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Injectable and microporous scaffold of densely-packed, growth factor-encapsulating chitosan microgels

Michael S. Riederer[a], Brennan D. Requist[a], Karin A. Payne[b], J. Douglas Way[a], Melissa D. Krebs[a,*]

[a] Department of Chemical & Biological Engineering, Colorado School of Mines, 1613 Illinois Street, Golden, CO 80401, United States
[b] Department of Orthopedics, University of Colorado Anschutz Medical Campus, 12800 E. 19th Avenue, Aurora, CO 80045, United States

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ABSTRACT

In this work, an emulsion crosslinking method was developed to produce chitosan-genipin microgels which acted as an injectable and microporous scaffold. Chitosan was characterized with respect to pH by light scattering and aqueous titration. Microgels were characterized with swelling, light scattering, and rheometry of densely-packed microgel solutions. The results suggest that as chitosan becomes increasingly deprotonated above the pK_a, repulsive forces diminish and intermolecular attractions cause pH-responsive chain aggregation; leading to microgel–microgel aggregation as well. The microgels with the most chitosan and least cross-linker showed the highest yield stress and a storage modulus of 16 kPa when condensed as a microgel paste at pH 7.4. Two oppositely-charged growth factors could be encapsulated into the microgels and endothelial cells were able to proliferate into the 3D microgel scaffold. This work motivates further research on the applications of the chitosan microgel scaffold as an injectable and microporous scaffold in regenerative medicine.

1. Introduction

Hydrogels, which are water-swollen crosslinked polymer networks, have been widely explored as injectable biomaterials due to their ability to act as both cellular scaffolds and drug delivery systems (Bae, Wang, & Kurisawa, 2013; Hoare & Kohane, 2008; Kretlow, Klouda, & Mikos, 2007; Lin & Anseth, 2009; Nguyen & Alsberg, 2014). Many types of injectable hydrogels, including those with stimuli-responsive physical or chemical gelation, have the potential to be delivered in a minimally-invasive manner (Bhattarai, Ramay, Gunn, Matsen, & Zhang, 2005; Jeon, Powell, Solorio, Krebs, & Alsberg, 2011). However, most injectable hydrogels are not microporous and require enzymatic or hydrolytic degradation prior to cellular infiltration (Lin & Anseth, 2009; Zhu & Marchant, 2011). One approach to create an injectable, microporous scaffold is to condense microparticle hydrogels, microgels, into a scaffold so cells can migrate through the void spaces (García Cruz et al., 2008; Griffin, Weaver, Scumpia, Di Carlo, & Segura, 2015). These concentrated microgel solutions exhibit large viscosities, shear-thinning behavior, and yield stresses (Ketz, Prud'homme, & Graessley, 1988); properties that are ideal for localized, surgical injections (Beck et al., 2015; Koetting, Peters, Steichen, & Peppas, 2015; Wang, Leeuwenburgh, Li, & Jansen, 2011).

Chitosan is a pH-responsive polysaccharide, composed of glucosamine (GlcN) and *N*-acetyl-glucosamine (GlcNAc) units, which has been widely used in tissue engineering applications (Muzzarelli, 2009; Rinaudo, 2006). Due to the weakly basic activity of GlcN, chitosan is soluble in dilute acidic solutions but becomes insoluble near pH 7.0 due to intermolecular hydrogen bonding and hydrophobic interactions (Schatz, Pichot, Delair, Viton, & Domard, 2003). Hydrogels and microparticles of chitosan have been widely used in drug delivery and often utilize the ability of chitosan to ionically and physically crosslink (Agnihotri, Mallikarjuna, & Aminabhavi, 2004; Bhattarai, Gunn, & Zhang, 2010; Sinha et al., 2004; Skop, Calderon, Levison, Gandhi, & Cho, 2013). However, these hydrogels are typically nano-porous and limit cellular infiltration (Mekhail & Tabrizian, 2014). Chitosan microparticles can provide a porous scaffold where cells proliferate within the void spaces (García Cruz et al., 2008), but a microporous chitosan microgel scaffold appropriate for localized surgical injections which is stable at physiological pH has not been demonstrated.

For protein release from covalently crosslinked chitosan hydrogels, post-fabrication encapsulation is beneficial to reduce

* Corresponding author.
E-mail addresses: mriedere@mines.edu (M.S. Riederer), brequist@mines.edu (B.D. Requist), Karin.payne@ucdenver.edu (K.A. Payne), dway@mines.edu (J.D. Way), mdkrebs@mines.edu (M.D. Krebs).

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undesirable conjugation (Solorio, Zwolinski, Lund, Farrell, & Stegemann, 2010; Yuan et al., 2007; Zhang, Zhu, Wang, & Ding, 2005). The small size of microgels provides high surface-area-to-volume ratio, allowing rapid loading of drugs post-fabrication (Malmsten, Bysell, & Hansson, 2010). Chitosan has been used to encapsulate a wide range of proteins (Muzzarelli, 2009). However, the post-fabrication encapsulation of proteins into chitosan-genipin microgels has not been commonly applied. Chitosan is often modified to provide increased affinity between polymer and drug, but this can result in microparticles with limited degradation (Skop et al., 2013). While genipin has been used to externally crosslink chitosan microparticles by soaking, this can lead to heterogeneous and dense crosslinking which limits lysosomal degradation (Skop et al., 2013; Yuan et al., 2007). In this work, we use the slow reaction kinetics of genipin to mix polymer and crosslinker prior to emulsification and form uniformly crosslinked microgels.

We hypothesized that a scaffold consisting of chitosan microgels which are densely packed can provide a microporous gel which maintains stability at physiological conditions due to microgel–microgel attractions and thus allows cellular infiltration. To understand factors which influence such a chitosan scaffold at the material and cellular levels, six different formulations of chitosan microgels were fabricated. The swelling of the microgels was studied with respect to pH and ionic strength, and the microgels encapsulated two oppositely-charged (Bergmann, Holz, & Kopitz, 2011) isoforms of vascular endothelial growth factor (VEGF): $VEGF_{121}$ and $VEGF_{165}$. Mechanical properties of the condensed microgels were assessed to develop a gel which would remain stable in physiological saline buffer. The ability for endothelial cells to interact with the VEGF-loaded microgels was examined through a metabolic assay and confocal fluorescent imaging. The work presented here shows that chitosan microgels can be sufficiently condensed into an injectable scaffold and motivates further research on this scaffold for tissue engineering.

2. Experimental

2.1. Materials

Chitosan (CS) ("medium molecular weight", 84.1% deacetylation) was purchased from Sigma-Aldrich (St. Louis, MO) and purified as described below. HEPES, Span 80 (sorbitan monooleate), and Tween 20 (polyethylene glycol sorbitan monolaurate) were purchased from Sigma-Aldrich and used as received. Genipin (Wako Chemicals USA; Richmond, VA), recombinant human $VEGF_{165}$ (R&D Systems; Minneapolis, MN), recombinant human $VEGF_{121}$ (Peprotech; Rocky Hill, NJ), Dulbecco's phosphate buffered saline (DPBS, HyClone; Logan, UT) and CellTracker Green 5-chloromethylfluorescein diacetate (CMFDA) dye (Thermo Fisher Scientific; Waltham, MA) were used as received. Anti-VEGF ELISA development kits (Peprotech), lysozyme (Thermo Fisher Scientific) and PrestoBlue Cell Viability Reagent (Thermo Fisher Scientific) were used as provided. Pooled-donor Human Umbilical Vein Endothelial Cells (HUVECs) isolated in media without exogenous VEGF, endothelial basal media (EBM), and endothelial growth media (EGM) were purchased from Lonza (Walkersville, MD). Ultrapure deionized water (diH_2O) was generated with a Milli-Q filtration system (EMD Millipore; Billerica, MA). PureCol collagen (3 mg/mL) was purchased from Advanced BioMatrix (San Diego, CA). Polytetrafluoroethylene (PTFE) release agent (MS-122AD) was generously provided by Miller-Stephenson (Morton Grove, IL).

2.2. Chitosan irradiation and purification

CS was irradiated (I-CS) to 5 Mrad total dose at 220 krad/h with Cobalt-60 irradiation (Michigan Memorial Phoenix Project, Univ. of Michigan, USA). CS and I-CS were purified separately by dissolving 10 g in 1 L of 1% acetic acid solution, followed by vacuum filtration through a 2.7 μm cellulose paper filter (GE Healthcare, Marlborough, MA) and a 0.45 μm PES membrane filter (Thermo Scientific). The solution was dialyzed (MWCO 3500; Fisher Scientific) for 4 days against diH_2O before the retentate was adjusted to pH 8.0 with 1 M NaOH and the polymer was separated by centrifugation (4000 × *g*, 5 min). The pellet was repeatedly resuspended in diH_2O and centrifuged until the supernatant was less than pH 7.2. The purified CS and I-CS were then lyophilized and stored in a desiccator prior to use.

2.3. Polymer characterization

CS and I-CS were dissolved in 0.5 M acetic acid/0.2 M sodium acetate buffer (pH 4.2) at concentrations between 0.8 and 10 mg/mL and intrinsic viscosity was measured with an Ubbholde viscometer (Schott-Gerate, No. II; Mainz, Germany). Mark-Houwink Parameters ($K = 3.5 \times 10^{-4}$ and $a = 0.76$) were applied to determine molecular weight (Terbojevich, Cosani, & Muzzarelli, 1996). Fourier Transform Infrared (FTIR) spectroscopy was performed on CS and I-CS using a Nicolet NEXUS 470 FTIR (Thermo) equipped with a Specac Attenuating Total Reflectance (ATR) attachment (Golden Gate; Kent, UK). Potentiometric titrations of CS and I-CS were performed by dissolving 50 mg in 50 mL of 10 mM HCl/1 mM NaCl and titrating with 0.101 M NaOH. Light transmittance measurements at 600 nm on CS and I-CS solutions (1 wt%) were performed at pH 6.0, 6.8, 7.4, and 8.0 ($n = 3$) using a UV–Vis spectrophotometer (Genesys 10S, Thermo Scientific).

2.4. Microgel production

Chitosan/genipin (CS/GP) microgels were formed by an *in situ* emulsion crosslinking method (recipes shown in Table 1).

CS was dissolved at 2, 4, or 6 wt% into 10 mL of 0.5% acetic acid solution by magnetic stirring overnight in a closed container. GP solution (100 or 500 mM; 0.5 mL in ethanol) was added dropwise into the stirring chitosan and allowed to mix for 5 min. The CS/GP mixture was emulsified by stirring in 90 mL of 5% Span 80 in mineral oil and homogenizing at 6000 RPM for 5 min (PROScientific). The emulsion was stirred at 650 RPM for 18 h in a 40 °C water bath. Then the microgel emulsion was separated to 25 mL aliquots and centrifuged (2000 × *g*, 5 min) to remove the mineral oil phase. The pellet aliquots were further washed by consecutive centrifugation (2000 × *g*, 5 min) and resuspension of the pellet in 40 mL of each of the following: hexane, 50% ethanol in diH_2O containing 1% Tween 20, and diH_2O (5 times). The microgels were then stored in excess diH2O at 4 °C. Prior to experiments, a 1:1 microgel dilution was prepared by mixing 1 mL diH_2O per gram hydrated microgel pellet (2000 × *g*, 5 min).

2.5. Microgel characterization

To measure CS/GP microgel diameters, 100 μL of each 1:1 microgel dilution was mixed with 1 mL of diH_2O and imaged with a

Table 1
Recipes for CS/GP microgels.

Condition	CS (g)	I-CS (g)	Acetic acid	GP (μmol)	Ethanol (mL)
CS-2/GP-5	0.2	0	10 mL, 0.5%	50	0.5
CS-4/GP-5	0.2	0.2	10 mL, 1.0%	50	0.5
CS-6/GP-5	0.2	0.4	10 mL, 1.5%	50	0.5
CS-2/GP-25	0.2	0	10 mL, 0.5%	250	0.5
CS-4/GP-25	0.2	0.2	10 mL, 1.0%	250	0.5
CS-6/GP-25	0.2	0.4	10 mL, 1.5%	250	0.5