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Title: 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.

Keywords: Alginate, Hydrogel, Click chemistry, Cell adhesion, Cell encapsulation, Tissue engineering

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 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 photoinitiated thiol-ene based modification of the norbornene groups to present thiol-bearing peptides or fluorescent dyes. 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)benzotrile hydrochloride and 150 mmol formamidine acetate were mixed while adding 1 mol of anhydrous hydrazine. The reaction was stirred at 80 °C for 45 minutes 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 NaHCO₃, 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 5x 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 hours, 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 diH₂O. 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 hours. For Young's modulus measurements click alginate hydrogels were formed under siliconized glass plates (Sigmacote; Sigma-Aldrich) with 2 mm spacers. After 2 hours of

crosslinking at room temperature, cylindrical disks were punched using an 8 mm biopsy punch, transferred to DMEM, and swollen to equilibrium for 24 hours 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 seconds at 10 mW/cm². 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 μ g/mL streptomycin (Gibco) at 37 °C, in a 5% CO₂ 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 hours 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 hours 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 minutes with a 4 μ M 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 μ m 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 CaSO₄ slurry (0.21 g CaSO₄/mL ddH₂O) 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 hour, after which 6 mm disks were punched and placed in culture medium where the crosslinking reaction was expected to proceed to completion.

2.8 3D in vitro cell assays

Cells were retrieved from alginate hydrogels by digestion in a 5 U/mL alginate lyase (Sigma-Aldrich) solution in HBSS for 20 minutes. For viability testing, cells were stained with a Muse Count and Viability Kit and tested on a Muse Cell Analyzer (EMD Millipore). To assess total cell metabolic activity, gels were transferred to wells containing 10% AlamarBlue (AbD Serotec) in cell culture medium and incubated for 4 hours. The reduction of AlamarBlue was assessed according to the manufacturer's instructions.

2.9 Mice

All work was done with BALB/cJ mice (female, aged 6-8 weeks; Jackson Laboratories) and was performed in compliance with National Institutes of Health and institutional guidelines.

2.10 In vivo hydrogel inflammatory response

Ultrapure alginate with low endotoxin levels (MVG alginate, ProNova Biomedical AS) was modified as described above with norbornene and tetrazine and subsequently prepared at 2% w/v in DMEM after purification. Click alginate hydrogels were prepared by mixing ultrapure Alg-N and Alg-T polymers with N:T = 1 by connecting two syringes with a luer lock. 15 minutes after mixing, 50 μ L of click alginate hydrogel was injected subcutaneously through an 18G needle. For ionic hydrogel samples, a 2% w/v ultrapure alginate solution was prepared in DMEM and similarly mixed in a syringe with a CaSO₄ slurry at a final concentration of 2%. 50 μ L of the ionically crosslinked gel was also injected subcutaneously in the same mice. Both gel samples were retrieved along with the surrounding skin after 1 week, 1 month, and 2 months of injection and fixed overnight in 10% neutral buffered formalin solution (Sigma-Aldrich). Samples were embedded in paraffin, sectioned, and stained with hematoxylin and eosin (H&E) by the Harvard Rodent Histopathology Core.

3. Results:

3.1 Synthesis, characterization, and crosslinking of click alginate polymers

To prepare click alginate polymers, norbornene or tetrazine groups were introduced to high molecular weight alginate biopolymers using conventional carbodiimide chemistry (Fig. 1-A). The degree of substitution of norbornene or tetrazine groups onto purified click alginate polymers was determined from ^1H NMR spectra (Fig. S-1). A 5% degree of substitution of norbornene (Alg-N) or tetrazine (Alg-T) on alginate carboxyl groups was obtained using this method, and these batches of click alginate polymers were used for all subsequent experiments.

To form click alginate hydrogels, Alg-N and Alg-T polymer solutions were prepared separately and mixed together to gel. Upon mixing of the two click alginate polymers, a stable gel was formed via an inverse electron demand Diels-Alder reaction between the two polymers, which releases nitrogen gas (Fig. 1-B). The nitrogen gas evolved from the crosslinking reaction does lead to the formation of a few small bubbles within the hydrogel. A stable gel was formed within 1 hour at 25 °C (Fig. 2-A), though the gelation kinetics could be tuned by varying the temperature or initial degree of substitution of the click alginate polymers (data not shown). The gelation kinetics at 25 °C are favorable because it allows the user to easily achieve a well-mixed polymer formulation before gelation, a common challenge with other alginate hydrogel crosslinking methods.

3.2 Compressive Young's modulus and swelling behavior

The mechanical properties of the extracellular matrix have been shown to affect cell fate and function in 2D and 3D environments [34-37]. In order to tune mechanical properties over a wide range, click alginate polymers were mixed at different ratios of Alg-N and Alg-T (N:T ratio) for a given polymer concentration between 2 and 4% w/v. These click alginate hydrogel samples were subjected to unconfined compression tests resulting in a compressive Young's modulus that predictably increased with increasing polymer concentration, and decreased as the ratio between the polymers deviated from the stoichiometrically balanced N:T ratio of 1 (Fig. 2-B, Table S-1, Table S-2). The ability to tune the mechanical properties of the resulting gel over a large range by simply changing the ratio of the two polymers allows control over gel stiffness while keeping other parameters such as polymer concentration, and ligand density constant which may be useful for studies of mechanobiology.

The swelling ratio of hydrogel systems can affect mechanical properties, mass transport, and the presentation of ligands on the gel surface. To investigate how volumetric swelling would change at different polymer concentrations and N:T ratios, click alginate hydrogels were made as previously described and allowed to swell for 24 hours at 37 °C. The swollen volume was measured and compared to the casted volume (Fig. 2-C). For a given polymer concentration, the volumetric swelling ratio increased as the N:T ratio deviated from 1, demonstrating an inverse relationship between mechanical properties and swelling ratio as expected. While the N:T ratio has a significant effect on the

swelling ratio, the polymer concentration does not have a significant effect, indicating that the swelling ratio of click alginate is dominated by crosslink density rather than polymer concentration (Table S-3).

3.3 Post-gelation modification of click alginate hydrogels

To explore if additional functionalities can be introduced to click alginate hydrogels after polymerization, we grafted thiol-containing molecules onto unreacted norbornenes in pre-formed click alginate hydrogels using a photoinitiated thiol-ene reaction (Fig. 3-A). Gels with N:T = 2 were used to ensure unreacted norbornenes were available to react after the initial gelation. RGD peptide solutions at high (2 mM) or low (0.2 mM) concentration were reacted onto the surface of these click alginate hydrogels and then gels were seeded with NIH 3T3 fibroblasts expressing a cytosolic fluorescent marker (EGFP). 3T3 cells readily adhered and spread on gels modified with RGD, while very few cells were able to attach or elongate on control gels with no RGD (Fig. 3-B). Cells on click alginate hydrogels presenting RGD were able to form branched interconnected networks, with a significant RGD density-dependent 2-3 fold increase in surface coverage over the 3 day culture, while unmodified click alginate gels were observed to be non-cell-adhesive and showed a decrease in surface coverage by cells over time (Fig. 3-C). After 3 days in culture, cells also showed an increase in spreading and actin stress fiber formation with higher RGD concentration (Fig. 3-

D). Additionally, the high viability of cells after 3 days of culture demonstrated the cytocompatibility of the click alginate hydrogels for 2D cell culture (Fig. 3-E).

3.4 Cell encapsulation in click alginate hydrogels

In order to demonstrate the utility of click alginate hydrogels for cell encapsulation, cell viability and metabolic activity of cells encapsulated in click alginate hydrogels were investigated over a 3 day culture period; ionically crosslinked hydrogels were used for comparison in these studies. Representative images of encapsulated cells stained with ethidium homodimer-1 show minimal cell death in both click and ionically crosslinked gels 4 hours and 3 days after encapsulation (Fig. 4-A). Quantification revealed that click alginate hydrogels resulted in significantly higher viability of encapsulated 3T3 cells both immediately after encapsulation ($93 \pm 1\%$ vs. $87 \pm 2\%$) and after 3 days of culture ($84 \pm 2\%$ vs. $79 \pm 4\%$) (Fig. 4-B). It should be noted that a loss in measured cell viability may occur during the cell retrieval process by enzymatic digestion of the hydrogels. The overall metabolic activity of the cells encapsulated in the different hydrogels was also analyzed, and noted to increase over the 3 day culture period for both hydrogel crosslinking chemistries (Fig. 4-C).

3.5 In vivo injection

The inflammatory response to the injection of click alginate hydrogels *in vivo* was investigated next. Click crosslinked and ionically crosslinked alginate

hydrogels were injected subcutaneously and retrieved after 1 week, 1 month, and 2 months. The gelation kinetics of click alginate hydrogels allows them to be mixed and readily injected, in a similar manner to ionically crosslinked hydrogels. A thin fibrous capsule was found to surround both types of gels 1 week after injection. H&E staining revealed a very thin capsule of collagen and fibroblasts surrounding the material throughout the duration of the study with minimal inflammation (Fig. 5). At 1 month, the ionically crosslinked gels were seen to lose structural integrity and allowed for infiltration of fibroblasts and immune cells into the gel, while the click crosslinked samples showed no evidence of breakdown nor cell infiltration into the material for up to 2 months (see Fig. S-2), and maintained a thin layer of fibroblasts surrounding the gel.

4. Discussion:

Our results show that alginate polymers can be modified with norbornene and tetrazine to create alginate hydrogels with a wide-range of mechanical properties without the input of external energy, crosslinkers, or catalysts. While recent work has used similar click chemistry for localized drug delivery, this work presents the first use of the tetrazine-norbornene click reaction to covalently crosslink polysaccharides into hydrogels [29,38]. Crosslinking of alginate by different methods has been extensively explored to make covalently crosslinked hydrogels that are mechanically robust, but these chemistries lack the cytocompatibility inherent in the bioorthogonal click reaction reported here [19,21,39]. The

simplicity of this crosslinking modality provides the opportunity to control the mechanical properties of the click alginate hydrogel by adjusting the ratio of the polymers, rather than changing the total concentration of polymers in the system. This could potentially allow for the decoupling of material variables such as gel architecture, stiffness, and ligand density in further applications of click alginate hydrogels.

Click crosslinked alginate hydrogels were used to form a cytocompatible 2D cell culture substrate that can be modified to display cell adhesion peptides at varying concentrations. Alginate hydrogels must display cell adhesive ligands in order for mammalian cells to attach, spread, and proliferate on the surface of the hydrogel. Without ligands such as RGD presented from the hydrogel surface, few cells will attach, and those that do will retain a spherical morphology and undergo apoptosis [21]. Unfortunately, the carbodiimide chemical reaction most commonly used to attach RGD peptides to the backbone of alginate is slow and requires lengthy purification and lyophilization time [40]. In this work, photoinitiated thiol-ene chemistry between norbornene and cysteine-bearing RGD peptides was employed to rapidly modify click alginate hydrogels to present adhesion ligands on the surface of the gel. This thiol-ene reaction is a powerful light-mediated click reaction that is simple, reproducible, fast, and highly efficient – achieving conversions nearing completion in aqueous media [41]. Although we did not investigate the thiol-ene reaction conversion as a function of hydrogel depth specifically, several recent papers have reported the ability to functionalize the

interiors of hydrogels using this method [28,30,42,43]. When click alginate hydrogels were modified with RGD peptides using this strategy, fibroblasts seeded on the gels responded with increased attachment and spreading as RGD density was raised, over a 3 day culture period. In addition to the simple and rapid coupling reaction, the thiol-ene based strategy for modifying alginate hydrogels also presents a straightforward method to change the ligand density on hydrogels of otherwise equal composition. Altogether, these data demonstrate the flexibility of click alginate hydrogels for culturing cells in 2D and allowing independent control over the presentation of bioactive ligands on the gel surface.

Furthermore, click crosslinked alginates can be used *in vitro* to encapsulate cells in 3D with high viability, providing a covalent alternative to conventional ionically crosslinked alginate hydrogels. A variety of cell types have been encapsulated in ionically crosslinked RGD modified alginates with high viability *in vitro* [11,35,44-46]. However, encapsulation of cells in covalently crosslinked RGD modified alginates is limited by the potential incompatibility of the available crosslinking chemistries [47,48]. The data shown here establishes the ability to encapsulate fibroblasts in covalently crosslinked RGD modified click alginate hydrogels while maintaining cell viability at a high level. The aforementioned ability to independently tune the microenvironment mechanical properties and adhesion ligand density can be exploited with the click crosslinked 3D cell culture system in the future to probe cell responses to a variety of stimuli *in vitro*.

In vivo testing showed that click alginate hydrogels can crosslink *in situ*, provoke minimal inflammatory response, and resist fragmentation and cell infiltration when injected subcutaneously. Histology revealed minimal acute inflammation in the tissue surrounding the injected gel in both click crosslinked and ionically crosslinked alginate. As is typical with many biomaterials, a small fibrotic capsule was formed around the hydrogel periphery in both cases [49]. When compared to ionically crosslinked alginate, click alginate hydrogels demonstrate superior long-term structural integrity. Ionically crosslinked samples fragmented significantly after 1 month *in vivo*, resulting in cell infiltration, whereas the click alginate hydrogels remained intact during the 2 month study and were highly resistant to cell infiltration. In tissue engineering applications where cell trafficking within the hydrogel is desirable, click alginate hydrogels could be processed using existing techniques to introduce microscale porosity to the hydrogels [50,51]. Alternatively, click alginate polymers could be crosslinked using tetrazine or norbornene-modified matrix metalloproteinase-degradable peptide sequences to allow cell-mediated degradation [29,52]. The use of partially oxidized alginate polymers would also allow degradation of the hydrogel over controlled time scales for *in vivo* tissue engineering applications [20,53]. The tissue compatibility and stability of click alginate hydrogels could make it particularly useful for applications where isolation from host immune cell infiltration is required [54,55].

5. Conclusions:

Click alginate polymers are synthetically accessible and can be crosslinked in biological media at physiological pH to create tunable hydrogels with a wide range of mechanical properties. The rapid, bioorthogonal, and cytocompatible click crosslinking reaction makes click alginate hydrogels favorable for cell engineering applications. Click alginate hydrogels can be quickly modified to be cell adhesive and used for 2D or 3D cell culture. Additionally, click alginates have a minimal inflammatory response and high stability *in vivo*, making them attractive materials to use for long-term cell encapsulation and biomaterials-based tissue engineering applications.

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Appendix

Supplementary data

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Figure Legends:

Fig. 1. Fabrication of click alginate hydrogels. Schematic of click alginate polymer synthesis. Aqueous carbodiimide chemistry is used to modify alginate backbone carboxylic acids with tetrazine or norbornene, resulting in Alg-T or Alg-N polymers respectively (A). Alg-T and Alg-N polymers are mixed together to create a covalently crosslinked click alginate hydrogel network, with the loss of N₂ (B).

Fig. 2. Click alginate hydrogel mechanical properties. Representative *in situ* dynamic rheometry plot at 25 °C for 3% w/v click alginate at N:T = 1, demonstrating modulus evolution with time (A). Compressive Young's modulus (B) and volumetric swelling ratios (C) for 2%, 3% and 4% w/v click alginate hydrogels at varying N:T ratio. Values represent mean and standard deviation ($n = 4$).

Fig. 3. Cell adhesion, spreading, and proliferation on click alginate hydrogels modified with RGD peptides after synthesis. Schematic of CGGGGRGDSP peptide coupling reaction onto click alginate hydrogel surface using photoinitiated thiol-ene chemistry (A). Representative images of 3T3 fibroblast adhesion, spreading, and proliferation on click alginate hydrogels with varying RGD peptide density (scale bar = 200 μm) (B), and quantification (Two-Way ANOVA with Turkey's post-hoc test, * $p < 0.05$, **** $p < 0.0001$ relative to No RGD control;

Values represent mean and standard deviation, $n = 4-7$) by endogenous EGFP expression (green) over 3 days (C). Phalloidin (red) and Hoescht 33342 (blue) staining of F-actin filaments and nuclei at 3 days for cells adherent to RGD modified click alginate hydrogels (scale bar = 100 μm) (D). Representative fluorescent images of EGFP (green) 3T3 cells cultured on click alginate hydrogels with varying ligand density for 3 days and stained with ethidium homodimer-1 (red) (scale bar = 100 μm) (E). The High, Low, and No RGD conditions refer to the 2 mM, 0.2 mM, and 0 mM peptide solutions used to modify the click alginate hydrogel surface.

Fig. 4. Cell encapsulation in click crosslinked and ionically crosslinked alginate hydrogels. 3T3 fibroblasts were encapsulated in 2% w/v click crosslinked (N:T = 1) and ionically crosslinked alginate hydrogels and stained with ethidium homodimer-1 (red) for dead cells at 4 hours and 3 days post encapsulation (scale bar = 100 μm) (A). Quantitative analysis of cell viability (Two-Way ANOVA with Sidak's post-hoc test, ** $p < 0.01$, *** $p < 0.001$; Values represent mean and standard deviation, $n = 4$) and overall metabolic activity as measured by reduction of AlamarBlue over time in culture ($n = 6$) (B).

Fig. 5. Tissue response following subcutaneous injection of click and ionically crosslinked hydrogels *in vivo*. Representative hematoxylin and eosin (H&E) stain of tissue sections at 1 week, 1 month, and 2 month following injection into

BALB/cJ mice (scale bar = 150 μm). Images focus on the gel-tissue interface, with dashed lines indicating the border between the hydrogel and the surrounding tissue. Asterisks indicate the location of the click alginate hydrogel, which separates from the tissue during histological analysis with no cell infiltration.

Supplementary Information

Supplementary Methods:

¹H NMR

Alg-N, Alg-T, and unmodified alginate polymers were dissolved in deuterium oxide (Sigma-Aldrich) at 1.5% w/v. ¹H NMR spectra were obtained on a 400 MHz NMR spectrometer (Varian). The degree of substitution was calculated by comparing the integral of the alginate backbone proton peaks at δ 5.0 and δ 4.5 with either the alkene proton peaks of norbornene at δ 6.2-5.9 (m, 2H) or the aromatic proton peak of tetrazine at δ 10.4 (s, 1H).

Supplementary Figure Legends:

Fig. S-1. ¹H NMR spectra of unmodified alginate and click alginate polymers. Blue box highlights the appearance of alkene protons in Alg-N spectra and red box highlights aromatic protons in Alg-T spectra after coupling reaction of norbornene and tetrazine onto alginate.

Fig. S-2. H&E of click and ionically crosslinked alginate hydrogel. Images focus on interior of the hydrogel at 2 months following subcutaneous injection *in vivo* (scale bar = 200 μm).

Table S-1. Young's modulus statistical differences between polymer concentration at each N:T ratio. Values calculated using Two-Way ANOVA with Turkey's post-hoc test, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, x = not significantly different.

Table S-2. Young's modulus statistical differences between N:T ratio at each polymer concentration. Values calculated using Two-Way ANOVA with Turkey's post-hoc test, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, x = not significantly different.

Table S-3. Swelling ratio statistical differences between N:T ratio at each polymer concentration. Values calculated using Two-Way ANOVA with Turkey's post-hoc test, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, x = not significantly different.