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Versatile click alginate hydrogels crosslinked via tetrazine–norbornene chemistry

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ABSTRACT

Alginate hydrogels are well-characterized, biologically inert materials that are used in many biomedical applications for the delivery of drugs, proteins, and cells. Unfortunately, canonical covalently crosslinked alginate hydrogels are formed using chemical strategies that can be biologically harmful due to their lack of chemoselectivity. In this work we introduce tetrazine and norbornene groups to alginate polymer chains and subsequently form covalently crosslinked click alginate hydrogels capable of encapsulating cells without damaging them. The rapid, bioorthogonal, and specific click reaction is irreversible and allows for easy incorporation of cells with high post-encapsulation viability. The swelling and mechanical properties of the click alginate hydrogel can be tuned via the total polymer concentration and the stoichiometric ratio of the complementary click functional groups. The click alginate hydrogel can be modified after gelation to display cell adhesion peptides for 2D cell culture using thiol-ene chemistry. Furthermore, click alginate hydrogels are minimally inflammatory, maintain structural integrity over several months, and reject cell infiltration when injected subcutaneously in mice. Click alginate hydrogels combine the numerous benefits of alginate hydrogels with powerful bioorthogonal click chemistry for use in tissue engineering applications involving the stable encapsulation or delivery of cells or bioactive molecules.

1. Introduction

Hydrogels are highly hydrated, crosslinked polymer networks that resemble the environment of natural soft tissue, making them attractive materials for a variety of biomedical applications such as tissue engineering, drug delivery, and vaccines [1–7]. Alginate biopolymers are versatile, naturally derived linear polysaccharides comprised of repeating (1,4)-linked β-D-mannuronic and α-L-guluronic acid, and can be crosslinked to form hydrogels via a variety of ionic and covalent crosslinking methods [8,9]. Alginate hydrogels can be engineered to release small molecules and proteins, present bioactive ligands to cells, and degrade at a tunable rate [10–12]. Furthermore, ionically crosslinked alginates have been used extensively for drug delivery, cell encapsulation, and tissue engineering because ionic crosslinking can be largely benign to cells and encapsulated molecules [13].

The encapsulation of various small molecules, proteins, and cells in alginate hydrogels has thus far been largely limited to the reversible ionic crosslinking method which uses divalent cations, such as Ca^{2+}, to form ionic bridges between adjacent polymer chains. These gels have been shown to be weak and to lose mechanical integrity over time *in vitro* and *in vivo* due to the reversible nature of the crosslinking and subsequent outward flux of ions from the hydrogel [14]. Calcium crosslinked alginate gels can yield non-uniform physical properties, due to extremely rapid crosslinking with certain ions [15]. Moreover, leached calcium from calcium crosslinked alginate gels can be immunostimulatory, which is unfavorable in many *in vivo* applications [16]. While alginate is well

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characterized in its ability to quantitatively couple small molecules, peptides, and proteins to the polymer backbone, these reactions (e.g. carbodiimide couplings) are typically limited in efficiency by slow reaction kinetics under aqueous conditions [17].

To overcome many of the challenges associated with ionic crosslinking, alternative covalent crosslinking strategies have been developed, though none are completely biologically inert [18–21]. Many of these covalent crosslinking strategies produce stable and uniform gels with mechanical properties that are controllable over a wider range compared to ionically crosslinked gels, but they may not be optimal for protein or cell encapsulation due to the cross-reactivity of the crosslinking chemistry with cells and proteins. Additionally, as the quantity and length of the crosslinker increases, the properties of the resulting hydrogel are significantly altered, making it difficult to compare such gels to alginate-based ionically crosslinked hydrogels [22].

Click chemistry has recently emerged as an alternative approach to synthesize covalently crosslinked hydrogels with high chemoselectivity and fast reaction rates in complex aqueous media, at physiologically relevant pH and temperature ranges both *in vitro* and *in vivo* [23]. Recent findings have established a set of bioorthogonal click reactions that do not require the cytotoxic copper catalyst used in early reports. These copper-free chemistries include strain-promoted azide-alkyne cycloaddition (SPAAC) and the inverse electron demand Diels–Alder reaction between tetrazine and norbornene [24,25]. Previous reports have used these click reactions primarily to crosslink click end-functionalized branched polyethylene glycol (PEG) with linear crosslinkers composed of either PEG or linear peptides terminated with the appropriate click reaction pair [26–29]. The mechanical properties and swelling behavior of these click crosslinked PEG hydrogels could be tuned by varying the linear crosslinker concentration [30,31].

We hypothesized that a simpler and more robust click crosslinked biomaterial could be designed to exhibit stable and tunable mechanical properties, present bioactive ligands to cells, and encapsulate those cells in a cytocompatible covalent crosslinked alginate hydrogel. In this report, we modified alginate biopolymers with tetrazine or norbornene functional groups, allowing for covalent crosslinking without the need for external input of energy, crosslinkers, or catalysts, using the bioorthogonal inverse electron demand Diels–Alder click reaction. In addition to the crosslinking reaction, the click alginate system exploits photoinitated thiol-ene based modification of the norbornene groups to present thiol-bearing peptides. We investigated cell adhesion on the hydrogel surface and cell growth and viability when encapsulated in 3D in click alginate hydrogels. In addition, we studied the host inflammatory response to click alginate hydrogels that are injected *in vivo*.

2. Materials and methods

2.1. 3-(*p*-benzylamino)-1,2,4,5 tetrazine synthesis

3-(*p*-benzylamino)-1,2,4,5-tetrazine was synthesized according to an established protocol [32]. Briefly, 50 mmol of 4-(aminomethyl)benzonitrile hydrochloride and 150 mmol formamidine acetate were mixed while adding 1 mol of anhydrous hydrazine. The reaction was stirred at 80 °C for 45 min and then cooled to room temperature, followed by addition of 0.5 mol of sodium nitrite in water. 10% HCl was then added dropwise to acidify the reaction to form the desired product. The oxidized acidic crude mixture was then extracted with DCM. After discarding the organic fractions, the aqueous layer was basified with $NaHCO_3$, and immediately extracted again with DCM. The final product was then recovered by rotary evaporation, and purified by HPLC. All chemicals were purchased from Sigma-Aldrich.

2.2. Click alginate polymer synthesis

Click alginate biopolymers were modified with either 1-bicyclo[2.2.1]hept-5-en-2-ylmethanamine (Norbornene Methanamine; Matrix Scientific) or 3-(*p*-benzylamino)-1,2,4,5-tetrazine by first allowing high molecular weight alginate, M_w = 265 kDa (Protanol LF 20/40; FMC Technologies) to dissolve in stirred buffer containing 0.1 M MES, 0.3 M NaCl, pH 6.5 at 0.5% w/v. Next, *N*-hydroxysuccinimide (NHS; Sigma-Aldrich) and 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC; Sigma-Aldrich) were added in 5× molar excess of the carboxylic acid groups of alginate. Either norbornene or tetrazine was then added at 1 mmol per gram of alginate to make Alg-N or Alg-T, respectively. The coupling reaction was stirred at room temperature for 24 h, after which the reaction was quenched with hydroxylamine (Sigma-Aldrich) and dialyzed in 12–14 kDa MWCO dialysis tubing (Spectrum Labs) for 4 days against a decreasing salt gradient from 150 mM to 0 mM NaCl in diH_2O. The purified Alg-N and Alg-T polymers were treated with activated charcoal, sterile filtered (0.22 μm), and freeze-dried. This resulted in purified Alg-N or Alg-T polymers with a 5% degree of substitution of the available carboxylic acid groups of alginate (Fig. S-1).

2.3. Preparation and characterization of click alginate hydrogels

Click alginate hydrogels were prepared by first separately dissolving freeze-dried Alg-N and Alg-T polymers to final desired concentration (2–4% w/v) in Dulbecco's Modified Eagle Medium (DMEM; Gibco). For gelation kinetics measurements, Alg-N and Alg-T polymer solutions were mixed at a desired ratio (i.e., 0.5–4:1 N:T) and directly pipetted onto the bottom plate of a TA Instruments ARG2 rheometer equipped with 8 mm flat upper plate geometry. A Peltier cooler was used to control the temperature for temperature dependent experiments, and mineral oil was applied to the gel periphery to prevent the hydrogel from drying during testing. Hydrogel samples were subjected to 1% strain at 1 Hz, and the storage and loss moduli (G′ and G″) were monitored for 4 h. For Young's modulus measurements click alginate hydrogels were formed under siliconized glass plates (Sigmacote; Sigma-Aldrich) with 2 mm spacers. After 2 h of crosslinking at room temperature, cylindrical disks were punched using an 8 mm biopsy punch, transferred to DMEM, and swollen to equilibrium for 24 h at 37 °C. Swollen hydrogel sample dimensions were measured using calipers for volumetric swelling ratio measurements, and then subjected to unconfined compression testing (1 mm/min) using a 10 N load cell with no preload (Instron Model 3342). The Young's modulus, E, was calculated as the slope of the linear portion (first 10%) of the stress vs. strain curves.

2.4. Post-gelation thiol-ene photoreaction onto click alginate hydrogels

Click alginate hydrogels were made as previously described (2% w/v, N:T = 2) and then a cell adhesive CGGGGRGDSP peptide (Peptide2.0) solution at 0.2 or 2 mM containing 0.5% w/v photoinitiator (Irgacure 2959; Sigma-Aldrich) was pipetted on top and the gel was covered with a glass coverslip. Gels were irradiated at 365 nm for 60 s at 10 mW/cm^2. The gels were washed several times with DMEM to remove excess photoinitiator and unreacted peptide and swollen to equilibrium at 37 °C before seeding with cells.

2.5. EGFP 3T3 cell culture

NIH 3T3 (ATCC) cells were transduced with lentivirus produced from an EGFP-containing lentiviral vector (pLCAG EGFP, Inder Verma lab, Addgene plasmid 14857) [33] and were selected for 7 days in 1 μg/mL puromycin dihydrochloride (EMD Millipore). EGFP-expressing 3T3 fibroblast cells were cultured in DMEM supplemented with 10% (v/v) fetal calf serum, 100 U/mL penicillin, and 100 μg/mL streptomycin (Gibco) at 37 °C, in a 5% CO_2 environment. Cells were passaged approximately twice per week.

2.6. Cell adhesion

For cell adhesion studies, slabs of click alginate hydrogels were modified with cell adhesion peptides as described above. 6 mm disks were punched, placed in DMEM, washed several times, and swollen for 4 h prior to seeding with cells at 5×10^4 cells/mL at a depth of approximately 1 mm above the surface of the gel. Cells were given 24 h to adhere and spread and then visualized via EGFP fluorescence using an epifluorescence microscope. EGFP images were used to quantify total cell area using ImageJ software. After 3 days of culture, cells were fixed and stained using Alexa Fluor 594 phalloidin (Molecular Probes) and Hoescht 33342 (Molecular Probes) to visualize F-actin filaments and nuclei respectively. To visualize cell death, gels were incubated for 20 min with a 4 μM ethidium homodimer-1 (Molecular Probes) solution in Hanks Buffered Saline Solution (HBSS) and imaged using an epifluorescence microscope.

2.7. Cell encapsulation

For cell encapsulation studies, Alg-N polymers were modified to have approximately 20 cell adhesive GGGGRGDSP peptides (Peptide2.0) per alginate chain as previously described [17]. 600 μm thick click alginate hydrogels at 2% w/v, N:T = 1, were then made containing cells at 3×10^6 cells/mL. Ionically crosslinked hydrogels were similarly prepared at 2% w/v using the same cell density and backbone RGD modified Alg-N polymers. A $CaSO_4$ slurry (0.21 g $CaSO_4$/mL ddH_2O) at a final concentration of 2% w/v was used to crosslink the ionically crosslinked hydrogel samples so as to match the mechanical properties of the two substrates as closely as possible. To minimize the time in which cells did not have access to culture media, gels were allowed to crosslink at room temperature for 1 h, after which 6 mm disks