red (excitation/emission: 528/617 nm). Dead controls were exposed to 70% methanol prior to staining, while live controls were cultured in normal culture media without CS particles. Fluorescent images were captured using an inverted microscope, and total fluorescence of each well was measured in a SpectraMax M2^e plate reader (Molecular Devices, Toronto, Canada) at the wavelengths specified above (n=3). Relative viability was analyzed in comparison to live controls, using the following equation:

$$\text{Fraction of live cells} = \frac{F(517)_{\text{sample}} - F(517)_{\text{DEAD}}}{F(517)_{\text{LIVE}} - F(517)_{\text{DEAD}}},$$

where $F(517)_{\text{sample}}$, $F(517)_{\text{LIVE}}$, and $F(517)_{\text{DEAD}}$ are the fluorescence at 517 nm of the sample, live controls, and dead controls, respectively.

2.3.2. In Vitro Cytotoxicity of CS Microspheres when Incorporated within 3D ESC Embryoid Bodies—Undifferentiated D3 murine embryonic stem cells (ESCs) were maintained on gelatin-coated tissue culture dishes in DMEM media supplemented with 15% FBS and 10^3 U/ml leukemia inhibitory factor (LIF; Millipore, Billerica, MA). Embryoid bodies (EBs) were formed using a single-cell suspension by forced aggregation in AggreWell™ 400 inserts (Stem Cell Technologies, Vancouver, CA) [19]. Briefly, 1.2×10^6 cells in 0.5 ml of medium were inoculated into AggreWell™ inserts, containing approximately 1,200 wells per insert, and centrifuged at 200 RCF for 5 minutes to cluster cells in the wells. Subsequently, 200 μ l of CS microsphere solution was added in a 4:1 microsphere-to-ESC ratio, and a second centrifugation was performed at 200 RCF for 5 minutes. After 24 hours of culture, aggregates were removed from the wells using a wide-bore pipette and transferred to suspension culture on a rotary orbital shaker (40 RPM) to maintain the homogeneity of the population [20].

CS microsphere incorporation within EBs was examined through histological sections of EBs after 3 days of culture. EBs were sampled and fixed in a 10% formalin solution. The EBs were washed in PBS and resuspended in HistoGel (Thermo Richard-Allan Scientific, Kalamazoo, MI) prior to paraffin processing. Sections of 5 μ m were stained using Safranin-O (Sigma-Aldrich) for GAG detection, Fast Green (Sigma-Aldrich) for cytoplasm, and Weigert's hematoxylin (Sigma-Aldrich) for cell nuclei and imaged using a brightfield microscope (Nikon Eclipse 80i).

EBs with and without CSMA microspheres were also stained with LIVE/DEAD stain (30 minutes) and imaged by laser-scanning confocal microscopy (LSM 510; Carl Zeiss, Oberkochen, Germany) to examine the cytotoxicity of CSMA microspheres on ESCs within 3D EBs.

2.4. Statistical Analysis

Statistical significance was determined using Minitab 15 Statistical Software (State College, PA) with a one-way analysis of variance (ANOVA) with Tukey's multiple comparison test ($p < 0.05$) for swelling ratio and cytotoxicity analysis and with a 2-sample t-test ($p < 0.05$) for growth factor loading efficiency and cumulative release. All values are reported as mean $\pm$ standard deviation.

3. RESULTS

3.1. Characterization of CS Nano- and Microspheres

3.1.1. Proton Nuclear Magnetic Resonance Spectroscopy— ^1H NMR spectra indicate that APMAM was successfully conjugated to CS by the EDC reaction. CSMAm product synthesized in the presence of sulfo-NHS clearly showed the appearance of vinyl groups, found in the APMAM moiety (Figures 2b-d; peaks B₁ and B₂, corresponding to the labeled protons in Figure 1a). The modified CS product also possessed proton peaks C and E, as labeled in Figures 2b-c, which were present in APMAM. ^1H NMR spectra also suggested that the degree of modification increased with increased ratio of starting reagents

per CS disaccharide equivalent. While vinyl groups were clearly visible in 4:1, 3:1, and 5:2 molar ratio reactions, vinyl peaks were barely distinguishable in the 7:10 reaction product (Figure 2c,d, and data not shown). At 1:5 molar ratio and when synthesized at 4:1 ratio without sulfo-NHS, vinyl peaks were not visible (Figure 2e,f). Similarly, peaks C and E did not appear in these reaction products. This indicates that despite a modification of the CS from the original reagent (as indicated by the light scattering data below), APMAM protons were not visible by ^1H NMR at 1:5 molar ratio and without sulfo-NHS. Successful methacrylation of CS to form CSMA via reaction with methacrylic anhydride was also verified by ^1H NMR (data not shown).

3.1.2. Hydrogel Formation and Swelling—CSMAm crosslinking and swelling results in PBS also indicated that the degree of modification increased with increasing ratio of starting reagents per CS disaccharide equivalent. While 1:5 and 7:10 ratio reactions and the reaction without sulfo-NHS resulted in products that did not form robust hydrogels, at 5:2 molar ratio and above CSMAm crosslinked to form bulk hydrogels (Table 1). As molar ratio increased from 5:2 to 3:1 to 4:1, swelling ratio significantly decreased from 39.8 ± 1.8 to 20.4 ± 1.2 to 12.8 ± 0.6 , respectively. Decreased swelling is indicative of increased crosslinking density [21], likely as a result of higher degree of substitution of the methacrylamide in the EDC reaction. For comparison, crosslinked CSMA materials used for microsphere fabrication possessed a swelling ratio of 31.3 ± 3.6 (data not shown).

3.1.3. Size Distribution of CS Micelles—Highly substituted CSMAm synthesized in the presence of sulfo-NHS did not appear to form stable micelles in an aqueous environment; therefore, only the 4:1 molar ratio without sulfo-NHS was used for micelle characterization. In order to accurately characterize the size of the CSMAm micelles, DLS analysis was performed. Micelle formation was examined by DLS for independent synthesis batches ($n=3$) and was verified to possess similar diameters (data not shown). CSMAm micelles formed with an average diameter of 324.1 ± 8.5 nm in ddH₂O (Figure 3). A lower intensity collection of smaller CSMAm micelles appeared to form at 73.2 ± 4.4 nm, as well. Unmodified CS at the same concentration, did not produce detectable scattering by DLS. Zeta potential measurements indicated that the CS micelles possessed a zeta potential of -38.7 ± 1.1 mV.

3.1.4. Morphology and Size Distribution of CS Microspheres—The CS microspheres had a smooth, round morphology, and Coulter Counter analysis indicated that the microspheres exhibited a unimodal size distribution with an average diameter of 4.3 ± 0.93 μm (Figure 4a,b). Additionally, the microparticles stained positively by DMBB for sulfated GAG, appearing purple (Figure 4a).

3.1.5. Complexation and Release from CS Microspheres—To verify that a model positively charged growth factor was able to electrostatically complex with CS, the ability of CS microspheres to bind TGF- β 1 in solution was examined. When the CS microspheres were incubated overnight with 25 ng of TGF- β 1 (500 μl at 50 ng/ml), $97.4 \pm 1.3\%$ of free TGF- β 1 in solution became incorporated with the CS microspheres (data not shown). Additionally, in the following release study, positively charged TGF- β 1 exhibited no appreciable release after loading in CS microspheres. Only $1.4 \pm 0.3\%$ (0.34 ± 0.08 ng per mg microspheres) of loaded TGF- β 1 was observed in solution at time 0, representing a low amount of unloaded or loosely affiliated growth factor and high loading efficiency, and only 0.2% (0.04 ng) additional release was detected over the following 5 days (Figure 4c). In contrast, negatively charged TNF- α , loaded identically to TGF- β 1, demonstrated $17.7 \pm 3.5\%$ (4.43 ± 0.87 ng per mg microspheres) of loaded TNF- α in solution at time 0, representing a significantly lower loading efficiency than TGF- β 1, and $43.9 \pm 9.1\%$

cumulative release (10.97 ± 2.27 ng) over the first 15 hours from CS microspheres with no further detectable release after 15 hours (Figure 4c). Cumulative release of TNF- α from 3 hours to 5 days, correcting for differences in loading, was significantly greater than TGF- β 1 released. Overall, after 5 days, significantly more release of TNF- α was observed compared to TGF- β 1.

3.2. Cytocompatibility of CS Nano- and Microspheres

3.2.1. In Vitro Cytotoxicity of CS Micelles and Microspheres on 2D MSC Monolayers

—LIVE/DEAD staining of bovine MSCs cultured in monolayer in the presence of increasing concentrations of CSMAm micelles and CSMA microspheres revealed that cells remained largely viable at the lowest concentrations of 1 and 10 mg/10⁶ cells with 1.21 ± 0.14 and 0.77 ± 0.05 viability, respectively, for CSMAm micelles and 1.09 ± 0.11 and 1.12 ± 0.29 viability, respectively, for CSMA microspheres, compared to live controls which possessed normalized viabilities of 1.00 ± 0.08 and 1.00 ± 0.13 for CSMAm and CSMA experiments, respectively (Figure 5a). Viability of all 1 and 10 mg/10⁶ cells samples was not statistically different from live controls. Cells appeared mostly green (live) and possessed a spread morphology, characteristic of MSCs (Figure 5b,c). At 100 mg/10⁶ cells, a significant decrease in viability was observed in the presence of CSMAm with 0.38 ± 0.06 viability, compared to live controls, as MSCs experienced a large decrease in cell density and spreading (Figure 5a,d), while viability did not significantly decrease in CSMA-containing media (0.98 ± 0.16 viability; Figure 5a,d). At the 320 mg/10⁶ cells concentration, very few cells remained adhered to the surface (Figure 5e) in both CSMAm and CSMA samples, resulting in a significant decrease in viability to 0.18 ± 0.07 and -0.05 ± 0.06 , respectively.

3.2.2. In Vitro Cytotoxicity of CS Microspheres when Incorporated within 3D ESC Embryoid Bodies

—CS microspheres were successfully incorporated within embryoid bodies using the forced aggregation method described. The microspheres stained positively in histological sections for sulfated GAG by Safranin-O, appearing red (Figure 6b). Surrounding ESCs also appeared to be morphologically normal, compared to control EBs (Figure 6a), with no signs of cell death or adverse cell response after 3 days, suggesting that the incorporation of CS microspheres within 3D stem cell aggregates does not negatively impact the extracellular microenvironment. Additionally, LIVE/DEAD staining of EBs observed similar degrees of viability with CSMA microspheres incorporated (Figure 6d), when compared to control EBs (Figure 6c), indicating that ESCs remained largely viable when CSMA microspheres were incorporated within 3D EBs.

4. DISCUSSION

In the first part of this study, a model reaction using EDC (and sulfo-NHS) to conjugate a methacrylamide moiety to CS was used to determine the effects of degree of modification on the properties of the resulting GAG-based material. This approach can be applied to any polysaccharide possessing a carboxylic acid moiety, including dermatan sulfate, heparin, heparan sulfate, and hyaluronic acid [22]. The resulting CSMAm material demonstrated increasing degrees of modification with greater molar ratios of starting reagents, resulting in higher degrees of crosslinking, as verified by NMR data and significantly decreased swelling in bulk hydrogels (Figure 2 and Table 1).

Interestingly, at lower degrees of modification, CSMAm self-assembled into micelles in aqueous solution. When reacted at 1:5 molar ratio and without sulfo-NHS, proton peaks associated with APMAm were not visible by ¹H NMR (Figure 2e,f), though a modification of CS was apparent by the formation of nanospheres, as confirmed by DLS analysis (Figure

3). It is likely that the APMam groups were not visible by ^1H NMR, because these hydrophobic moieties were internalized within the micelle structure that formed in the NMR solvent (water). Similar results were observed in ^1H NMR spectra of CS-PLLA micelles in D_2O , where internal protons were not visible due to limited mobility within the micelle core [10].

In this study, EDC chemistry was used to confirm that the degree of conjugation, and resulting molecular structure, could be controlled by altering the ratios of the starting reactants during synthesis; however, in order to efficiently scale up the size of the reaction while maintaining crosslinking capabilities to form microparticles, methacrylate groups were added to CS by reacting the GAG with methacrylic anhydride. Resulting CSMA was crosslinked in a water-in-oil, single-emulsion to form CS microspheres. While the microspheres described here had an average diameter of $4.3 \pm 0.93 \mu\text{m}$ (Figure 4a,b), the size of the microparticles could be easily modified by altering the emulsion conditions, such as speed of mixing and CSMA concentration, prior to crosslinking [23]. The results of this work suggest that by altering the degree of conjugation, CS-based materials can be developed that can be presented to cells on different size scales, including nanoscale micelles and larger microspheres, as well as controlled degree of crosslinking within particles. Therefore, this set of materials provides a versatile platform to explore presentation and release of various growth factors because the basic chemistry of the delivery vehicle is not altered, while a wide range of particle sizes is achieved.

Zeta potential results indicate that CSMA micelles possessed of zeta potential of $-38.7 \pm 1.1 \text{ mV}$, confirming that the micelles were negatively charged and stable in solution and suggesting that these micelles may have application in delivery of various cationic factors. Similarly, CSMA microspheres were shown to electrostatically complex with TGF- β 1 at physiological pH. TGF- β 1 was capable of a higher loading efficiency than TNF- α , as indicated by the time 0 data point, despite identical loading conditions, likely due to electrostatic interactions. Considerable release of TNF- α was also observed from CS microspheres over the first 15 hours; however, minimal TGF- β 1 release was seen over 5 days, indicating that TGF- β 1 remained bound to the CS microspheres during this time (Figure 4c). TGF- β 1 possesses an isoelectric point (pI) of 9.5 and thus was selected to represent a positively charged growth factor [24], while TNF- α has a pI of 5.3, making it negatively charged at physiological pH [25]. Such results are comparable to other CS-based hydrogels and microspheres, which have been reported to retain positively charged lysozyme, aprotonin, and vasopressin [11, 26]. TGF- β isoforms are important for a number of processes in mammalian development [27–28], and have been particularly implicated in chondrogenesis *in vivo* [29] and *in vitro* [30–31], making this a relevant growth factor to investigate for directing differentiation of stem cells. While 100% of loaded TNF- α was not released over the time course of this study, as might be expected with little affinity between the cytokine and the CS matrix, this could be explained by the potential degradation of the protein over time in solution, resulting in artificially low ELISA readings after release [32].

Cumulative release data from these experiments suggest that significantly more TNF- α was liberated than TGF- β 1, thus supporting the use of these materials for controlled delivery via electrostatic interaction with growth factors. Delivery kinetics can be further tailored by customizing the size and crosslinking density of the particles by altering the synthesis and fabrication parameters [33–34]. In combination with the release of growth factors through dissociation from the CS-based carrier, these biomaterial carriers could potentially be enzymatically cleaved by chondroitinase, a naturally secreted enzyme that digests chondroitin sulfate [35], thereby providing a second method for localized delivery.

In addition to control over size and the potential for degradation, the CS-based particles presented here are particularly advantageous because both types of particles are fabricated almost entirely from only CS molecules, for nearly uniform biochemical presentation to target cells. In previous work, CS has been combined with synthetic polymers like PLLA and PLGA, as well as natural materials including chitosan, alginate, and gelatin [10,12,36–38] to produce micron-scale particles. However, to the best of our knowledge, this is the first time that CS microspheres and micelles have been synthesized without significant modification with other large polymers, making this system beneficial for specifically probing GAG-stem cell interactions.

While CS plays a role in many developmental processes, CS delivery vehicles may be particularly useful to promote chondrogenesis. CS-containing proteoglycans versican and perlecan regulate mesenchymal condensation and growth factor signaling during cartilage development [39–40], and culture in CS-containing hydrogels upregulated expression and production of chondrocytic ECM by encapsulated mesenchymal stem cells [41]. Therefore, in the future, the use of CS-based materials for controlled growth factor delivery may be advantageous as a means to direct cell differentiation to promote regeneration of cartilaginous tissues.

As a next step in the development of these materials as bioactive factor delivery vehicles, the cytotoxicity of CS was investigated *in vitro* using bovine MSCs and murine ESCs. MSCs and ESCs were chosen for their multipotent and pluripotent potential, respectively [42–43]. In this study, MSC monolayers were cultured with the presence of CS micelles and microspheres in the media for 24 hours to determine cytotoxicity in 2D, and CS microspheres were incorporated into ESC embryoid bodies for 3 days to examine cytocompatibility within 3D cell spheroids. ESCs are often cultured and differentiated within embryoid bodies; however, these dense, multicellular spheroids possess numerous boundaries to diffusion [8]. CS particles could potentially be utilized to deliver growth factors throughout the EB to improve homogeneous differentiation, compared to diffusion of soluble factors from the media [8].

MSCs, cultured in 2D with moderate concentrations of CSMAM micelles and CSMA microspheres of 1 and 10 mg/10⁶ cells, remained viable with no statistical difference from live controls (Figure 5a-c), while CSMA microspheres also remained cytocompatible up to 100 mg/10⁶ cells. Similarly, ESCs surrounding CS microspheres incorporated into EBs appear to be morphologically healthy with no apparent cell death (Figure 6), suggesting that CS microspheres could potentially be used to deliver growth factors within dense cell aggregates. At the highest concentration tested, 320 mg/10⁶ cells (as well as 100 mg/10⁶ cells for CSMAM), MSCs experienced statistically decreased viability after 24 hours with the presence of CSMAM micelles and CSMA microspheres in the culture media (Figure 5a,d-e). Cell death may have resulted at high concentrations due to endocytosis of nanoscale CSMAM micelles by cells [9], or the high negative surface charge of CS particles may have interacted with receptors on the cell membrane [44] or altered the osmotic pressure of the media, though these mechanisms were not further explored in this study. The range of concentrations in these studies, equivalent to 0.064, 0.64, 6.4, and 20.5 mg/ml, includes and exceeds previously reported values used for cytotoxicity testing of CS-based microspheres and were defined according to ASTM International Standards F1903 and F813 regarding cytotoxicity testing of particles and materials, respectively [10, 12, 45–46]. Only 1 and 10 mg CS/10⁶ cells samples (0.064 and 0.64 mg/ml, respectively), which experienced no significant difference in cell viability from live controls, fell within previously reported ranges for cytotoxicity testing (0–5 mg/ml) of CS-PLLA microspheres [12]. Taken together, these results indicate that CS materials did not appear to affect the morphology of stem cells when cultured at moderate concentrations.

Overall, the results of these studies demonstrate that CS chains were successfully modified to form amphiphilic self-assembling CS micelles on a nanoscale (~73 nm and ~320 nm), as well as microscale fabrication of CS microspheres (~4 μm) for a wide range of sizes, and that CS microspheres retained their ability to bind positively charged growth factors. This flexibility in particle size can potentially provide a large degree of control for release of cationic factors from these CS materials, and the use of naturally-derived polysaccharide matrix without modification by other polymers may be especially advantageous in better understanding the role of GAGs in cell differentiation. Therefore, CS nano- and microspheres with the ability to deliver growth factors via electrostatic interaction provides a controlled, yet extremely versatile platform to further explore means to direct differentiation of stem cells for a variety of applications in tissue engineering and regenerative medicine.

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Figure 1.

Modification reactions of chondroitin sulfate. (a) Chondroitin sulfate A (CS) and N-(3-aminopropyl)methacrylamide (APMAm) were reacted in the presence of N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide (EDC) with or without N-hydroxysulfosuccinimide (sulfo-NHS) to yield chondroitin sulfate methacrylamide (CSMAm). Protons are labeled for ^1H NMR results in Figure 2. (b) CS and methacrylic anhydride were reacted in the presence of NaOH to yield chondroitin sulfate methacrylate (CSMA).

Figure 2.

^1H NMR spectra of (a) chondroitin sulfate, (b) APMAm, and reaction products synthesized at (c) 4:1, (d) 7:10, and (e) 1:5 molar ratios of APMAm, EDC, and sulfo-NHS to CS disaccharide, as well as (f) 4:1 ratio without sulfo-NHS. Protons are labeled according to corresponding diagrams in Figure 1a, and spectra indicate successful addition of methacrylamide moieties to the chondroitin sulfate.

Figure 3.

Size characterization of CSMAM micelles. Dynamic light scattering measurements of hydrodynamic diameter indicated that a bimodal distribution was present with average diameters of 324.1 ± 8.5 nm and 73.2 ± 4.4 nm. Particle sizing measurements were consistent for 4 separate samples (shown in red, blue, green, and orange).

Figure 4.

Characterization of CSMA microspheres. (a) CS microspheres possessed a smooth, rounded morphology and stained positively for sulfated GAG by DMMB (purple). Scale bar = 20 μm. (b) CS microspheres exhibited a unimodal distribution with an average diameter of 4.3 ± 0.93 μm. (c) Positively charged TGF-β1 (triangles) experienced very little release from negatively charged CS microspheres after 5 days; however, negatively charged TNF-α (squares) experienced large early release within 15 hours. * indicates statistically different loading efficiency (0 hours) compared to TGF-β1 loaded samples. + indicates statistically different cumulative release from 3 hours to 5 days compared to TGF-β1 release samples ($p < 0.05$).

Figure 5.

LIVE/DEAD cytotoxicity analysis of bovine marrow stromal cells cultured in 2D monolayer in the presence of CSMAM micelles and CSMA microparticles. (a) Fraction of live cells per well compared to live controls, as determined by fluorescence values recorded at 517 nm wavelength. LIVE/DEAD fluorescence images in the presence of (b) 1 mg, (c) 10 mg, (d) 100 mg, and (e) 320 mg CSMAM or CSMA per 10^6 cells for 24 hours. Live cells appear green, while dead cells appear red. Scale bar = 200 μ m. * indicates statistically different from live controls ($p < 0.05$).

Figure 6.

Cytotoxicity of mouse embryonic stem cells with CS microspheres incorporated within 3D embryoid bodies. (a) Cells in embryoid bodies without CS microspheres. (b) CS microspheres stained positively for sulfated GAGs by Safranin-O (red stain; black arrows). Surrounding cells appear morphologically normal, with no signs of cell death after 3 days. Scale bar = 50 μm. A boxed region of cells and microspheres is also shown at higher magnification. Scale bar = 20 μm. (c) LIVE/DEAD fluorescence images of embryoid bodies without CS microspheres and (d) with incorporated CS microspheres, showing similar degrees of viability. Live cells appear green, while dead cells appear red. Scale bar = 200 μm.

Table 1

Molar ratios of reactants in EDC modification of CS and resulting swelling ratios of crosslinked hydrogels in PBS

CS (by disaccharide MW)	Molar Ratio to CS			Swelling Ratio
	APMam	EDC	Sulfo-NHS	
2.5 mM	4:1	4:1	None	No Gel
2.5 mM	1:5	1:5	1:5	No Gel
2.5 mM	7:10	7:10	7:10	No Gel
2.5 mM	5:2	5:2	5:2	39.8 ± 1.8 *
2.5 mM	3:1	3:1	3:1	20.4 ± 1.2 *
2.5 mM	4:1	4:1	4:1	12.8 ± 0.6 *

* indicates statistically different from all other samples (p<0.05)