Alginate modification via click chemistry for biomedical applications

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Highlights

- The need for the modification of alginate properties was established
- Click chemistry reactions were discussed
- Functionality of using click chemistry for alginate-based materials was explored

Contents

Abstract

Alginate biopolymers are characterized by favorable properties, of biocompatibility, degradability, and non-toxicity. However, the poor stability properties of alginate have limited its suitability for diverse applications. Recently, click chemistry has generated significant research interest due to its high reaction efficiency, high selectivity for a single product, harmless byproducts, and processing simplicity. Alginate modified using click chemistry enables the production of alginate derivatives with enhanced physical and chemical properties. Herein, we review the employment of click chemistry in the development of alginate-based materials or systems. Various click chemistries were highlighted, including azide and alkyne cycloaddition (e.g. Copper-(I)-catalyzed azide-alkyne cycloaddition (CuAAC), Strain-promoted alkyne-azide cycloaddition (SPAAC)), Diels-Alder reaction (Inverse electron demand Diels-Alder (IEDDA) cycloaddition, Tetrazine-norbornene Diels-Alder reactions), Thiol-ene/yne addition (Free-radical thiol-ene addition click reactions, Thiol-Michael addition click reactions, Thiol-yne addition click reaction), Oxime based click reactions, and other click reactions. Alginate functionalized with click chemistry and its properties were also discussed. The present study shows that click chemistry may be employed in modifying the mechanical strength, biochemical/biological properties of alginate-based materials. Finally, the applications of alginate-based materials in wound dressing, drug delivery, protein delivery, tissue regeneration, and 3D bioprinting were described and the future perspectives of alginates modified with click chemistry, are subsequently presented. This review provides new insights for readers to design structures and expand applications of alginate using click chemistry

reactions in a detailed and more rational manner.

Keywords: click chemistry; alginate; biomedical applications; biomaterial engineering

1 Introduction

Alginate is a natural water-soluble polysaccharide. It can be extracted from some brown algae-cell walls and bacteria, such as *Ascophyllum nodosum*, and, *Pseudomonas* spp respectively (Lee & Mooney, 2012; Smidsrød & Skja˚k-Br˝k, 1990). Alginate contains β-D-mannuronic acid (M) and 1-4 linked α-L-guluronic residues (G). Typically, the blocks repeat and display homogenous chains of MMM and GGG, interdispersed with heterogeneous chains of MGM (Lee & Mooney, 2012; J. Sun & Tan, 2013).

Due to the excellent qualities of alginate, in terms of biocompatibility, biodegradability, and non-antigenicity (Paques, van der Linden, van Rijn, & Sagis, 2014), it has been extensively used in biomedical and pharmaceutical applications, including tissue engineering(Chawla, Kaur, Joshi, & Singh, 2020; J. Liu et al., 2020), drug delivery(Joshy et al., 2018; Yin, Wang, & Wang, 2018) and wound dressings(Zhang & Zhao, 2020; Zhao et al., 2020). Alginate can be transformed into several forms, such as hydrogels, microspheres, microcapsules, foams, sponges, and fibers, thus enhancing its applicability in various fields(Venkatesan, Bhatnagar, Manivasagan, Kang, & Kim, 2015). Although alginate is used in different applications, it still has some disadvantages, such as poor stability in aqueous conditions and uncontrollable degradation. Pure alginate also exhibits weak mechanical properties which leads to the rupturing of alginate hydrogels when stretched to ~ 1.2 times of its original length, thus

restricting the application of alginate hydrogels (J.-Y. Sun et al., 2012).

To obtain alginates with desirable properties, various strategies have been developed to synthesize functional alginates, including physical, chemical, and biological methods. The free functional moieties distributing along the backbone, hydroxyl and carboxyl, provide active sites. That is ideal for chemical functionalization and thus makes alginate a versatile material for numerous applications. Alginate is, therefore, able to readily form alginate derivatives, which are characterized by enhanced characteristics such as improved biodegradability (Gong et al., 2021), mechanical strength (H. Yan et al., 2016) and gelation property (Heo, Akimoto, Kobatake, & Ito, 2019). These alginate derivatives are also characterized by tunable cell affinity. A consideration of the methods employed in facilitating the improvement of alginate properties suggests that click chemistry constitutes a highly efficient procedure. At the time of preparing this review, there are a few papers covering the click reactions in polysaccharides (Liebert, Hänsch, & Heinze, 2006; Meng & Edgar, 2016). However, literature regarding alginate-based modification or functionalization, using click chemistry is sparse. It is, therefore, crucial to summarize these studies. This article aims to summarize the recent progress of alginate-based modification using click chemistry and highlight the recent applications of click chemistry. Herein, the prospects of alginate applications based on click chemistry are also discussed.

2 Alginate functionalized with click chemistry and its properties

Click chemistry, first coined by Sharpless, is a synthetic concept that describes a group of reactions that "…must be modular, wide in scope, give very high yields, generate only harmless byproducts that can be removed by nonchromatographic methods, and be stereospecific (but

not necessarily enantioselective)" (Kolb, Finn, & Sharpless, 2001; Kolb & Sharpless, 2003). These reactions require mild reaction conditions, are insensitive to oxygen and water, easy to perform, and require simple product isolation methods (Kolb et al., 2001; Kolb & Sharpless, 2003). Click chemistry includes the combination of activated molecules via a two-step coupling involving click functional groups, leading to the formation of a stable conjugate(Bilal, Rasheed, Zhao, Iqbal, & Cui, 2018). Furthermore, the high thermodynamic driving force (i.e. > 20 kcal/mol) that characterizes click chemistry reactions, leads to a high selectivity for the formation of a single product. (Kolb et al., 2001). These features make click chemistry reactions suitable for various applications. Click chemistry therefore opens an interesting prospect to design alginate for the preparation of functionalized materials. Recognizing therefore the importance of click chemistry, the major types of click chemistry reactions previously explored in the literature, will be introduced in the following subsections. The effects of alginate modified with different click reaction on properties, such as mechanical properties, drug delivery and antibacterial, are also discussed.

2.1 Copper-(I)-catalyzed azide-alkyne cycloaddition (CuAAC)

The click reaction involving azide and alkyne functional groups typically leads to a high yield of ~ 95% while under a mild temperature condition which ranges from 25-70 °C(Wolfgang & Christian, 2006). This click reaction can also tolerate functional groups which are firmly and covalently bonded to the backbone or substrate since the aromatic 1,2,3-triazole ring has high stability (Huang & Chang, 2009).

Cu(I)-catalysed [3+2] azido-alkyne cycloaddition (CuAAC) was developed by Sharpless et

al. and Meldal et al (Rostovtsev, Green, Fokin, & Sharpless, 2002; Tornøe, Christensen, & Meldal, 2002), and is based on Huisgen's 2,3-cycloaddition chemistry (Huisgen, 1963). This reaction forms a triazole from an azide and terminal alkyne and is activated using a Cu catalyst (Table 1) (Baskin & Bertozzi, 2009). CuAAC usually occurs in the richly functionalized biological environment at physiological temperatures (Agard, Prescher, & Bertozzi, 2004). It allows for high-sensitivity detection of azides and is often referred to as the most widely used click reaction. During the reaction of CuAAC, Cu(I) with catalytic characteristics is difficult to remove from the products. Cu(II) could form an excellent polymeric backbone with alginate (Bahsis et al., 2020) . Super porous hydrogels were prepared through coordination of copper (II) to a naturally occurring alginate biopolymer via CuAAC. There is an effective electrostatic interaction between copper (II) ions and alginate chains (Rui Rodrigues & Lagoa, 2006) , meanwhile, the guluronic units could capture divalent cations (Akamatsu, Maruyama, Chen, Nakao, & Nakao, 2011) . Therefore, the cross-linking structure was formed, and copper (II) ions act as cross-linking agents.

The versatility of the CuAAC reaction with highly reactive functional groups endows alginate-based materials with desirable properties. To enhance the long-term stability and mechanical strength of alginate hydrogels, the CuAAC reaction was used to control and improve structural stability through covalent crosslinking. Alginate functionalized with pendant alkyne groups or azide groups was synthesized to prepare gel capsules via click chemistry (Breger et al., 2015). These click capsules were permanent "click" crosslinks. Compared to traditional Ca^{+2} crosslinked alginate capsules, the gel capsules produced from click chemistry showed

improved stability in ionic media, consistent molecular weight cut-off (MWCO), increased permeability to diffusants, and water swelling characteristics (Figure 1). Alginate hydrogels employed in therapeutic drug encapsulation could also have enhanced stabilities when CuAAC chemistry method is used. The cross-linked alginate matrix is employed in encapsulating ionic or non-ionic drugs and presents its advantages. The research by Kumar et al showed that the alginate-*graft*-POEGMA materials, preparing with functionalized alginate and poly(oligo ethylene glycol methacrylate) (POEGMA) using alkyne and terminal azide groups respectively with the method of CuAAC, demonstrated an improved encapsulation efficiencies (up to 50%) and a enhanced anti-tumor performance, for the doxorubicin-loaded particles, specifically, such that tumors were almost eliminated (Kumar et al., 2019). In addition, the hydrogel-containing nanoparticles can be designed as drug carriers for the treatment of serious diseases, such as neurodegenerative disorders and cancer (Kishimoto et al., 2012). But the small size and large surface area of nanoparticles can lead to agglomeration, which results in limited drug loading and 'burst release' (Kurdtabar & Rezanejade Bardajee, 2019). Click reaction could be utilized to decorate molecules to solve the agglomeration. Crescenzo et al decorate alginate chains with Azido-homoalanine Kcoil (Aha-Kcoil) by azide-alkyne click chemistry, that form the hybrid hydrogel system, promoting the uniform dispersion and release of gold nanoparticles (Roth et al., 2019).

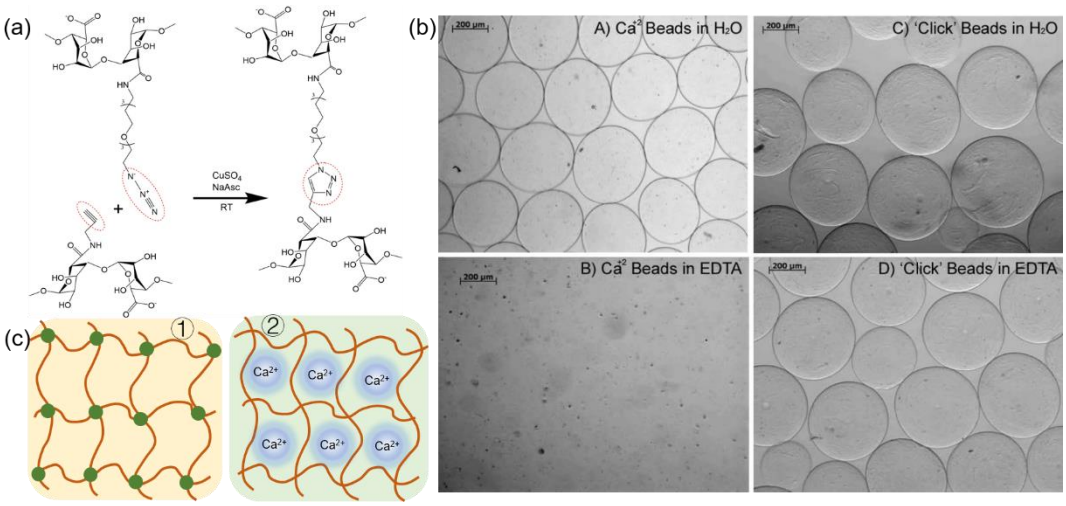


Figure 1. "click" alginate hydrogel capsules. (a) Schematics of click reaction between azide and alkyne functionalized alginate to fabricate "click" alginate hydrogels; (b) Optical microscopy images of Ca^{2+} and "click" crosslinked alginate capsules in d.i. H_2O (A, C) and after exposure to EDTA (B, D). "Click" capsules maintained the integrity for at least 1 month and up to 6 months when stored in water on the lab bench at room temperature. (c) Schematic of crosslinked alginate hydrogel. ① "click" crosslink; ② Ca^{2+} crosslink.(Breger et al., 2015)

For the polymer with specifical structures, 1,3-dipolar cycloadditions are the effective synthetic route to modify alginate chains to form block copolymers or biological hybrids with desired performance. Cyclic *c*RGD-pentapeptides were conjugated to biomacromolecule alginate on the hydroxyl group with Rutjes' method (Krause, Kirschning, & Dräger, 2012; Paleček, Dräger, Kirschning, 2011). The synthesized "smart" bioactive polymer may be employed in hydrogel development, which is suitable for therapies and tissue engineering applications. This method also could be utilized for chitosan biomaterials. In a previous work, the long-chain quaternary ammonium was grafted at 6-OH of chitosan to form a derivative via the protection-click reaction deprotection process. The hydrogels prepared with derivatives and sodium alginate exhibited controlled Tea tree oil releasing properties, the equilibrium swelling ratio was mainly affected by its sodium alginate content (Y. Chen et al., 2017). Additionally,

when bis-propargyl-succinate and bis-propargyl hexane urethane reagents are utilised, a hybrid hydrogel can be formulated using alkynated alginate or hyaluronic acid. The gelation behavior and swelling properties were characterized as a function of their composition and solution pH. When the gels were examined in PBS at 37°C, there was no significant weight loss during the initial 5 days. Subsequently, different weight loss occurs and leads to the change of hydrogel stability, due to the chemical structure involving in click reaction. Long alkylene groups with hydrophobic properties retard the hydrolytic degradation rate, while ester groups are more prone to hydrolytic decomposition than urethane groups. Therefore, the preliminary hydrolytic degradation of the hybrid hydrogel was also faster than that of urethane-containing gel (Bui, Jeon, Um, Chung, & Kim, 2015).

To track the status of alginate and confirm the clinical effect both in vitro and in vivo, the use of the fluorescence-labeling option may be valuable. Crucially, the lack of intrinsic fluorescent groups indicates that a chemical labelling procedure is necessary. For example, studies have shown that in the production of coumarin-grafted blue-emitting fluorescent alginate via carbodiimide coupling then alkyne-azide 'click' chemistry, the modified alginate retains the capability to create hydrogels that are mechanically stable and maintain fluorescence for long time periods (Araújo et al., 2020). CuAAC chemistry may be utilized to build synthetic molecular architectures with excellent properties.

Unfortunately, the copper catalyst is toxic to both bacterial (Link & Tirrell, 2003) and mammalian cells, and it is retained in the products, thus precluding the broad exploration (Tan, Rubin, & Marra, 2011). In order to overcome these problems, a series of noncopper-catalyzed

click reactions have been developed recently and used for highly efficient “click” conjugation, such as strain-promoted azide-alkyne cycloaddition (SPAAC), the inverse electron demand Diels-Alder reaction between tetrazine and norbornene, Michael addition, and Oxime (Devaraj, Weissleder, & Hilderbrand, 2008; Jewett & Bertozzi, 2010).

2.2 Strain-promoted alkyne-azide cycloaddition

The Bertozzi research group (Agard et al., 2004; Codelli, Baskin, Agard, & Bertozzi, 2008; Sletten & Bertozzi, 2008) initially detected the strain-promoted alkyne-azide cycloaddition (SPAAC) click reaction. It was observed that the alkyne-azide cycloaddition could be substantially promoted through bringing ring strain into the alkyne moiety rather than a metal catalyst (H. Jiang et al., 2015; Zheng et al., 2012). The click reactions of cyclooctyne derivatives are typically rapid with a constant reaction rate, k_2, of up to 2.3 $M^{-1}s^{-1}$ (Agard et al., 2004; Blackman, Royzen, & Fox, 2008). Crucially, this reaction type can occur under physiological conditions, without Cu(I) catalysts, thus preventing the risk of unwanted toxic effects (Baskin & Bertozzi, 2009; Saxon & Bertozzi, 2000). Due to its high reactivity, biorthogonality and little off-target reactivity, the SPAAC click reaction has attracted rapidly increasing interest and can be used to repeatedly refill drug-releasing depots at a tumor site, repeating the release of a drug at a site of tumor resection, leading to improvements in the efficacy and tolerability in tumor models (Agard et al., 2004; Brudno et al., 2018; Roy, Mondal, Hatai, & Bandyopadhyay, 2014). For instance, in the study by Brudno et al (Moody, Palvai, & Brudno, 2020), refillable hydrogel depots were created from highly modified alginate strands by using multi-arm cyclooctyne cross-linkers. Tetrabicyclononyne (tBCN) agents covalently

cross-link azide-modified alginate hydrogels. These alginate gels, produced via click-linking were altered using azide groups via an extended level of substitution, and caused negligible inflammatory responses in the host (Moody et al., 2020).

2.3 Diels-Alder reaction

The [4+2]-cycloaddition reaction between an electron-rich diene and electron-deficient dienophile was discovered by Otto Diels and Kurt Alder and bears their names “Diels-Alder” reaction (Sanyal, 2010). This discovery was the basis of their Nobel Prize award in 1950. The [4+2] cycloaddition, as the typical Diels-Alder reaction, contains a conjugated diene which is electron-rich, and electron-poor dienophile (such as alkene, maleic acid) to form a cyclohexene system (Meng & Edgar, 2016), that is well-known in facilitating hydrogel cross-linking (Fan et al., 2015). This reaction has several excellent features, such as mild reaction conditions, high efficiency, thermal reversibility, and excludes the involvement of any chemical initiator (Kirchhof, Brandl, Hammer, & Goepferich, 2013; Koehler, Alge, Anseth, & Bowman, 2013). In addition, water can be used as a solvent to enhance the reaction rate (Moulay & Touati, 2010).

Furan compounds are important heterocyclic compounds that facilitate easier cyclic reactions due to their low aromaticity (Oliver Kappe, Shaun Murphree, & Padwa, 1997). Furan/maleimide Diels-Alder adduct presents a relatively low temperature of decoupling through its retro-Diels-Alder reaction (wherein, retro-Diels-Alder is the reverse process of the Diels-Alder rection, specifically the dissociation the Diels-Alder adducts formed with diene

and the dienophile(Kwart & King, 1968)). It may be used for various interesting applications, such as recyclable and self-healing materials.

Calcium-binding derivatives of alginate were synthesized by partial substitution of its carboxyl functionalities with furan. Based on Ca^{2+} physical networks, a low density of covalent crosslinks with maleimide end groups and a four-arm poly(ethylene glycol) crosslinker were incorporated into a highly transient physical network to synthesize hydrogel (Ghanian, Mirzadeh, & Baharvand, 2018). The long chains of furan-alginate consisting of G-rich domains formed calcium-cross-linked stiff zones, which were surrounded by PEG-mediated covalent cross-links. The stiff zones dissipated energy through reversible dissociation. Permanent PEG cross-links, as elastically active zones, stored energy for rapid self-recovery upon unloading and prevented massive plastic deformation of chains (Figure 2). These hydrogels have interesting features, such as immediate self-recovery under cyclic loading, highly efficient and autonomous self-healing upon fracture, and capability for viable cell encapsulation.

Additionally, various bismaleimides and trismaleimides characterized by different molar masses were used in furan-modified alginate chains as cross-linkers. The hydrogels with tuning mechanical properties and pulsatile swelling behavior were fabricated with Diels-Alder chemistry (García-Astrain & Avérous, 2019). García-Astrain and Avérous successfully functionalized alginate with furfurylamine and then a series of cross-linked alginate hydrogels were formed using the reaction of furan-modified alginate and maleimide cross-linkers (García-Astrain & Avérous, 2018). After the conjunction of an antimicrobial peptide HHC10 with the oxy-norbornene group, the hydrogels fabricated with furyl-modified sodium alginate

and bimaleimide functional PEG molecule were demonstrated to present strong antibacterial properties and good biocompatibility (G. Wang et al., 2018). These hydrogels can be employed to support sustained mechanical functions, replace or repair load-bearing soft tissues, and provide good antimicrobial properties.

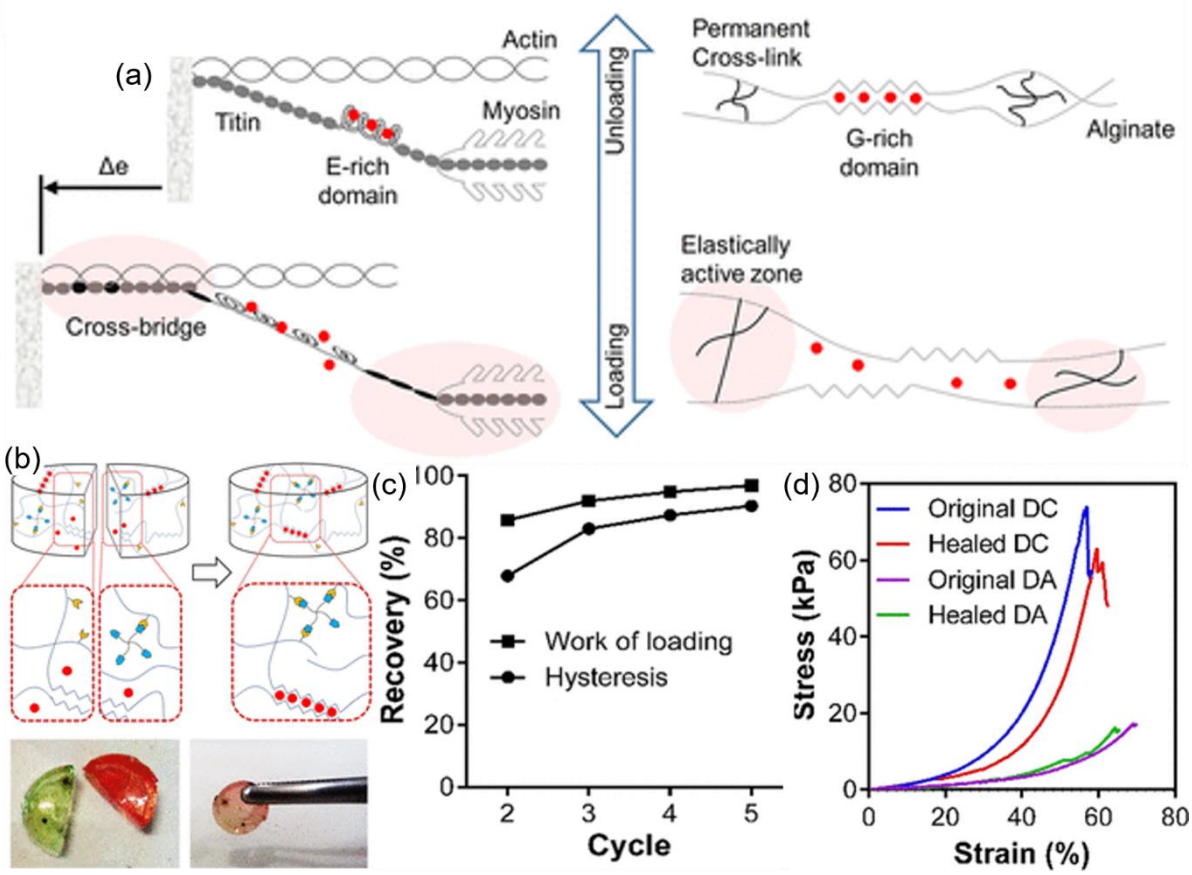


Figure 2. Design and self-recovery, self-healing properties of tough hydrogels based on Dual Cross-linked alginate. (a) Biologically Inspired Design of Tough Hydrogels; (b) Schematic representation and photographs of the healing process under physiological conditions for two colored cuts of the DC hydrogels; (c) Recovery efficiency of the hysteresis energy and work of loading after each cycle; (d) Compression stress-strain curves of the original and healed samples of the DA and DC hydrogels. (Ghanian et al., 2018)

2.4 Inverse electron demand Diels-Alder (IEDDA) cycloaddition

Inverse electron demand Diels-Alder (IEDDA) cycloaddition is a rapid chemical reaction, capable of achieving completion even under mild conditions. In the IEDDA reaction, a 1,2,4,5-

tetrazines (s-tetrazines) derivative performs as a ‘diene’ (norbornene, Nb) and an alkyne (or strained alkene) acts as a ‘dienophile’ (tetrazine, Tz) (Carboni & Lindsey, 1959). [4+2]-Cycloaddition occurs on $C^{3,6}$ carbon atoms of tetrazine, and the nitrogen molecule and oxidation provide the pyridazine cycle(Suvorov, Cheskov, Mironov, & Grin, 2019). The electron demand in the Diels-Alder reaction indicates that a diene that is rich in electrons reacts with an electron-poor dienophile. In the IEDDA, however, an electron-rich dienophile reacts with a diene that is electron deficient (Oliveira, Guo, & Bernardes, 2017). The reaction rate constant typically ranges from 10^3 to 10^6 $M^{-1}s^{-1}$, depending on the structures of the reactants (Izquierdo & Delgado, 2018; Oliveira et al., 2017; Selvaraj & Fox, 2013). IEDDA is suitable for biocompatible materials due to the capability of encapsulating drugs without causing damage(Desai, Koshy, Hilderbrand, Mooney, & Joshi, 2015).

Several Nb and Tz can be introduced into the alginate system to tune the crosslinking density and the properties of the matrix without changing the total amount of alginate. The results from Mooney et al’s study showed that Nb-Tz click chemistry has the ability to control stiffness and viscoelasticity of artificial extracellular matrix (ECM) hydrogels, without altering the diffusional nutrient transport or alginate architecture at the cellular scale, compared with ionic-only alginate hydrogels (Vining, Stafford, & Mooney, 2019). Tetrazine (Tz), transcyclooctene (TCO), and norbornene (Nb) were used to modify alginate by combining EDC/NHS (ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC), N-hydroxysuccinimide (NHS)) carbodiimide crosslinker via click chemistry. These single cell-encapsulated microscale hydrogels (25-30 μm microgels) have been fabricated to form structures with packing densities

comparable loose randomly packed configurations (Y. Hu et al., 2017). Norbornene groups, such as a 'diene', also could be utilized to decorate alginate to control properties. The reactions between norbornene-functionalized alginates (Alg-Nb) and tetrazine cross-linkers while also using IEDDA click chemistry, could prepare hydrogels with facilitating precise DOX release (Figure 3) (Anugrah, Ramesh, Kim, Hyun, & Lim, 2019). Under the trigger of NIR irradiation, the hydrogel was de-cross-linked to linear alginate chains. The various cross-linking densities were controlled by adjusting the feed ratio of the precursors, Alg-Nb, and diselenide-tetrazine (Se-Tz), then tuning the release rate of loaded DOX (Anugrah et al., 2019).

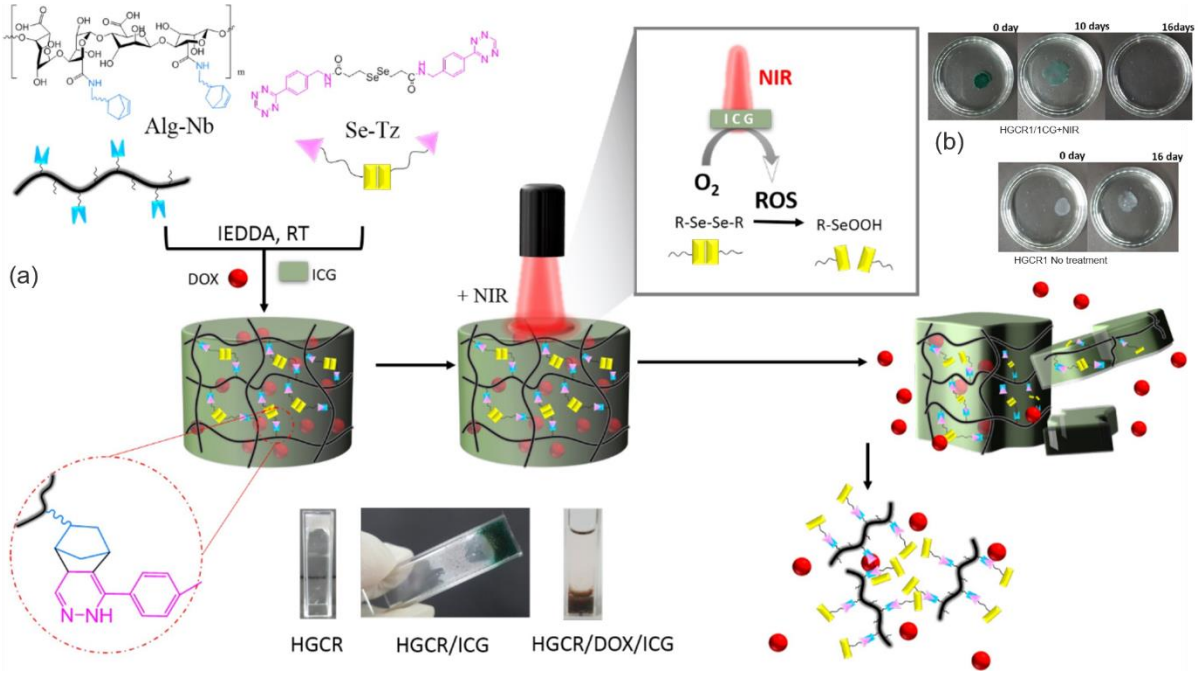


Figure 3. Near-infrared responsive alginate-based hydrogels via tetrazine-norbornene chemistry. (a) A schematic illustration of NIR-responsive alginate-based hydrogels; (b) Photos of the degradation process. (Anugrah et al., 2019)

The incorporation of norbornene (Nb) and tetrazine (Tz) endow more elastic properties to the alginate hydrogels (Gonzalez-Pujana et al., 2020). It has been demonstrated that G-blocks of alginate may be reinforced by permanent covalent crosslinking, such that IEDDA reaction

executes the "click" to connect the existing G-block ionic crosslinks (Vining et al., 2019), thus providing permanent covalent crosslinking to reinforce alginate. The modified alginate and cytokine-loaded heparin-coated beads prolonged the immunomodulatory licensing of hMSCs. In another study, Joshi (Desai et al., 2015) incorporated tetrazine and norbornene groups with alginate polymer chains to enable covalently crosslinked click alginate hydrogels formation (Figure 4). Mechanical properties and swelling properties were tuned by altering the total polymer concentration and by varying the complementary click functional group's stoichiometric ratio. The alginate hydrogels also can facilitate cell encapsulation without causing damage. Moreover, the rheological and mechanical properties of crosslinked alginate-based hydrogels were modified via changing the substitution degree of norbornene, oxidation state, and the proportion of norbornene to tetrazine integrated in the alginate-based hydrogels (Lueckgen et al., 2018).

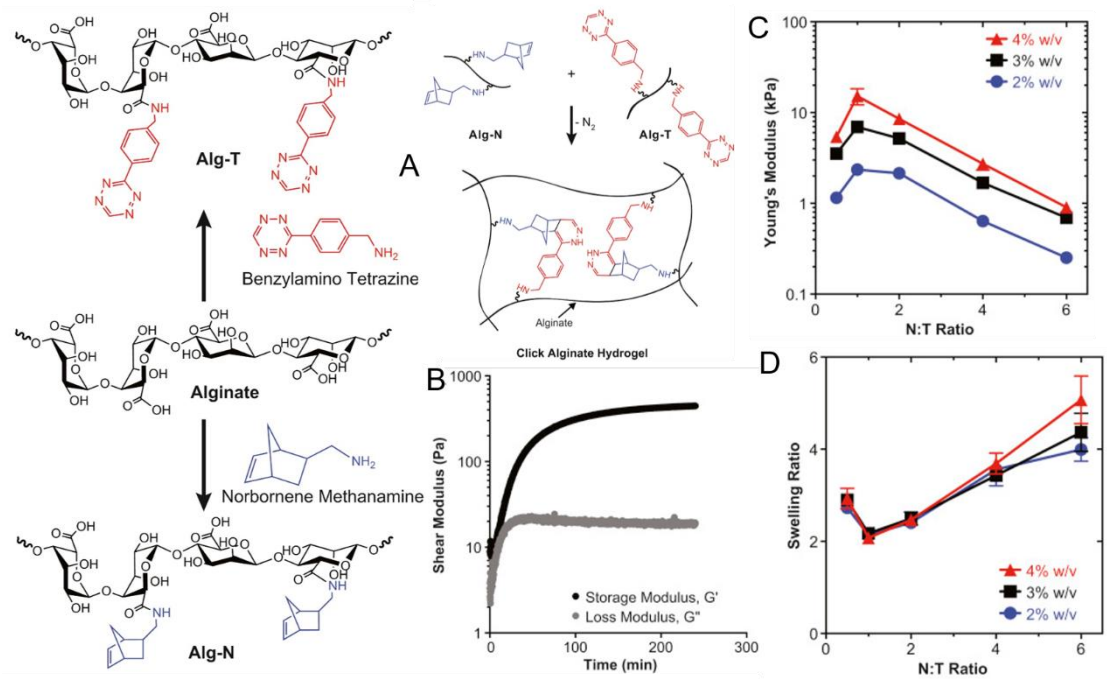


Figure 4. An illustration of click alginate polymer synthesis (A) and mechanical properties (B-D). (A) Click alginate hydrogels fabrication. Modification of alginate backbone carboxylic

acids with tetrazine or norbornene using aqueous carbodiimide chemistry, to produce Alg-T or Alg-N polymers, respectively. Alg-T and Alg-N polymers are mixed leading to the production of acovalently crosslinked click alginate hydrogel network, with the release of N_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 (B). Compressive Young's modulus (C) and volumetric swelling ratios (D) for 2%, 3% and 4% w/v click alginate hydrogels at varying N:T ratio. Values represent mean and standard deviation (n = 4)(Desai et al., 2015).

The tetrazine-based IEDDA reaction between a dipyridyl-functionalized tetrazine and trans-cyclooctene is biorthogonal ligation, which was first reported by Fox and co-workers (Blackman et al., 2008). The reaction rate of the tetrazine-based IEDDA reaction is three orders of magnitude greater than the optimized SPAAC reaction, leading to the fastest biorthogonal conjugation of tetrazine ligation (Patterson, Nazarova, & Prescher, 2014). The tetrazine-norbornene Diels-Alder reaction involves tetrazine and norbornene compounds that are asymmetric, leading to the production of several isomeric dihydropyridazine products, with rate constants for the second-order reaction of 1.9 and 1.6 $M^{-1}s^{-1}$ in an aqueous buffer and fetal bovine serum respectively (Devaraj et al., 2008). It has the potential to create functionalization and coupling of polymers without requiring additional additives, initiators, or catalysts.

For the degradation of tetrazine under physiological conditions, designated groups will be introduced into IEDDA reactions. That has been demonstrated and undertaken by Shoichet. IEDDA-crosslinked HA hydrogels were designed by replacing tetrazine with the more stable methylphenyltetrazine to eliminate reagent degradation (Delplace et al., 2020). The designed IEDDA hydrogel facilitates multiphoton imaging of embedded retinal explants in a duration longer than the duration required by agarose thermogel (Delplace et al., 2020). Furthermore,

when the trans-cyclooctene (TCO) moiety was incorporated into the sugar backbone, the alginate polymer (TCO-gel) was constructed. These gels will react with circulating in-Tz molecules through IEDDA reaction in a biorthogonal fashion, localizing the Tz molecules and their radioactive cargo to the TCO-gel. This approach could precisely regulate, when biochemical and/or physical signals are manifested in a biomaterial that is implanted and also improves the spatial site of systemic tiny molecules via vivo chemical delivery (Mejía Oneto, Gupta, Leach, Lee, & Sutcliffe, 2014).

2.5 Thiol-ene/yne addition

The thiol-click reaction is a well-expanded concept of click polymerization. The reaction of a thiol with carbon-carbon double bond, or simply "ene", as the general concept of thiol-click reaction, has been well known since the early 1900s (Posner, 1905). Many basic thiol-ene reactions have been defined since the early 2000s. The reactions of a thiol with ene, triggered by a radical (thiol-ene reaction) or anionic chain (thiol Michael addition), have the characters of click chemistry, such as being insensitive to ambient oxygen and water, single specifically products and rapid reaction rates. This significant versatility makes the thiol-ene reaction amenable to various applications, including biomedical, tissue engineering and bioorganic modification fields. These thiol reactions include thiol-ene free-radical addition, catalyzed thiol Michael addition, and thiol-yne addition reactions.

2.5.1 Free-radical thiol-ene addition click reactions

Free-radical thiol-ene reactions involve the formation of thiyl radical, which then propagates across the 'ene' functional group to produce the carbon-centered radical. The radical source, or

photo initiator under the light, extracts hydrogen from thiyl radicals to form thiyl in a chain transfer process which may be added to the carbon-carbon double bond (Aimetti, Machen, & Anseth, 2009; Cramer & Bowman, 2001). In other words, thiyl radical activates the carbon-carbon double bond by forming a carbon-based radical. A chain transfer reaction involving the carbon-based radical and another thiol group facilitates the generation of a new thiyl radical or propagate through carbon-carbon double bonds (Rydholm, Bowman, & Anseth, 2005).

This reaction involves the alternation of propagation and chain transfer events. UV light can be used to rapidly initiate Thiol-ene chemistry, under mild conditions and in the absence of complex reagents(Beria et al., 2014). The light mediated thiol-ene reaction can be effectively activated at the special location and time and combines the benefits of click chemistry with superiorities of photo-initiated processes. A homogeneous polymer network could subsequently be formed by tuning the combination of step-growth and chain-growth mechanisms (Cramer & Bowman, 2001). The distinct advantages of thiol-ene reaction, such as simplified polymerization kinetics, decreased shrinkage and stress, and lacked sensitivity to oxygen inhibition, solve the limitations of traditional photo-initiated systems(Hoyle & Bowman, 2010).

UV-directed thiol-ene click reaction, as an effective method, is extensively employed in the preparation of gels and post-modification of polymers, with the purpose of obtaining qualified hydrogel (Yap et al., 2020). Lang et al (Xu et al., 2020) functionalized sodium alginate backbone to synthesis SA derivatives via grafting vinyl ether (VE) side chains to form amido linkages (-CONH-). A dual crosslinking alginate hydrogel SA-VE/DTT was fabricated through

hydrogen-bonded along with thiol-ene click chemistry reaction under UV exposure (Xu et al., 2020). These systems maintained fast gelation, superior storage modulus, and long-term stability. Wang et al introduced cysteine-terminated antimicrobial peptide HHC10-CYS (HHC10) into sodium alginate hydrogel via the photoinitiated thiol-ene reaction. The antibacterial activity was up to 100% after culture for 24h, and the cytocompatibility was improved (G. Wang et al., 2018). In the process of preparation, the efficient photo-click reaction provides spatiotemporal control through a step-growth mechanism. This photo click chemical reaction affords sites for cell attachment and embedment with enhanced the quality (Pereira, Barrias, Bártolo, Granja, 2018). After implantation in the backs of mice (C57/B16) for 8 weeks, hydrogel modified with thiol-ene chemistry by UV irradiation improved the tissue and cell infiltration, with *in vivo* implantation resulting in degradable materials rather than non-degradable controls.

Thiol-ene click reaction could manipulate antimicrobial properties via decorating hydrogel. Various cellulose derivatives were prepared via the thiol-ene click reaction between cellulose and the thiol compounds (H. Hu, You, Gan, Zhou, & Zhang, 2015). The micelles through combining derivatives and Ag nanoparticles displayed good antimicrobial activities to both *S. aureus* and *E. coil* (H. Hu, Wu, Wang, Wang, & Zhou, 2019). The optimal preparation route and excellent biological performance of the above hydrogels may bring about potential biomedical applications in wound dressing materials.

2.5.2 Thiol-Michael addition click reactions

Thiol-Michael addition type reactions refer to reactions between thiols and electron deficient

enes (Allen & Happ, 1964). Allen et al first reported these reaction types (Allen & Happ, 1964). The most widely used enes are (meth) acrylates, maleimides, acrylonitrile, cinnamates, crotonates, fumarate esters, and α,β-unsaturated ketones (Hoyle & Bowman, 2010). Maleimide as ene has been most widely used in the thiol-Michael reaction (M. Li, De, Gondi, & Sumerlin, 2008). Catalysts, such as metals, organometallics, Lewis acids, are utilized to initiate the thiol-Michael reaction (Mather, Viswanathan, Miller, & Long, 2006). Most of the thiol-Michael reaction focuses on the addition of thiol-groups to acrylic compounds (Çakmakçi, Yuce-Dursun, & Demir, 2017; Kröger, Boonen, & Paulusse, 2017; Moon, Pekkanen, Long, Showalter, & Libby, 2017). Meanwhile, Michael acceptors also contain maleimides, vinyl sulfones, fumarates, crotonate, ynones and propiolates (Nair et al., 2014; Stolz & Northrop, 2013). Accompanied by the initiation of terminating chains in thiol-Michael reactions, there are no anionic coupling processes compared to the thiol-ene radical reaction (Hoyle & Bowman, 2010). This reaction can occur under mild conditions in short reaction times i.e. minutes or even seconds. Thiol-Michael addition type reactions are therefore suitable for functionalization of polymer or preparing biomaterials.

Michael addition reaction is selective for the formation of hydrogels, *in situ,* and is well-known as a viable polymer synthesis strategy (Z. Q. Liu et al., 2015). The *in situ* cross-linking of hydrogels using the Michael addition reaction between thiol-modified chitosan and poly(propylene oxide) poly(ethylene oxide)- poly(propylene oxide) (PPO-PEO-PPO) was undertaken by Gabilondo et al (Guaresti, Basasoro, González, Eceiza, & Gabilondo, 2019). These hydrogels are characterized by high sensitivity to variations in pH and also present

complete degradation in lysozyme solution after 24 h of immersion. Hydrogel with bifunctional cross-linker in 1:3 ratio has a more cross-linked network, showing lower swelling ratios than other tested hydrogels. With the addition of the cross-linker, the decrease of mean storage and loss modulus was also observed. Therefore, the swelling and rheological behaviors were regulated by altering the cross-linking agent in the networks.

2.5.3 Thiol-yne addition click reaction

Thiol-yne addition click reaction is similar to thiol-ene chemistry in that thiol groups react with carbon-carbon triple bonds (Truong, Tsang, & Forsythe, 2017). Thiyl radical addition to an 'yne' functional forms a vinyl sulfide radical, and then chain transfers to a thiol group, to regenerate a thiyl radical and form the vinyl sulfide addition product (Lowe & Bowman, 2013; Minozzi et al., 2011). A carbon-centered radical is formed through thiyl radical addition to vinyl sulfide group. Then the chain transfers to another thiol functional group, leading to the regeneration of the thiyl radical and formation of the thiol-vinyl sulfide addition product (Lowe & Bowman, 2013). The thiol-yne addition click reaction could progress via a radical or nucleophilic mechanism (Macdougall, Truong, & Dove, 2017). The radical method has been utilized in polymer science to synthesize a series of materials, including dendrimer (G. Chen, Kumar, Gregory, & Stenzel, 2009), multifunctional brush polymers (Hensarling, Doughty, Chan, & Patton, 2009), and block polymers (Chang & Dong, 2013). The nucleophilic method has been adopted to synthesize hydrogel materials (Cai et al., 2016).

Thiol-yne click-reaction is a suitable approach to prepare robust click-hydrogels. According to Dove et al injectable alginate hydrogels, fabricated via the thiol-yne click reaction, had

exceptional mechanical performance and were capable of retaining their mechanical properties even after being immersed in a cell culture media for three weeks (Pérez-Madrigal et al., 2020). The extended stability enhanced cytocompatibility, and sufficient stiffness was also retained.

2.6 Oxime based click reactions

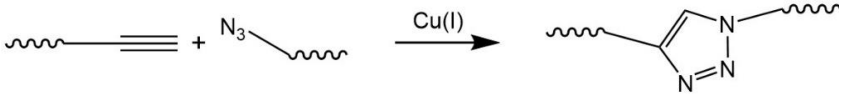
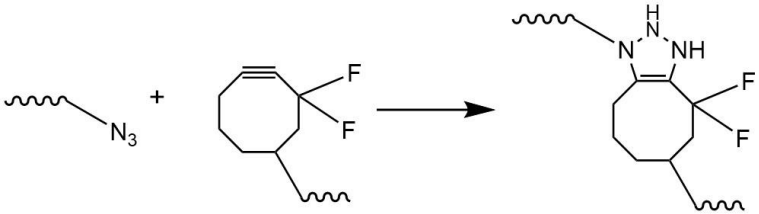
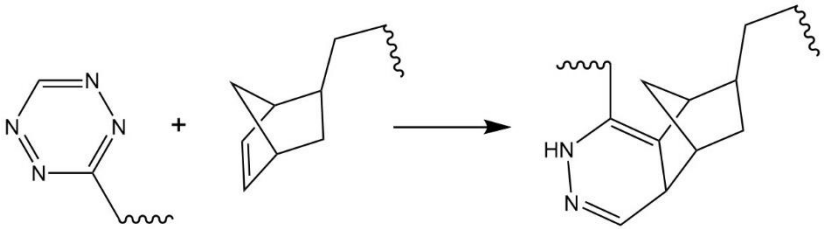
Oxime click reactions facilitate oxime bond formation and involve reactions between a substituent of aminooxy and an aldehyde or ketone moiety to produce imine hydrazone and chemical bonds of oxime (Kalia & Raines, 2008). Since oxime bonds have higher stabilities compared to bonds in thiol groups, it has emerged as a robust strategy in fields such as bioconjugation (Ulrich, Boturyn, Marra, Renaudet, & Dumy, 2014) and biomacromolecules (Christman et al., 2011). Oxime reactions have the properties of click reactions, such as high reaction rates, water production as a by-product, and orthogonality to molecules present in the cellular environment(Kalia & Raines, 2008). Thus, Oxime click reactions have been extensively employed in modifying surfaces of materials (Zeng, Ramya, Dirksen, Dawson, & Paulson, 2009) and bequeaths the unique properties of tuning and reversibility, to varying degrees (Grover, Lam, Nguyen, Segura, & Maynard, 2012; Lin et al., 2013).

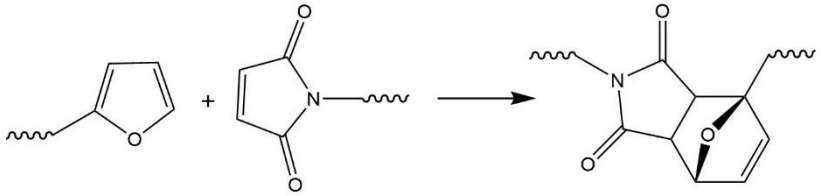
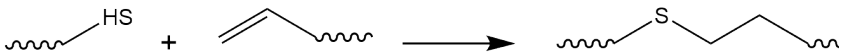
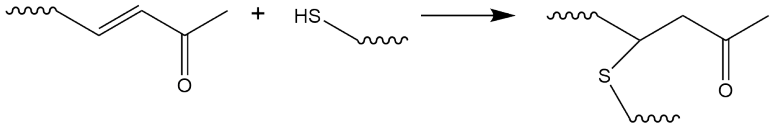
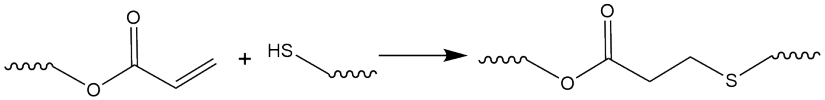
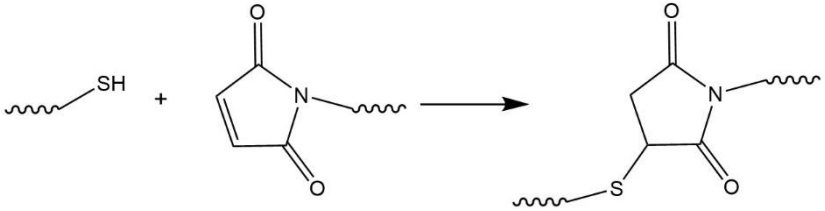
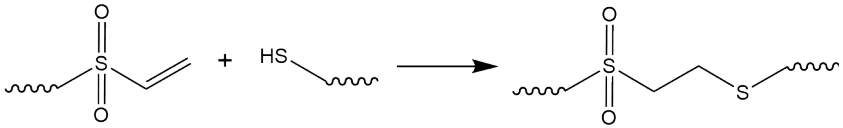
2.7 Other click reactions

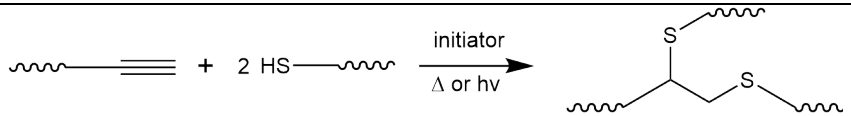
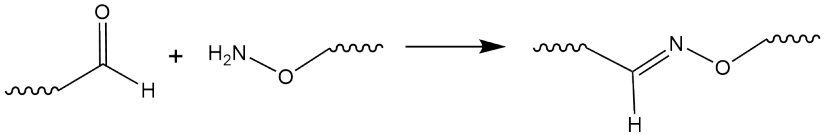
Beyond the click reactions mentioned above, three other click reactions have been developed recently. Firstly, the spontaneous amino-yne click reaction was reported by Tang et al (B. He et al., 2017; B. He et al., 2016). It involves reactions of amines and propiolate, and can proceed

in a regio- and stereospecific fashion under mild conditions without any photoinitiator or catalyst(B. He et al., 2017; B. He et al., 2016, Oktay, Demir, & Kayaman-Apohan, 2020). As a high reactivity reaction, hydroamination between dipropiolate and secondary diamine can occur, leading to products with high molecular weights, characterized by excellent yields. Second, nitrile-click chemistry, as an effective and novel strategy, has been received attention (Y. Li et al., 2019; Oktay, Zhang, You, & Hong, 2018). The nitrile-containing polymers, especially based on C≡N groups, have been devoted to click reaction (Zil'berman, 1986). The click reaction with sodium azide and zinc chloride was utilized to modify acrylonitrile polymers(Tsarevsky, Bernaerts, Dufour, Du Prez, & Matyjaszewski, 2004). Therefore, polyacrylonitrile (PAN) is completely suitable for nitrile-click chemistry (W. Wang et al., 2017). Surface modification of PAN using nitrile-click chemistry has constituted the research focus in recent times. And third, novel click chemistry is the protection-click reaction-deprotection process. During the reaction, phthalic anhydride was used to protect 2-NH_2, and azid group was utilized to replace 6-OH group of chitosan. The click chemistry reaction was occurred between an azide group and an alkynyl terminated quaternary ammonium salt. The final product was obtained along with the deprotection of phthalic group (Y. Chen et al., 2017). This preparation method improves the thermal stability of products and water solubility. These click reactions are useful for modifying functional molecules due to its chemo selectivity.

527 Table 1. Major click chemistry reaction types and their applications in biomaterials

Click reactions	Reacting functional groups	Mechanism	Advantages	Disadvantages	Properties	Applications
CuAAC	azide+alkyne; e.g., azide and alkyne end group		Cu catalyst; Reversible; Bioorthogonal; no by-products	Cytotoxicity of Cu and difficult to remove completely	Structural stability; promotes the uniform dispersion(Roth et al., 2019);	Cell encapsulation(Breger et al., 2015), drug delivery(Kumar et al., 2019), antibacterial materials(Y. Chen et al., 2017)
SPAAC	azide+alkyne ring; e.g., azide and cyclooctyne		No catalyst;	Disturbing cross-linking reaction due to hydrophobicity of the cyclooctane(S. Fu, Dong, Deng, Zhuo, & Zhong, 2017; Truong et al., 2015)	Tuning mechanical properties; decrease inflammatory(Moody et al., 2020);	Tissue engineering; drug delivery;
IEDDA	Dienophile+diene; e.g., Tetrazyne and norbornene		No catalyst; Faster rate of reaction than Cu-free click reaction;		Control stiffness and viscoelasticity(Vining et al., 2019); rheological and mechanical properties; prolong immunomodulatory properties(Gonzalez-Pujana et al., 2020);	Cell encapsulation, drug delivery, targeted delivery of systemic small molecules(Mejía Oneto et al., 2014);
Diels-Alder	diene+alkene; e.g., furan and maleimide		No catalyst; Thermally reversible(Wei, Yang, Zheng, & Shen, 2009);	Longer reaction time (gelation time is 1.5-24h)(Fisher, Anandakumaran, Owen, &	Self-healing(Ghanian et al., 2018); mechanically properties; swelling	Soft tissue engineering, drug delivery(García-Astrain & Avérous,

				Shoichet, 2015);	behaviour; antibacterial properties(G. Wang et al., 2018);	2018),
Thiol-ene	alkene+thiol; e.g., Methacrylate group and dithiothreitol		Using photoinitiatorr; Spatial and temporal control(Rydholm et al., 2005); Insensitivity to oxygen(Shih & Lin, 2012)	Radical is toxic to encapsulated cell in initiation step(A. Fu, Gwon, Kim, Tae, & Kornfield, 2015; Rydholm et al., 2005)	Mechanical and permeability; stability; antimicrobial capacity(H. Hu et al., 2019); degradation behaviour(Lueckgen et al., 2019);	Wound dressing; drug delivery; tissue engineering
		Alkene-thiol Michael addition  Methacrylate-thiol Michael addition				
Thiol-Michael	Thiol+α,β-unsaturated carbonyl group; maleimide+thiol; e.g., maleimides, acrylate esters	 Thiol-maleimide Michael addition	No catalyst; Mild reaction condition	The reaction is disturbed by thiols of protein(Pupkaite, Rosenquist, Hilborn, & Samanta, 2019)	Control rheological and swelling behavior(Guaresti et al., 2019);	Tissue engineering
		 Thiol-vinysulfone Michael addition				
						

Thiol-yne	alkynes+thiol		Initiator		Mechanical performance; stability; cytocompatibility;	Soft tissue regeneration(Pérez-Madrigal et al., 2020);
Oxime coupling	Aminooxy+aldehyde/ketone; e.g., Aldehyde+hydroxylamine		No catalyst; Fast reaction kinetics;	Challenge of synthesize precursor(Grover et al., 2012)		

528

3 Biomedical Applications

The excellent biocompatibility and promising physicochemical properties of alginate have promoted various biomedical applications, including wound dressing, pharmaceutical, tissue regeneration, renewable energy, and 3D bioprinting applications. The following sections describe the recent advances and summary of applications of alginate-based materials modified via click reactions.

3.1 Wound dressing

The skin, as the largest organ in human body, is a natural barrier that protects the internal organs against pathogens and dehydration from environmental aggressions (Hoque & Haldar, 2017). When the surface of skin is interrupted via dermal wounds (acute and chronic), protection from the pathogens is a significant clinical challenge for health services, due to poor vascularization, protease susceptibility, and microbial invasion at the wound site (Parani, Lokhande, Singh, & Gaharwar, 2016). The function of wound dressing is to protect the damaged area from bacterial infection, and to provide an appropriate environment to encourage the re-establishment of the skin integrity and homeostasis thus accelerating the healing process (Kujath & Michelsen, 2008; Ma et al., 2019). Due to its biocompatibility, biodegradability, non-immunogenicity, affordability, and water content, alginate and alginate-based materials are of considerable attention and attractive for use as wound dressing. Alginate dressings can absorb wound fluid in the dry state and form hydrogels. That provides a moist environment and reduces bacterial infections for wound healing. However, pure

alginates are generally limited by their poor mechanical stability when in the swollen state and may dehydrate if not covered. It is difficult to secure skin, prevent bacterial infection, and promote bioactivities, especially in chronic wound healing. To overcome the poor mechanical stability of hydrogels, functionalized alginate dressings have been developed.

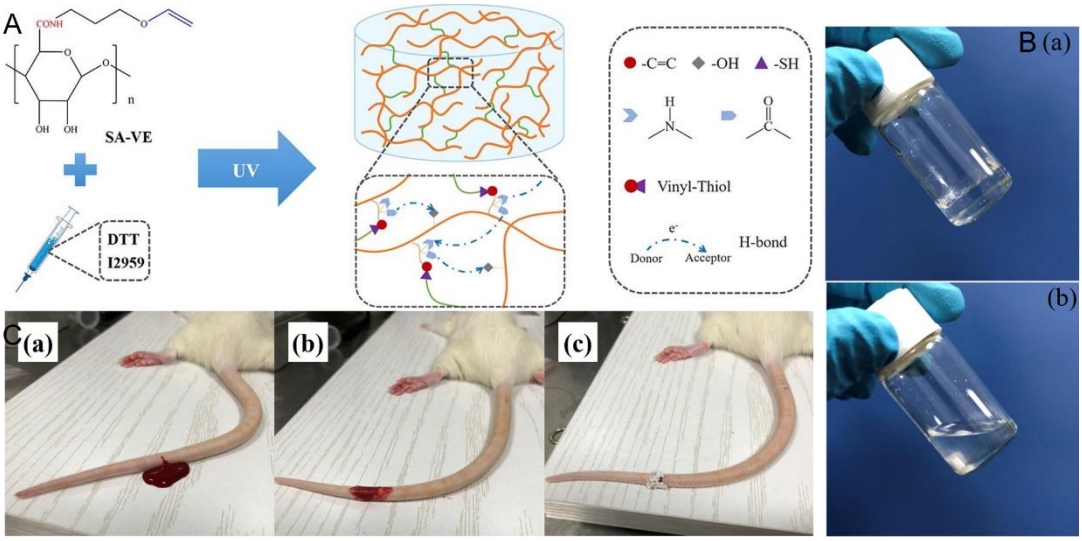


Figure 5. crosslinking alginate hydrogel with superior gel properties based on viny ether sodium alginate. (A) Synthesis diagram of SA-VE/DTT hydrogel; (B) Hydrogen bonding verification: SA-VE/H_2O hydrogel added with (a) DI water; (b) NaSCN aqueous solutions (3 mol/L); (C) Rat tail hemostasis test results: (a) natural hemostasis; (b) Ca-Alg; (c) SA-VE.(Xu et al., 2020)

Different types of functional groups have been introduced to produce alginate-based wound dressing materials. Mixing of functional alginates with other biopolymers will facilitates the formation of a structure that is characterized by a crosslinked network. Vinyl ether side chains were grafted into sodium alginate backbone, thus providing new reaction sites for further cross-linking. The functionalized sodium alginate subsequently formed a series of dual crosslinking hydrogel sodium alginate-vinyl

570 ether/dithiothreitol (SA-VE/DTT) with dithiothreitol under UV exposure (Figure 5)(Xu
571 et al., 2020). The hydrogel system displayed superior mechanical strength, long-term
572 stability, as well as a fast hemostasis behavior when applied for 26 s in rat tail wounds.
573 By increasing the amount of charged groups inside the hydrogel, the internal structure
574 was resembled to form double network hydrogel with high toughness (Benselfelt &
575 Wågberg, 2019).

576 Additional, antibacterial properties can ensure the success rate of medical supplies.
577 The conventional strategies are using in loading antibacterial substances (such as
578 antibacterial particles or groups, antibiotic, or antimicrobial agents) into hydrogels to
579 possess antibacterial activity (M. Chen et al., 2019; M. He, Wang, Zhang, Zhao, & Zhao,
580 2017). However, their application is restricted because of the risk of temporary
581 antimicrobial activity, gel formation, and cytotoxicity of nanoparticles (D. Jiang et al.,
582 2016). Chemical grafting of agents can enhance antimicrobial efficacy, reduce
583 cytotoxicity, and prolong biostability (Ng et al., 2014). The antimicrobial peptide
584 HHC10 was introduced into SA/PEG hydrogels via a photoinitiated thiol-ene click
585 reaction, showing strong antibacterial properties and desirable biocompatibility (G.
586 Wang et al., 2018). Chemical reaction strategies can effectively immobilize
587 antibacterial agents in medical devices, enhancing the antibacterial properties, and thus
588 have significant application potential.

589 **3.2 Pharmaceutical applications**

590 Alginate is the backbone of different pharmaceutical applications, including

thickening, gel-forming, encapsulation. It performs a major role in regulated drug release. However, there are some restrictions for the conventional drug delivery systems, including low drug efficacy, poor targeting, poor distribution, uncontrolled pharmacokinetics, and serious side effects in non-target tissues (Aw, Addai-Mensah, & Losic, 2012). Nowadays, hydrogels as depots for tissue localized drug delivery have been accepted as a proper solution to address these problems. Hydrogel delivery systems can transport the drugs to the targeted sites. Here, we describe progress in the pharmaceutical application using alginate or alginate derivatives.

3.2.1 Drug delivery

Alginate hydrogels constitute well-researched technologies for drug delivery and have been explored in the regulation of drug release. The most important advantages of using alginate to encapsulate drugs are its excellent hydrophilicity and the efficiency of the gelation process which occurs under mild condition (Erik, Aase, Paul, Anders, & Maria, 2010). Diffusion is the dominant release mechanism in controlled drug release from hydrogels (Peppas, Bures, Leobandung, & Ichikawa, 2000). Alginate hydrogels have a typically porous structure, leading to the rapid diffusion of drug molecules.

Injection hydrogels are used ubiquitously as cell and drug carriers. A click cross-linking strategy is established to be adequate for injectable hydrogels and was employed in the development of functionalized alginate hydrogels (Kim et al., 2016). Injection hydrogels may be used as refillable hydrogel depots, for targeting drug-carrying nanoparticles refills to a device placed within a tumor site (Brudno et al., 2014).

612 Refillable hydrogel depot system based on biorthogonal click chemistry capture
613 prodrug refills from the blood and then sustainably release active drugs locally (Brudno
614 et al., 2018). The use of biorthogonal click chemistry in targeting circulating small
615 molecules to alginate hydrogel resident intramuscularly in diseased tissues was
616 demonstrated in the literature (Brudno et al., 2015). These small molecules were shown
617 to be capable of repeatedly targeting the diseased area in a sustained manner for about
618 one month. The click-mediated targeting exhibited high specificity for the target sites
619 and enhanced the delivery of suitable small molecules (Brudno et al., 2015; Mejía
620 Oneto et al., 2014). Brudno et al introduced tetrabicyclononyne (tBCN) agents to
621 fabricate cross-link azide-modified alginate hydrogels (Moody et al., 2020). tBCN click
622 cross-linked gels improved click-mediated capture of small molecules drugs from the
623 blood, and this *in vivo* targeting was sustained over multiple weeks and with multiple
624 rounds of systemic capture (Figure 6). For several special environment, stimuli-
625 responsive triggers have been introduced to alginate hydrogels to facilitate smart
626 hydrogels under physiological conditions. The utilization of smart hydrogels for drug
627 delivery applications, has been receiving increasing interest in recent times. Under
628 external stimuli, these smart hydrogels can release entrapped drug molecules in a
629 controlled and targeted manner while also minimizing unwanted intrusiveness of the
630 procedure (Matricardi, Di Meo, Coviello, Hennink, & Alhaique, 2013; Qiu & Park,
631 2001). The improvement in the molecular mobility was achieved when comb-type
632 grafted hydrogels with network-graft architecture and dangling chains were

incorporated into a cross-linked network, for enhanced conformational adjustment ability of the incorporated chains when responding to external stimuli (S.-Q. Chen, Li, Pan, Li, & He, 2016).

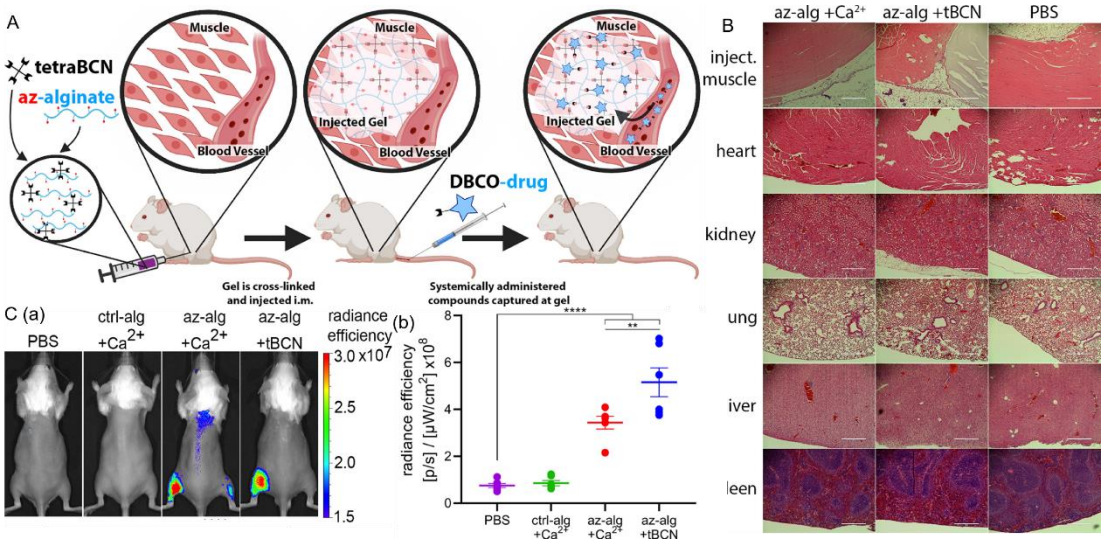


Figure 6. tBCN cross-linked azide-alginate gels and refillable depot stability for uses in the body. (A) Using click chemistry to cross-link refillable depots. Left to Middle: Azide-alginate strands are added and injected into target tissues and cross-link *in situ.* Middle to Right: Intravascular administration of cyclooctyne-conjugated therapeutics permits selective capture and display of drug at gel site. (B) Biocompatibility of tBCN cross-linked azide-alginate gels. Model/reference images of H&E stained sections from injection site and five major organs four weeks after intramuscular injection of calcium and tBCN cross-linked azide-alginate hydrogels and PBS-injected controls. Scale bar = 400 µm. (C) Cross-linking with tBCN improves on-target capture of circulating DBCO fluorophores. Reference images (a) and quantitation (b) of fluorophore capture by intramuscularly-injected alginate hydrogels. Azide-alginate cross-linked with either tBCN or calcium were compared to control gels that did not contain azide groups and a PBS injection. DBCO-Cy7 was administered i.v. 24 hours following depot implantation. One week following i.v. administration, mice were imaged to assay capture and retention of fluorescent signal at the target site. Samples show mean ± SEM. Statistical significance represented as **$p < 0.01$, and ****$p < 0.0001$ between groups by multiple unpaired t-tests with Holm-Sidak correction for multiple comparisons. N=6. (Moody et al., 2020)

Polymeric microspheres are effective delivery vehicles, which provide the potential for greater suspension stability, high drug loading, and lower burst release (Mohanraj & Chen, 2006). Encapsulating the drug in polymeric microspheres is an effective strategy that can control and sustain drug release. It can encapsulate the drugs in the hydrophilic alginate polymer, protect encapsulated drugs from harsh physiological microenvironments including moisture, heat, oxidation and 'mask' their horrible taste and odour (Sharma, Purwar, & Gupta, 2015), and deliver hydrophobic drugs to a specific treatment target in aqueous systems. For instance Rashidan et al encapsulated synthesized anti-cancer compounds into Na-alginate microspheres (Rashdan, Farag, El-Gendey, & Mounier, 2019). The study showed that Fickian diffusion law described the drug release mechanism while also highlighting that sustainable drug release was achieved. This is an effective solution to the hydrophobic defect of these compounds by encapsulation in hydrophilic Na-alginate polymer microspheres. Pore size and crosslinking density determine the release rate of the encapsulated drug. Increasing polymer crosslinking density, accompanied by the decrease of equilibrium swelling and average pore size, leads to the decrease in the drug diffusion rate (Korsmeyer & Peppas, 1981). When introducing Ca^{2+} to alginate solution, a reticulated structure, known as the "egg-box" model, was formed by the interaction of Ca^{2+} ions and electronegative alginate molecules. Hence, it is easier to control and sustain the drug delivery system. Compared to traditional ionic crosslinked alginate capsules, click capsules showed

improved permeability to diffusants of small size and superior stability, which have the capability to encapsulate drug and cells (Breger et al., 2015; Gattás-Asfura, Valdes, Celik, & Stabler, 2014).

3.2.2 Protein delivery

Protein encapsulated polymeric microspheres have proved effective in releasing even very labile bioactive moieties (R. R. Chen & Mooney, 2003; Saltzman & Olbricht, 2002), thus have drawn attention for many years. Proteins, including enzymes, growth factors, hormones, and interleukins, are employed in several biomedical applications as therapeutic agents (Dimitrov, 2012). Protein pharmaceuticals have high specificity and activity at relatively low concentrations, making them indispensable in combating human diseases. However, some harsh microenvironments, such as changes in pH, temperature, and ionic strength, can trigger the denaturing or alteration of protein structures, leading to the loss of therapeutic qualities (Z. Li et al., 2013; Wells & Sheardown, 2007). Alginate is an excellent candidate for protein delivery because it cannot be degraded in the human body via enzyme-catalyzed processes (Shalaby & Burg, 2003). Alginate is therefore attractive for encapsulation protein to minimize denaturation and prevent degradation. Several strategies have been assessed to enhance and regulate protein release from alginate-based formulations.

Due to the porous structure and inherent hydrophilic properties, hydrogels have been chosen to tune the release rates of protein. However, hydrogels, with larger mesh sizes

than typical proteins, easily lead to the rapid release by diffusions (Boontheekul, Kong, & Mooney, 2005). Alginate can be utilized to prepare crosslinked hydrogels with a suitable structure to encapsulate proteins. For instance, I. Noh et al prepared functionalized alginate-based terpolymeric semi-interpenetrating (semi-IPN) hydrogel with small pore sizes (9.4±3.1 μm, 5.2±1.1 μm, 1.8±0.4 μm) (Das, Pham, Lee, & Noh, 2019). These pores led to a sustained release of bovine albumin serum (BSA) for a period of 5 days and the sustained release of 5-amino salicylic acid (5-ASA) for a period of 30 h. The affinity and interaction of heparin-binding proteins and alginate contributed to the regulated containment and discharge of proteins (Ruvinov, Freeman, Fredo, & Cohen, 2016; Zuo et al., 2015). However, the cross reaction occurs between the crosslinking chemistries and encapsulated proteins, leading to the loss of bioactivity (McCall & Anseth, 2012). The bioorthogonal click chemistry between tetrazine and norbornene can form covalently crosslinked hydrogels, meanwhile, keep the integrity of encapsulated cargo (Alge, Azagarsamy, Donohue, & Anseth, 2013; Koshy et al., 2016). When Laponite was incorporated into alginate substitute via spontaneous tetrazine and norbornene ligation, the interactions of alginate and Laponite could alter the crosslinking and swelling behavior. This hydrogel avoided protein denaturation and sustained the release of the encapsulated protein. The interactions of positively charged domains on proteins and negatively-charged Laponite played a crucial role (Koshy, Zhang, Grolman, Stafford, & Mooney, 2018).

Alginate microspheres can efficiently encapsulate and protect proteins from

721 degradation, allowing for continuous protein release over a prolonged period.
722 Crosslinking interactions between sodium alginate and proteins enabled the efficient
723 loading of protein to alginate microspheres (Wells & Sheardown, 2007). In another
724 study, alginate microspheres, with a diameter of 1-10 μm, effectively sustained the
725 release of recombinant human bone morphogenic protein-2 (rhBMP-2) and also
726 improved cell growth (Quinlan et al., 2015). Amino-yne click reaction was developed
727 for enzyme immobilization due to its sustainability (Oktay, Demir, & Kayaman-
728 Apohan, 2019). The immobilized enzyme using amino-yne was observed to preserve
729 100% of its optimal activity at pH and temperature of 6.5 and 55 ºC respectively (Oktay
730 et al., 2020). Furthermore, a suitable environment was provided for the encapsulated
731 live cells to grow, while also providing covalent modification on the cell-load
732 microcapsules.

733 **3.3 Tissue regeneration**

734 Cartilage is a highly hypocellular tissue without self-repair abilities due to the
735 absence of vascularization and chondrocytes density (Costantini et al., 2016; Kesti et
736 al., 2015). Sports injuries or trauma result in focal lesions of the tissue, that may lead
737 to the degeneration of the surrounding tissue. Surgical intervention is an effective
738 method to treat and repair cartilage defects. Tissue engineering technique, combining
739 stem cells, engineering technologies, and scaffolds, can fabricate biological constructs
740 and thus support the regeneration of cartilage (J. R. Choi, Yong, & Choi, 2018). The
741 hydrogels, formed with alginate and alginate-based materials, are three-dimensional

742 cross-linked networks, providing the microenvironment to facilitate the migration,
743 adhesion, proliferation, and delivering cells and bioactive molecules (B. Choi, Kim, Lin,
744 Wu, & Lee, 2014). Therefore, these hydrogels have often been utilized in tissue
745 engineering approaches.

746 Nowadays, some alginate-based hydrogels have gained a lot of interest, because these
747 hydrogels can form the desired shapes to match irregular defects and transplant
748 chondrogenic cells via a minimally invasive method (Ren, He, Xiao, Li, & Chen, 2015).
749 Injectable hydrogel application has also generated a lot of attention in cartilage- and
750 bone tissue-engineering applications. Click chemistry methods have been utilized to
751 prepare injectable hydrogels, such as Michael addition-mediated hydrogels (Tan &
752 Marra, 2010) and click chemistry-mediated hydrogels (Takahashi et al., 2013). For
753 instance, Dove et al found that improved stiffness values and enhanced stability
754 properties in aqueous media (Figure 7) were displayed in injectable click-hydrogels,
755 ALG/HA-SH:2_{1A} click-hydrogels (Pérez-Madrigal et al., 2020), relative to gelatin-
756 based hydrogels cross-linked using the thiol-yne click reaction (Truong et al., 2017).
757 These hydrogels maintained the important properties of injectability, cytocompatibility,
758 and long-term stability in the soft 3D scaffold. In another study, the addition of calcium
759 alginate microsphere with leptin to hydrogel, promoted cartilage restoration when it
760 was transplanted into cartilage defects in rabbit femurs (R. Fu et al., 2019).

761 Hydrogel loading cell growth factors and bioactive drugs have been employed as the
762 medium of delivery in bone tissue engineering applications (H. Chen et al., 2017), to

763 maintain transfected chondrocytes for cartilage regeneration and regulate the discharge
764 of tissue induction factor, and antibacterial drugs (Fernandez, Tierney, Cunniffe,
765 O'Brien, & Kelly, 2016; Orth et al., 2011). X. D. Cao et al choose Diels-Alder click
766 chemistry and the thiol-ene reaction to fabricate sodium alginate based antibacterial
767 hydrogels SA/PEG-HHC10 hydrogels. The sterilization rate reached 100% when a
768 sufficient amount of HHC10 was incorporated (G. Wang et al., 2018). In another study,
769 Etienne et al (Mateescu et al., 2015) designed two hydrogels, based on alginate
770 modified with catechol moieties and the mixture of alginate catechol and thiol-
771 terminated Pluronic (AC/Plu-bisSH). Cytocompatible cross-linked HA-PEG hydrogels
772 were produced by employing furan functionalized HA and dimaleimide modified poly
773 (ethylene glycol) using the Diels-Alder click reaction, which is adequate for soft tissue
774 engineering applications (Figure 5) (Nimmo, Owen, & Shoichet, 2011). These
775 hydrogels can be injected and also 'jellify' in a few minutes. Moreover, the introduction
776 of CTL in these gels inhibited *P. gingivalis* development in the surrounding living
777 environment.
778 Alginate-based dual-crosslinked hydrogels with two orthogonal crosslinking
779 mechanisms, which are the spontaneous Diels-Alder reaction and the ultraviolet light-
780 initiated thiol-ene reaction were also employed by Cipitria et al (Lueckgen et al., 2020).
781 These mechanisms facilitated the hydrogels characterized with configurations in
782 stiffness, biomolecule presentation and degradation, granting localised regulation of
783 cell behavior. To endow cell activity of pure alginate, alginate was chemospecifically

functionalized with thiol-ended bioactive peptides (Bubenikova et al., 2012). The alginate-based biomimetic matrices with multiple peptide signals promoted specific cell interaction on the functionalized areas and avoided non-specific adhesion due to the inert pure alginate. Therefore, alginate hydrogels have broad potential applications for tissue engineering.

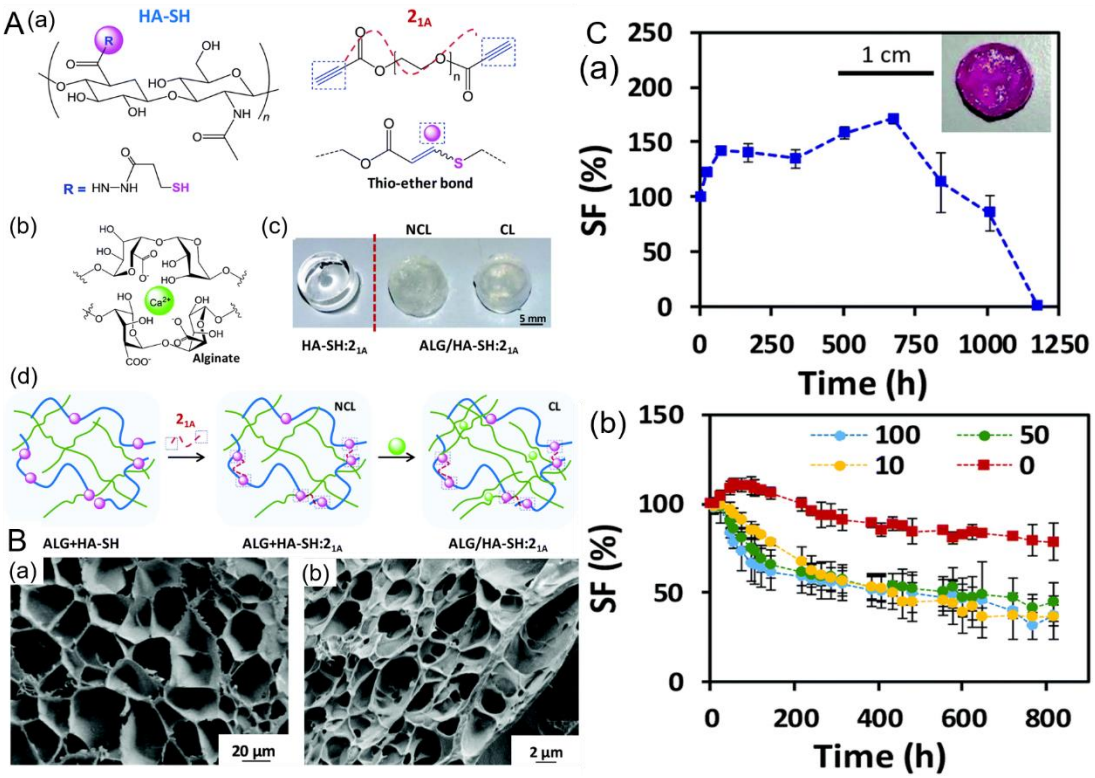


Figure 7. Robust alginate/hyaluronic acid thiol–yne click-hydrogel scaffolds with superior mechanical performance.

(A) Preparation of ALG/HA-SH:2_{1A} click-hydrogels. Schematics illustrating the composition and cross-linking of the dense HA-SH: yne network (a) and the alginate-based network (b). (c) Photographs of as prepared click-hydrogels: HA-SH:2_{1A} (no alginate in the composition) and ALG/HA-SH:2_{1A} (NCL = not cross-linked with Ca^{2+}; CL = cross-linked with Ca^{2+}). (d) Schematics illustrating the preparation steps of ALG/HA-SH:2_{1A} click-hydrogels;

(B) Cryo-SEM images taken for HA-SH:2_{1A} (left) and ALG/HA-SH:2_{1A} (right) click-hydrogels;

(C) Long-term stability of ALG/HA-SH:2_{1A} click-hydrogels at 37 °C in different environments. Swelling factor (SF) values recorded for click-hydrogels immersed in (a)

cell culture media with 1.8 mM Ca^{2+} (inset shows photo of ALG/HA-SH:2_{1A} after 14 days of immersion; (b) Ringer's solution with 8 mM of Ca^{2+} at various concentrations of hyaluronidase (100 U mL^{-1}, 50 U mL^{-1}, or 10 U mL^{-1}, and 0 U mL^{-1}). Error bars: SD with n = 4. .(Pérez-Madrigal et al., 2020)

3.4 3D Bioprinting

Three-dimensional (3-D) bioprinting is a rapid and effective approach for fabricating functional tissues *in vitro* (Seliktar, Dikovsky, & Napadensky, 2013). This approach facilitates the generation of complex geometry characterized by spatial heterogeneity that is not granted when traditional scaffold-based techniques are utilised. 3-D bioprinting addresses critical challenges facing current tissue engineering approaches. Bioinks (cell-laden biomaterials), may be employed forencapsulated cell deposition in a fabricated 3D construct and in developed complex structures of natural systems and tissues (Groll et al., 2016; Moroni et al., 2018). Extrusion, inkjet bioprinting, and laser mechanisms are common bioprinting technologies (Hölzl et al., 2016; Malda et al., 2013). The most commonly employed technique is the extrusion bioprinting method due to its comprehensive structure, operational simplicity, low cost, and printing functionality of cells with high densities (Murphy & Atala, 2014). Continuous bioink filament extruding from the nozzle is the most important property for extrusion. For inkjet bioprinting, bioink with picoliter-size droplets with low viscosity are used to construct higher resolution samples. For laser-assisted printing, higher concentration sodium alginate solution (6% and 8%) yielded a better printing quality and printed well-defined tubular constructs (J. Yan, Huang, & Chrisey, 2013). It reduced shear and intensity impact/damage.

The ink constituents and quality are critical for printing structures to meet both mechanical and biological requirements. Alginates have received much attention because of their ability to robustly form cell-compatible hydrogels in mild conditions. To facilitate tissue formation, alginates were used as bioinks to provide a matrix scaffold to direct a specific 3-D cell growth (Jia et al., 2014). Daly et al., (Daly, Critchley, Rencsok, & Kelly, 2016) determined that alginate was most preferable to facilitate improvement of the hyaline-like cartilage for 3D bioprinting relative to other bioinks such as GelMA and BioINK^{TM}. The viscosity and density are essential physical properties for alginate bioink. High viscosity bioinks provide integrity structures and support their own weight. However, gelation hinders the movement of encapsulated cells and reduces the capability to enable surrounding matrix re-structuring. Low viscosity bioinks provide a spacious and reconfigurable environment, lacking printability and integrity. Shear-thinning properties are possessed by alginate which also present high viscosities at relatively low concentrations of the alginate solution (Rezende, Bártolo, Mendes, & Filho, 2009). Bioprinting of fibroblast-laden alginate via extrusion printing revealed that even at a high concentration (10 wt.%) favorable printability (5 layers) was achievable, whereas for longer term cell culture (2 wt.%) the single layer may correlated with concentration (Shi, Laude, & Yeong, 2017). Rheological modifiers were utilized to prepare bioink, endowing desirable properties for the formation of free-standing structures (Leppiniemi et al., 2017; H. Li, Tan, & Li, 2018). Apeldoorn et al (Marchioli et al., 2015) observed that 4% alginate/5% gelatin

was a suitable hydrogel solution for plotting of islet and β-cells, which does not compromise the viability and morphology. However, high viscosity resulted in a dense mesh size, which impairs glucose diffusion and limits islet functionality, leading to high shear stresses on cells and subsequently, cell death (Marchioli et al., 2015; Ning, Guillemot, Zhao, Kipouros, & Chen, 2016). The concentration of alginate solution influences cell migration and morphology. Figure 8, shows a modular cell-laden bioink based on a norbornene functionalized alginate system, which is characterized by a rapid UV-induced thiol-ene cross-linking mechanism that prevents acrylate kinetic chain formation(Ooi et al., 2018). This system was developed by Baker et al., (Ooi et al., 2018). This altered bioink enabled printability and high cell survivability even at lower concentrations and produced 3-dimensional constructs that were stable. A novel crosslinking strategy was introduced into the bioink comprising of catechol modified hyaluronic acid (HACA) and alginate, involving ionic crosslinking, catechol mediated crosslinking, and Michael addition (Zhou, Yue, Chen, & Wallace, 2020). This bioink was easily extruded and crosslinking occurred when the two solutions from core and shell were in contact. During printing, proteins with cell adhesion motifs (gelatin) can be integrated with HACA/alginate hydrogel to improve cell interactions, and thus obtain high cell viability.

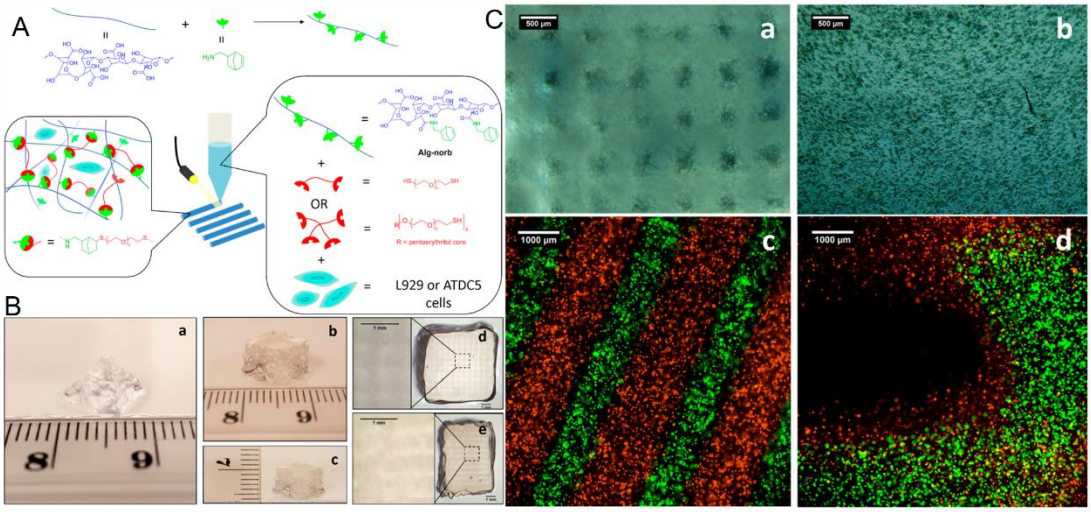


Figure 8. Norbornene functionalized alginate system as a cell-laden bioink for extrusion-based bioprinting. (A) Schematic overview of the strategy employed to develop photoactive alginate bioink (Alg-norb) for bioprinting of hydrogels reported in the current work. (B) Scaffolds bioprinted in a) the geometry of a pyramid. b) and c) the geometry of a cube. Porous-like structures can be seen in the cube scaffold shown in d) X-Y and e) Z planes when imaged between two glass coverslips. Of note, the bioprinting conditions used to produce these scaffolds match those optimized for high-cell viability. These scaffolds have shown stability in PBS for over two months. Theoretical side length = 6.9 mm (13 strands, 0.53 mm between strands), total height = 5.2 mm (200 µm/layer, 26 layers). (C) Images of 3D bioprinted hydrogels loaded with cells at a) day 0 and b) day 7. Green and red cell tracker labeled L929 as two different bioinks printed as alternating fibers c) in the X-Y plane and d) in the Z direction.(Ooi et al., 2018)

4 Conclusions and Outlook

Efficient and orthogonal click chemistries are critical for the functionalization of alginate molecules to produce well-defined structures and macromolecules. This review highlighted some exciting perspectives regarding the exploration of click reaction chemistry loaded novel cues in the application of alginate. Click modified alginate and alginate-based systems have been applied in different fields. Alginate and its derived polymers were discussed to demonstrate the feasibility of using click functionalization for various applications, along with their features. Some new types of materials with

novel functionalities, physiochemical stability, mechanical stability, cytocompatibility, and antibacterial properties, can be regulated to significance level with applied perspectives. Those constructs significantly reduce the cost in some applications, such as pharmaceutical, tissue engineering, wounding dressing, and 3D bioprinting.

The future success of alginate or alginate-based systems is mainly dependent on rational structure and properties, designed via click chemistry, and involving chemical, physical, and biological properties. These properties can be adjusted by careful selection of molecules with appropriate molecular weights, regulating the proportion of reactive functional groups, types of functional groups, and constructive mechanism. Multi block copolymers are expected to be synthesized by standard chain extension via click reactions. It is also important for click reaction alginate to optimize fabrication procedures, adopt green preparation process, and improve cytocompatibility. However, unreacted initiators left behind may diffuse out of prepared products, leading to undesirable impacts on humans i.e., toxicity. For the application of hydrogel to cell attachment, improving cell binding and modulating degradation via click chemistry should be investigated. Click chemistry, as the cross-linking reaction, can generate micells for targeted delivery of therapeutic compounds. For 3D bioprinting, tuning viscosity and density of bioink, controlling gelation time, and techniques to obtain high cell survivability, should be studied. Due to continuous investigations into its potential uses, the future role of functionalized alginate and alginate systems remains noteworthy, with new treatment options in future research anticipated.

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6 Conflict of Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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