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Alginate-chitosan hydrogels provide a sustained gradient of sphingosine-1-phosphate for therapeutic angiogenesis

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Abstract

Sphingosine-1-phosphate (S1P), a bioactive lipid, is a potent candidate for treatment of ischemic vascular disease. However, designing biomaterial systems for the controlled release of S1P to achieve therapeutic angiogenesis presents both biological and engineering challenges. Thus, the objective of this study was to design a hydrogel system that provides controlled and sustained release of S1P to establish local concentration gradients that promote neovascularization. Alginate hydrogels have been extensively studied and characterized for delivery of proangiogenic factors. We sought to explore if chitosan (0, 0.1, 0.5, or 1%) incorporation could be used as a means to control S1P release from alginate hydrogels. With increasing chitosan incorporation, hydrogels exhibited significantly denser pore structure and stiffer material properties. While 0.1 and 0.5% chitosan gels demonstrated slower respective release of S1P, release from 1% chitosan gels was similar to alginate gels alone. Furthermore, 0.5% chitosan gels induced greater sprouting and directed migration of outgrowth endothelial cells (OECs) in response to released S1P under hypoxia *in vitro*. Overall, this report presents a platform for a novel alginate-chitosan hydrogel of controlled composition and *in situ* gelation properties that can be used to control lipid release for therapeutic applications.

Key Terms:

Composite hydrogel; controlled release; lipid; outgrowth endothelial cell; homing; proangiogenic factors; sphingosine-1-phosphate (S1P)

1. Introduction

Hydrogels are appealing biomaterials for therapeutic applications given their low toxicity and strong biocompatibility.^{2,30,46} In particular, alginate hydrogels have been widely used for a broad range of tissue engineering strategies.^{1,10,18,36–38} Alginate polymers beneficially enable *in situ* gelation and thus may be delivered in a minimally invasive manner with known and tunable degradation kinetics.⁴ Alginate is an anionic copolymer derived from brown seaweed containing blocks of (1,4)-linked β -D-mannuronate and α -L-guluronate

residues.¹⁸ Another extensively studied polysaccharide for tissue engineering applications is chitosan, a cationic copolymer derived from chitin and composed of β -(1–4) linked 2-acetamido-2-deoxy- β -D-glucopyranose and the same de-N-acetylated sugar unit.^{16,30} Chitosan and alginate are known to form polyelectrolyte complexes and have been combined to create hybrid material systems with a variety of methods for tissue engineering purposes.^{2,8,16,24,43,48} Work in this field has traditionally focused on biomaterial delivery of large cargoes (i.e. growth factors and cytokines), but lipid mediators such as S1P have gained recent attention as equally suitable targets.^{5,28} Thus, there is a growing need for a better understanding of how these material systems may be created and tuned to control bioactive lipid release.

S1P boasts a prime pharmacological agent for promoting recruitment of blood vessel forming endothelial progenitor cells (EPCs) and overall therapeutic angiogenesis within damaged or ischemic tissues.^{19,45} S1P is a potent regulator of neovascularization in both tissue development and repair and is a crucial mediator of EPC trafficking.¹⁹ This bioactive lipid is upregulated under ischemic conditions and has been shown to stimulate greater angiogenic activity by outgrowth endothelial cells (OECs), a subset of EPCs that directly participate in blood vessel development, under hypoxic conditions characteristic of ischemia.⁴⁷ Endogenous S1P gradients are highly regulated and are critical for the lipid's essential role in spatial guidance and signaling for regulating cellular trafficking.²⁸ Therefore, sustained delivery is paramount to locally establishing favorable concentration gradients. However, designing biomaterial systems for controlled delivery of S1P remains both an engineering and biological challenge given its small size, strong hydrophobicity, and rapid metabolic turnover.^{5,28}

Thus, the objective of this study was to design a biomaterial system that could provide sustained delivery of S1P in a manner that would locally present gradients favorable for promoting angiogenesis and recruitment of OECs. In this work, we hypothesized that chitosan incorporation within alginate hydrogels would potentially provide electrostatic interactions with S1P thus increasing retention of the lipid and providing a means to control release. Alginate hydrogels were incorporated with varying degrees of chitosan and the impacts on network structure and mechanical properties were evaluated. Furthermore, the ability to use chitosan to control S1P release was tested and the subsequent potential to induce therapeutic angiogenesis was measured in terms of OEC sprouting and directed migration/invasion under ischemic conditions *in vitro* and blood vessel development in a chick chorioallantoic membrane (CAM) assay *in vivo*.

2. Materials and Methods

2.1 Hydrogel formulation

Alginate and chitosan polymers were purchased from Novamatrix. MVG alginate, LF20/40 polymer, containing a higher G-block content (> 60% as specified by the manufacturer) was used as the high molecular weight (HMW) component to prepare gels. LF10/60 polymer was used as the low molecular weight (LMW) component. Ultra-pure (UP) alginate polymer was used for *in vitro* cellular and *in vivo* assays. UP MVG LMW alginate was prepared by gamma (γ)-irradiating the HMW UP MVG alginate as previously described.^{37,38} Both of the

HMW polymers and LMW polymers were of comparable molecular weights (~250 kDa and ~120–150 kDa, respectively). All alginates were oxidized with sodium periodate (Sigma) to an extension of 1% of the sugar residues in the polymer. The oxidized alginate solutions were dialyzed, sterile filtered, lyophilized, and subsequently stored at -20°C . To prepare hydrogels, modified alginates were reconstituted to 2.667% w/v in phosphate buffered saline containing calcium and magnesium ions (PBS++; Life Technologies). UP chitosan CL 113 (~50–150 kDa and 70–90% de-acetylated) was used for the chitosan component. Chitosan was first dissolved at 8% w/v in 0.066 M glacial acetic acid (Sigma) for at least 3h then further diluted to desired % w/v with PBS++ and sterile filtered. To make hydrogels, 1.125 mL of LMW and 0.375 mL of HMW alginate solutions were first taken up into separate syringes, mixed together using a syringe connector, and collected into one syringe. This 75/25 (LMW/HMW) alginate solution was then mixed with 0.5 mL of 0.4, 2, or 4% w/v chitosan held in a separate syringe in order to achieve alginate-chitosan hydrogels at 0.1, 0.5, and 1% total chitosan content, respectively, and 2% total alginate content. For alginate hydrogels without chitosan, the solution was mixed with 0.5 mL of PBS++ instead. The syringes were then disconnected, 80 μL of calcium sulfate (Sigma) slurry was added, and the contents of the syringes were again mixed in order to ionically crosslink the hydrogels. The mixture was then dispensed onto a glass plate set with 1-mm spacers, sandwiched with another glass plate, and incubated for at least 25 minutes at room temperature. Disks were then punched out using a 10-mm biopsy punch (Acuderm Inc.). For S1P-loaded hydrogels, 20 μL S1P (1 mM in methanol) was added prior to mixing the alginate and chitosan solutions together. For hydrogels without S1P, methanol was incorporated as a carrier control. Hydrogels containing 0.1% chitosan were used as the blank control for all S1P-related experiments. A new set of hydrogels was prepared for each experiment.

For characterization of hydrogels of varying alginate polymer content, LMW alginate was dissolved at 2, 2.1, 2.5, or 3% w/v in PBS++. In each of two syringes, 1 mL of the designated alginate solution was collected and cross-linked together with calcium sulfate slurry using a syringe connector. The disks were then cast as previously described above.

2.2 Hydrogel characterization

Hydrogel disks were fabricated as described above using ultra-pure water and prepared for scanning electron microscopy (SEM) observation as previously described.^{3,16} Briefly, after lyophilization, disks were dissected in half using a razor blade, and then mounted on carbon tape with the cross section facing upwards. The samples were then coated with a thin layer of gold/palladium, placed in a vacuum chamber, and imaged with the XL30 Scanning Electron Microscope using a field emission gun electron source (Philips/FEI).

In order to qualitatively evaluate chitosan incorporation within alginate hydrogels, gels were prepared and immersed in Orange II dye solution (Acros Organics) as previously described with minor modifications.^{21,42} Briefly, hydrogel disks were immersed in Orange II solution (0.5 mg/mL in 0.066 M acetic acid) and incubated overnight at room temperature. Control alginate gels were immersed in 0.066 M acetic acid. The gels were then washed, placed in PBS++ for 24 hours, and excess liquid was removed using a damp kimwipe prior to photographing.

The equilibrium swelling ratios (Q_s) were determined as previously described with minor modifications.^{6,15} Briefly, hydrogels were cast and then swelled in PBS++ for 24 hours. Excess fluid was removed with a damp kimwipe and the mass of the swollen gel was determined. The gels were then frozen and lyophilized, and the dry weight was obtained. The swelling ratio was defined as:

$$Q = (W_s - W_d)/W_d$$

where W_s is the weight of the swollen gel and W_d is the weight of the dry gel.

To measure the storage moduli (G') of the hydrogels, the gel disks were first swollen in PBS++ overnight (room temperature) and excess fluid was removed with a damp kimwipe. The gel disks were then trimmed to a diameter of 8mm using a biopsy punch (Miltex) and placed between parallel plates (gap distance set at 0.9mm) in a rheometer (HR2, TA Instruments). The gels were then strained over a range within 0.4–5% at a frequency of 10 rad/sec and values for G' were obtained within the linear viscoelastic region. At least 9 points were used to obtain an average G' value for each disk and then an average G' value was reported for each hydrogel condition ($n=5-6$). In order to measure changes to the storage moduli over time, the gels immersed in PBS++ were incubated at 37°C, and measurements were recorded at the indicated time-points.

The initial mesh sizes (ξ) of the hydrogel networks were calculated as previously described using the measured values of storage moduli and swelling ratios at 24h.^{17,22,27} In particular, the molecular weight between crosslinks (M_c) was determined by:

$$M_c = c_p RT / G'$$

where c_p is the total concentration of the polymer solution, R is the gas constant ($8.314 \text{ m}^3 \cdot \text{Pa} \cdot \text{mol}^{-1} \cdot \text{K}^{-1}$), and T is the temperature at which the measurement was performed (296.15 K). The polymer volume fraction (v_2) was determined by:

$$v_2 = 1 + Q \rho_p / \rho$$

where ρ_p is the density of the polymer ($1.601 \text{ g} \cdot \text{cm}^{-3}$)¹⁷ and ρ is the density of water ($1.0 \text{ g} \cdot \text{cm}^{-3}$). The mesh size was then calculated as:¹⁷

$$\xi = v_2^{-1/3} L / (2M_c / M_r)^{1/2} C_n^{1/2}$$

where M_r is the molecular weight of the monomer units (390.1 g/mol), L is the carbon-carbon bond length of the monomer unit (5.15 Å), and C_n is the characteristic ratio ($C_n = 0.021M_n + 17.95$ calculated as for alginate). For all hydrogels, the characteristic values for alginate were also used for chitosan for simplicity considering widely known analogies between two polysaccharides.^{27,34}

2.3 S1P³³ release from hydrogels

In order to determine S1P release from the various hydrogels, S1P³³ (Perkin Elmer) was quantified via liquid scintillation counting. Hydrogels were loaded with S1P³³:S1P in a ratio of 1:3, immersed in eluent buffer consisting of PBS++ with 5% fatty acid free bovine serum albumin (FAF-BSA; Gemini Bio Products), and incubated at 37°C and 5% CO₂. At each time point, the eluent buffer was collected and 1 mL was reacted with 3 mL of scintillation fluid (EcoLite+) prior to reading in a liquid scintillation counter (Packard). Samples taken from blank hydrogels immersed in eluent buffer were used to subtract background readings. A mock solution was used to calibrate the amount of S1P present at each time point.

2.4 Cell culture

Endothelial Cell Growth Media –2 Microvascular (EGM-2MV) was prepared as instructed by the vendor (Lonza). N media, defined as EGM-2MV without the addition of growth factors, was used as the control medium in all experiments. For studies under hypoxia (1% oxygen), cells were cultured as previously described.⁴⁷

2.5 Isolation and characterization of OECs

OECs were isolated from human umbilical cord blood obtained from the UC Davis Umbilical Cord Blood Collection Program (UCBCP) and used within 12 hours of collection. OECs were isolated from female cord blood (one donor) following protocols approved by the UC Davis Stem Cell Research Oversight Committee and as previously described.^{12,13,36} The cells were confirmed to be OECs based on cobblestone morphology and marker identification via flow cytometry. Briefly, cells were blocked with human IgG (1 µg IgG/10⁵ cells) and then stained with phycoerythrin (PE) conjugated antibodies for human CD31 (Clone 9G11), CD34 (Clone QBEnd10), CD146 (Clone 128018), and CD45 (Clone 2D1), and fluorescein conjugated antibodies for human vascular endothelial growth factor receptor 2 (VEGFR2, Clone 89106), CD105 (Clone 166707), CXCR4 (Clone 44717), and CD14 (Clone 134620; R&D Systems). The cells were then analyzed using flow cytometry (FACScan; BD). Unstained cells or cells stained with an appropriate isotype control antibody were used for comparison. OECs were used between P3 and P5 for all experiments.

2.6 Angiogenic sprouting in response to released S1P

Cytodex 3 microcarrier (MC) beads (GE Healthcare Life Sciences) were hydrated, sterilized, and seeded with OECs as previously described in detail.⁴⁷ For analysis of 3D sprout formation in response to S1P released from the hydrogels of interest, the cell-laden MC beads were first incorporated within fibrin gels as previously described.^{36,37} Briefly, beads suspended in N media were combined with fibrinogen (Sigma) solution supplemented with aprotinin (Sigma) and distributed in 24-well plates. A second solution containing thrombin (Sigma) was then added at a 4:5 ratio and the plates were incubated at 37°C for 30 minutes. The rightmost third of the gel was then gently removed via aspiration and 50 µL of alginate or alginate-chitosan hydrogel loaded with S1P (as described earlier) was dispensed through a needle into the newly made space. Blank hydrogel containing 0.1% chitosan served as the negative control. The hydrogel was then covered with a fibrin solution without cell-laden beads in order to fill any air gaps and was incubated at 37°C for 30 minutes to allow for

gelation. The sealed gels were then covered with 0.5 mL of N media and incubated under hypoxia for 4 days without media changes. At this experimental endpoint, the gels were washed with PBS and fixed overnight at 4°C in 4% formaldehyde. For fluorescent imaging, the cells were stained with Hoechst 33342 (Molecular Probes). The average number of sprouts per bead was then calculated as previously described⁴⁷ and normalized to the average value for the negative control (n=4).

2.7 Directed migration/3D matrix invasion in response to released S1P

A modified Transwell chemotaxis assay was used to assay directed migration/3D matrix invasion of OECs in response to S1P released from the various hydrogels of interest as previously described with minor modifications.⁴⁷ Transwell inserts were pre-incubated in Endothelial Basal Media-2 (EBM-2; Lonza) overnight in order to prime the filter membranes. Either alginate or alginate-chitosan hydrogels loaded with S1P were prepared. Blank hydrogels containing 0.1% chitosan served as the negative control. Hydrogel disks were placed in the center at the bottom of each well in a 24-well plate, covered with 0.5 mL of fibrin gel solution, and incubated at 37°C for 40 minutes. The gels were then topped with 0.5 mL of N media. A transwell insert was then placed within the well, making slight contact with the fibrin gel, and seeded with 30,000 cells (labeled with Hoechst 33342) in 0.1 mL of N media. The plates were then incubated under hypoxia for 4 days without media changes. The inserts were removed and a cotton swab was used to remove any non-migrated cells in the top chamber. The inserts and gels were then fixed with 4% formaldehyde overnight at 4°C. In order to quantify the number of cells that migrated through the transwell membrane, the entire insert was fluorescently imaged at 20X to detect Hoechst staining (nuclei) using a Nikon Eclipse TE2000-S microscope. In order to quantify the number of cells that invaded the fibrin matrix, each fixed gel was removed from the well and sandwiched between two glass slides (VWR) prior to imaging. The total number of cells was quantified using ImageJ (n=6).

2.8 CAM assay

The *in vivo* activity of S1P released from hydrogels was assessed via a modified open-shell CAM assay.³⁹ Briefly, fertilized hy-line white leghorn chicken eggs (E0) were purchased from the UC Davis Avian Facility and were incubated vertically at 37.8°C for 3 days with 55–65% humidity and six rotations per day. On the third day (E3), the eggs were placed horizontally and the top of the egg was marked with a pencil line. The eggs were then held horizontally with the pencil mark facing upwards and were cracked open into 88.9 × 88.9 mm weigh boats (Fisher Scientific) in a laminar flow hood with aseptic techniques. The petri dishes containing the embryos were then incubated at 37.8°C with 90% humidity in aseptic conditions. On day 10 (E10), either blank (negative control) or S1P-loaded hydrogel disks were placed on the CAM away from major blood vessels. Pictures were taken with a 12-megapixel camera (iPhone 6S; Apple Inc.) of the region of the hydrogel location at 0h and after 24h of incubation (n=3). The angiogenic effects were assessed by quantifying the number of blood vessels surrounding the gel within a 2 mm radius as previously described.⁷ The percent change in the number of blood vessels over 24 hours was then calculated for each CAM. The percent difference from the blank control was then reported for each hydrogel condition.

2.9 Statistical analysis

Results are shown as the mean values with standard deviations. Comparisons were assessed by one-way analysis of variance (ANOVA) according to experimental design, followed by Tukey's test for multiple comparisons. In all cases, significance was asserted at $P < 0.05$. All analyses were performed using GraphPad Prism software (GraphPad Software Inc.).

3. Results

3.1 Physical and mechanical characterization of alginate-chitosan hydrogels

Alginate-chitosan hydrogels were ionically cross-linked to generate hybrid hydrogels with varied physical and mechanical properties. Figure 1A provides a proposed schematic of the hydrogel network and hypothesized electrostatic retention of S1P. Chitosan was incorporated within this hydrogel system at varying concentrations (0, 0.1, 0.5, 1 wt%). At all levels of chitosan incorporation, the hydrogels were first verified to retain injectability through an 18G needle and thus allow for minimally invasive delivery for therapeutic applications in the future (Fig. 1B). Next, the physical properties of the different hybrid alginate-chitosan hydrogels were monitored via SEM and Orange II dye incorporation (Fig. 1C–D). SEM was particularly useful to investigate the morphological changes in the hydrogel network upon incorporation of varying degrees of chitosan. Overall, the different hydrogels displayed a highly porous network with similar pore morphologies. The pore structure of the hydrogels became denser with increasing amounts of chitosan incorporation.

We next evaluated the ability to control the equilibrium swelling ratios and initial storage moduli properties of the hydrogels by the presence of chitosan within the polymer network. As such, increasing chitosan incorporation resulted in lower swelling ratios (Q) and higher storage moduli (G') indicative of increasing stiffness (Fig. 2A). Hydrogels of comparable alginate content alone did not exhibit this drastic increase in storage modulus (Fig. 2B). Correspondingly, the mesh size (ξ) decreased with increasing chitosan content with values of 7.9 ± 0.9 , 6.4 ± 0.6 , 4.2 ± 0.3 , and 2.8 ± 0.2 nm for 0, 0.1, 0.5, and 1% chitosan incorporation, respectively (Fig. 2C). Furthermore, degradation rates of hydrogel elasticity based on storage modulus were similar for all of the various hydrogel compositions with a more rapid decline exhibited within the first week (Fig. 2D).

3.2 OEC isolation and characterization

Clinically relevant vascular progenitor cells, human OECs, were used to investigate the effect of locally generated gradients of S1P from these hybrid hydrogels. OECs were isolated from cord blood following a widely established protocol^{12,13,36} and displayed characteristic cobblestone morphology (Fig. 3A). While there is no defined set of cell surface markers currently utilized to universally identify an OEC, it has been previously shown and confirmed here that these cells are positive for CD31, VEGFR2, CD34, CD105, CD146, and CXCR4, and negative for CD14 and CD45 (Fig. 3B).^{13,36,49}

3.3 Chitosan incorporation modulated S1P release over time and altered OEC sprouting and migration in vitro

The modified properties of hybrid hydrogels allowed for sustained and tunable release of S1P *in vitro*. 0.5% chitosan incorporation resulted in significantly slower release of hydrophobic S1P as compared to alginate hydrogels (Fig. 4A). However, 1% chitosan resulted in similar release properties as alginate alone with roughly 1.3- and 1.8-fold faster release than 0.1% and 0.5% chitosan gels, respectively, after 2 weeks. The ability for locally generated gradients of S1P to stimulate OECs was evaluated under hypoxia in order to identify the hydrogel capable of inducing the greatest response in ischemic conditions. OECs significantly sprouted in response to S1P released from hydrogels as compared to the blank control (Fig. 4B). There was not a significant difference in sprouting between alginate hydrogels alone or those with chitosan. Hydrogels containing 0.5% chitosan resulted in significantly more sprouting than either 0.1% or 1% chitosan hydrogels, in accordance with the observed slower S1P release. Additionally, S1P released from all gels containing chitosan resulted in significantly enhanced OEC migration through the transwell insert and invasion into the tissue-like fibrin matrix below compared to the blank control (Fig. 4C). Furthermore, hydrogels containing 0.5% chitosan exhibited an increased trend in migration compared to the other conditions.

3.4 Alginate-chitosan hydrogels exhibiting slower release of S1P maximally enhance blood vessel formation in a CAM assay

The optimal release rate and best-suited hydrogel composition for stimulating blood vessel development was tested via a CAM assay. Overall, S1P release from all hydrogels resulted in an increase in angiogenesis over a 24-hour period (Fig. 5A). Consistent with a slower release rate, the delivery of S1P from 0.5% chitosan gels showed maximal angiogenic potential with statistically significant increases in blood vessel formation as compared to all of the other conditions (Fig. 5B). Furthermore, the ability of S1P to induce blood vessel development appeared to be slightly impaired when delivered from gels with 1% chitosan as compared to all of the other hydrogel compositions.

4. Discussion

This study investigates the utility of alginate-chitosan composite hydrogels for tunable and controlled delivery of a bioactive lipid, S1P. The results of this work confirm our hypothesis that varying the chitosan content within alginate hydrogels controls the release of S1P. Moreover, this work highlights the dynamic nature in which the local gradient of S1P that is established dictates the cellular response and physiological outcome. Interestingly, the findings of this study indicate that slower lipid release results in greater angiogenic activity and recruitment of vascular progenitor cells. Overall, this work provides a strategy for using alginate-chitosan systems with *in situ* gelation properties that could be used for controlled hydrophobic lipid and drug release applications.

The alginate and chitosan gelation procedure described in this work allowed us to create a tunable and injectable hydrogel system with *in situ* gelation. *In situ* gel systems prompt great clinical advantages including ease of administration, lower cost due to often less complex

production processes, and improved patient comfort and compliance.²⁰ Thus, the development of *in situ* forming polymeric drug delivery systems has recently received great attention.²⁰ The use of composite alginate-chitosan systems for material release of encapsulated factors is a rapidly growing field in which considerable work has been focused on the creation of microcapsules^{8,9,11,24} and scaffolds/sponges.^{16,43,48} More recently, different combinations of alginate and chitosan have been tested to create three-dimensional networks formed by the associative forces between the two polymers.^{2,14} However, these systems typically do not enable *in situ* gelation and characterization in the context of hydrophobic lipid delivery is still lacking.

We sought to create a highly tunable and injectable system that can be used and altered as a delivery platform for diverse biomedical applications, including therapeutic angiogenesis. Alginate hydrogels with a bimodal weight distribution and 2 wt% polymer concentration have previously been shown to allow for the creation of hydrogels with low stiffness without impairing the gelling capacity.⁶ This study showed that the addition of a cationic polysaccharide, chitosan, at varying degrees (0.1, 0.5, or 1%) altered the material properties in a manner that resulted in stiffer, yet still injectable, hydrogels with lower swelling ratios and higher storage moduli. Correspondingly, increasing chitosan content resulted in more densely arranged polymer networks with smaller mesh sizes that are within the same overall range as those reported for alginate hydrogels of similar composition using varied cross-linking methods.^{17,22}

Importantly, this study suggests that release of S1P from alginate-chitosan hydrogels can be controlled by the chitosan content to effectively enhance therapeutic benefit via enhanced blood vessel formation. The use of 2% bimodal 75:25 (LMW:HMW) alginate hydrogels has been extensively characterized for the delivery of growth factors including VEGF,^{1,10,36–38} platelet derived growth factor –BB (PDGF-BB),¹⁰ stromal cell derived factor-1 (SDF-1),¹ and others, given its binding capacity to sequester heparin-binding proteins. However, release of lipids has not been widely studied and, in particular, the use of alginate systems for the delivery of S1P has never before been reported. Previous work has shown short term sustained release of S1P from poly(lactic-co-glycolic acid) (PLGA)-based systems,^{28,31,32} hollow cellulose fibers,⁴¹ and PEG bound with albumin which is known to specifically bind S1P.⁴⁴ Notwithstanding, the controlling mechanism by which S1P was released was not fully elucidated. Structurally, S1P displays a hydrophobic lipid tail and a negatively charged phosphate head group.²⁶ We thus hypothesized that the incorporation of a cationic polymer, such as chitosan, might provide electrostatic interactions between encapsulated S1P and the hydrogel system in order to control and sustain release.

Alginate hydrogels indeed provide sustained release of the hydrophobic lipid S1P to promote pro-angiogenic responses by both human OECs under hypoxia *in vitro* and in an *in vivo* CAM assay. Furthermore, increasing the chitosan content from 0 to 0.5% retarded the S1P release profile. A similar result has been observed in previous work where chitosan incorporation delayed drug release from alginate-based systems,^{16,33,35} but lipid release had not been demonstrated. Interestingly, gels with 1% chitosan content demonstrated faster release similar to that established with 0% chitosan gels (alginate alone). A similar phenomenon has been observed in previous work with alginate-chitosan films, wherein the

relative compositions of alginate and chitosan differentially altered release of silver sulfadiazine, a negatively charged drug of similar molecular weight as S1P.²³ However, further work is required to understand the molecular interactions involved that are controlling S1P retention and release. Regardless, the addition of chitosan in forming composite hydrogels has strong potential for allowing more spatial and temporal control of S1P release.

This work suggests that the slower S1P release regimen was more favorable for inducing angiogenesis and migration of OECs under hypoxia as compared to S1P gradients established from a faster release regimen. It is well established that S1P gradients are crucial to its function, particularly with regards to regulating cellular trafficking.¹⁹ However, the target release profile that is required in order to therapeutically manipulate cellular recruitment or angiogenesis has not been defined as the effect of altering lipid signaling gradients on host responses to implanted biomaterials has not been thoroughly characterized.²⁸ Numerous works have demonstrated that S1P delivery stimulates therapeutic angiogenesis,^{29,31,32,44} but the ability to enhance the angiogenic response by altering the S1P release regimen has yet to be shown. Indeed, previous work with PLGA based polymers has shown that sustained gradients of S1P can be achieved by altering the degradation kinetics of the polymeric systems.²⁸ However, the two distinct S1P gradients described in the previous work resulted in similar angiogenic responses *in vivo* (i.e. the murine dorsal skinfold window model), while the *in vitro* endothelial cell response in the presence of different S1P gradients was not evaluated.²⁸ In contrast, the four discrete S1P gradients generated here while using different alginate-chitosan gel formulations are shown to indeed induce different endothelial cell responses *in vitro*.

While the 3D angiogenic sprouting and directed migration assays tested here provide a beneficial means of determining the optimal delivery system for inducing therapeutic angiogenesis *in vitro*,^{25,38,47} the CAM assay is a step closer to the intricate microenvironment of *in vivo* systems.⁴⁰ Herein, S1P released from all of the tested hydrogels induced blood vessel formation as compared to normal development shown by the blank control as expected based on previous work.⁴⁴ However, 0.5% chitosan hydrogels induced roughly 15% more blood vessel formation as compared to control than alginate hydrogels did after 24h. In support of the *in vitro* data discussed previously, these results suggest that the manner in which S1P is delivered is crucial for inducing an enhanced angiogenic response. Thus, this assay highlights how this alginate-chitosan hydrogel system can be used and manipulated to control release of S1P in a manner that provides gradients suitable for inducing greater blood vessel formation.

In summary, this work confirms that the addition of chitosan polymer chains into alginate hydrogels allows one to control S1P release. Specifically, this study provides proof of concept that an injectable material system may be used in the potential future for delivering S1P in a localized, spatiotemporally controlled manner within ischemic tissue for local recruitment and enhanced angiogenic function of vascular progenitor cells for therapeutic angiogenesis.

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Fig. 1.

Chitosan incorporation alters alginate hydrogel microstructure. A proposed schematic of the hydrogel network interactions (A). Photographs depict that all hydrogel compositions remain injectable through an 18-G needle after at least 30 minutes of gelation. Scale bar represents 1 cm (B). SEM images of 75:25 alginate (first row) and alginate hydrogels further incorporated with 0.1% (second row), 0.5% (third row), and 1% (bottom row) chitosan. Images were acquired at 500X (left column) and 200X (right column); scale bars represent 200 μm (C). Distribution of chitosan (labeled with Orange II dye) incorporated at 0.1%, 0.5%, or 1% within alginate hydrogels (D).

Fig. 2.

The addition of chitosan to alginate hydrogels results in stiffer material properties. Swelling ratios (blue) and storage moduli (red) of the various hydrogel compositions (A). Storage moduli of hydrogels with increasing alginate polymer content (B). The mesh sizes of the hydrogel networks decrease with increasing chitosan content (C). Degradation of the material integrity of the various hydrogels over time in terms of storage moduli. Data represent mean $\pm$ SD (indicated by shaded areas) (D).

Fig. 3.

Characterization of OECs isolated from human umbilical cord blood. Isolated OECs show characteristic cobblestone morphology. Scale bar represents 200 μ m (A). Cells display commonly identified OEC surface markers assessed via flow cytometry. Shaded green histograms represent cells stained with the fluorescently conjugated antibody of interest; dark gray lines represent cells stained with the appropriate isotype control antibodies; light gray lines represent unstained cells (B).

Fig. 4. S1P released from alginate-chitosan hydrogels retains bioactivity and stimulates OEC angiogenic activity. Varying the degree of chitosan incorporation within alginate hydrogels results in altered release of S1P over time. Data represent mean $\pm$ SD (indicated by shaded areas) (A). Polymer release of S1P induces both OEC angiogenic sprouting (B) and directed migration (C) under hypoxia *in vitro*. Data represent mean $\pm$ SD and asterisks indicate statistically significant differences ($P < 0.05$) as compared to the negative (blank) control unless otherwise indicated.

Fig. 5. Sustained delivery of SIP from 0.5% chitosan hydrogels induced maximal blood vessel formation in a CAM assay. Representative images of the various hydrogels placed on the CAMs at 0h and after 24h of incubation. Scale bar represents 5 mm (A). Quantified blood vessel development showed that 0.5% chitosan hydrogels resulted in a statistically significant increase in response. Data represent mean $\pm$ SD and asterisks indicate statistically significant differences ($P < 0.05$) as compared to alginate hydrogels alone (B).