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REVIEW

Thiol–Norbornene Photoclick Hydrogels for Tissue Engineering Applications

Chien-Chi Lin,[1,2] Chang Seok Ki,[1,3] Han Shih[2]

[1]Department of Biomedical Engineering, Purdue School of Engineering and Technology, Indiana University–Purdue University Indianapolis, Indianapolis, Indiana 46202

[2]Weldon School of Biomedical Engineering, Purdue University, West Lafayette, Indiana 47907

[3]Department of Biosystems and Biomaterials Science and Engineering, Seoul National University, Seoul 151-742, Republic of Korea

Correspondence to: C.-C. Lin (E-mail: lincc@iupui.edu)

ABSTRACT: Thiol–norbornene (thiol–ene) photoclick hydrogels have emerged as a diverse material system for tissue engineering applications. These hydrogels are crosslinked through light-mediated orthogonal reactions between multifunctional norbornene-modified macromers [e.g., poly(ethylene glycol) (PEG), hyaluronic acid, gelatin] and sulfhydryl-containing linkers (e.g., dithiothreitol, PEG–dithiol, biscysteine peptides) with a low concentration of photoinitiator. The gelation of thiol–norbornene hydrogels can be initiated by long-wave UV light or visible light without an additional coinitiator or comonomer. The crosslinking and degradation behaviors of thiol–norbornene hydrogels are controlled through material selections, whereas the biophysical and biochemical properties of the gels are easily and independently tuned because of the orthogonal reactivity between norbornene and the thiol moieties. Uniquely, the crosslinking of step-growth thiol–norbornene hydrogels is not oxygen-inhibited; therefore, gelation is much faster and highly cytocompatible compared with chain-growth polymerized hydrogels with similar gelation conditions. These hydrogels have been prepared as tunable substrates for two-dimensional cell cultures as microgels and bulk gels for affinity-based or protease-sensitive drug delivery, and as scaffolds for three-dimensional cell encapsulation. Reports from different laboratories have demonstrated the broad utility of thiol–norbornene hydrogels in tissue engineering and regenerative medicine applications, including valvular and vascular tissue engineering, liver and pancreas-related tissue engineering, neural regeneration, musculoskeletal (bone and cartilage) tissue regeneration, stem cell culture and differentiation, and cancer cell biology. This article provides an up-to-date overview on thiol–norbornene hydrogel crosslinking and degradation mechanisms, tunable material properties, and the use of thiol–norbornene hydrogels in drug-delivery and tissue engineering applications. © 2014 Wiley Periodicals, Inc. J. Appl. Polym. Sci. **2015**, *132*, 41563.

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INTRODUCTION

Hydrogels are hydrophilic polymeric networks capable of imbibing large quantities of water without dissolving. A typical hydrogel can swell and hold water up to more than 90–99% of its mass. Because of this high degree of swelling, hydrogels are ideal for a variety of biomedical applications.[1] Recent efforts have focused on the use of hydrogels as material platforms for three-dimensional (3D) tissue cultures and for repairing damaged tissues.[2,3] Additionally, hydrogels can serve as carriers for the delivery of synthetic drugs or biological macromolecules (i.e., proteins and nucleotides).[4,5] Both natural and synthetic polymers can be used to fabricate hydrogels, as long as the materials do not elicit adverse biological response. Natural polymers or macromolecules (e.g., collagen, gelatin, laminin, and alginate) often contain bioactive motifs for cell–matrix interactions that are critical in the promotion and maintenance of cell phenotype and function. On the other hand, synthetic polymers, such as poly(ethylene glycol) (PEG), provide controllable material properties (e.g., elasticity, degradability) that may be more beneficial in the fabrication of matrices with desired functions and properties.[6] Taking advantages from both classes of materials, recent work has focused on the synthesis of hybrid hydrogels with both natural and synthetic components.[7,8]

In addition to material selection, the method by which the initially viscous precursor solution crosslinks into an elastic and insoluble hydrogel also affects the performance and utility of the hydrogels. For example, pure collagen and gelatin hydrogels can be prepared by adjusting the temperature of the precursor

Dr. Chien-Chi Lin is an assistant professor in the Department of Biomedical Engineering at Indiana University–Purdue University Indianapolis. He received his Ph.D. in Bioengineering from Clemson University in 2007 and completed his postdoctoral training at the University of Colorado at Boulder in 2010. His main research focus is on the development of light-cured polymeric hydrogels for type 1 diabetes and pancreatic cancer applications. He is also interested in functional liver regeneration and is studying liver hepatitis *in vitro*. He has published 40 peer-reviewed articles (including five book chapters), and his work has been cited approximately 1700 times.

Dr. Chang Seok Ki is an assistant professor in the Department of Biosystems & Biomaterials Science and Engineering at Seoul National University (SNU), South Korea. After receiving his Ph.D. from SNU (2009), he worked at SNU (postdoc, 2009–2010), Amorepacific Corp. (senior researcher, 2010–2012) and Indiana University–Purdue University Indianapolis (postdoc, 2012–2014). His current research focuses on nature-derived biomaterials and hydrogel scaffolds for 3D tissue cultures.

Han Shih is currently a Ph.D. student in the Weldon School of Biomedical Engineering at Purdue University, working in the laboratory of Professor Chien-Chi Lin. She earned her B.S. and M.S. in Biomedical Engineering from Purdue University at Indianapolis in 2010 and 2012, respectively. Her research focuses on the design and synthesis of multiscale hydrogels for pancreatic tissue engineering.

solution, whereas anionic alginate can be gelled by adding divalent cations (e.g., calcium, barium). Some synthetic amphiphilic polymers [e.g., poly(ethylene oxide)–poly(propylene oxide)–poly(ethylene oxide)] can also undergo sol–gel transition upon temperature change. The preparation of these physically gelled hydrogels does not involve chemical reactions, and thus, these hydrogels possess a high degree of cytocompatibility and biocompatibility. However, these purely physical hydrogels can be mechanically weak and may not be ideal for applications where a high mechanical strength is needed. Alternatively, hydrogels can be formed by the covalent crosslinking of soluble polymer chains into insoluble networks that may be more appropriate for applications requiring extended material stability. In general, covalent hydrogels can be formed via either radical-mediated polymerizations or bio-orthogonal click reactions.[9–11] Radical-mediated polymerizations are initiated by radicals that are generated from initiators excited or decomposed by an appropriate initiation energy source, such as photons, heat, redox potential, or enzyme activity. These radical species can propagate across multiple vinyl moieties on macromers. As a result, these chain-growth polymer networks formed by radical-mediated polymerization usually contain heterogeneous and high-molecular-weight crosslinks. Radical-mediated polymerizations are typically fast, and in some cases, the reaction kinetics can be controlled spatiotemporally. Alternatively, covalent hydrogels can also be formed through the crosslinking of mutually reactive macromer species, such as copper-catalyzed azide–alkyne cycloaddition,[12] copper-free azide–cyclooctyne cycloaddition,[13–16] thiol-based Michael-type conjugation,[17–23] norbornene–tetrazine click reaction,[24] oxime-based click reaction,[25–27] native chemical ligation,[28–30] and Diels–Alder reaction.[31] Although these step-growth polymerized networks possess no heterogeneous crosslinks, the crosslinking reaction starts soon after the mutually reactive components are mixed together. Therefore, the crosslinking reactions cannot be controlled spatiotemporally.

Among the commonly used covalent crosslinking methods, photopolymerization is one that permits facile control over the polymerization kinetics because the initiation and termination of crosslinking reaction can be precisely modulated by light irradiation.[32] For example, hydrogels based on vinyl derivatives of PEG (e.g., PEG–acrylate, PEG–methacrylate) or other macromers (e.g., gelatin–methacrylate) prepared by chain-growth photopolymerization have been used in tissue engineering applications for many decades. To circumvent the challenges associated with random chain polymerization while still retaining the benefits of photochemistry, Anseth et al.[33] developed a light- and radical-mediated step-growth polymerization scheme based on the orthogonal reaction between thiol and norbornene. The thiol–norbornene (or thiol–ene) photoclick reaction is one unique polymerization mechanism that combines advantages from both radical-mediated polymerization and a bio-orthogonal click reaction. Thiol–norbornene hydrogels are diverse in gelation and are highly cytocompatible and biocompatible for biomedical applications. The step-growth thiol–norbornene reaction occurs

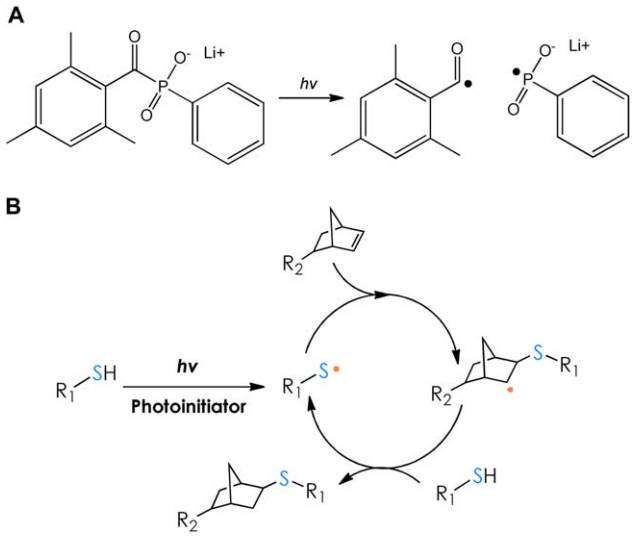

Figure 1. (A) Photocleavage of the type I photoinitiator lithium arylphosphinate into radicals. (B) Schematics of a radical-mediated step-growth thiol–norbornene photoclick reaction with a thiol–containing molecule (R1–SH) and a norbornene-functionalized macromer (R2–norbornene). hv indicates light irradiation. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

in a stoichiometric ratio.[33] It is usually performed in physiologically relevant conditions with a lower radical concentration in the initiation step compared with a chain-growth polymerization process.[34] It minimizes chemical toxicity that may cause undesired damage to the biological components present in the reaction mixture. Furthermore, the biophysical (e.g., crosslinking density) or biochemical (e.g., integrin-binding motifs) properties in the thiol–norbornene hydrogel can be modified with additional light exposure in the presence of cells.[33] This unique light-dependent feature permits the dynamic modification of material properties for guiding the cell fate process in a spatiotemporally regulated manner. In this review article, the gelation mechanisms of radical-mediated thiol–norbornene hydrogels are first described. The chemistry of norbornene-functionalized macromers [e.g., PEG, hyaluronic acid (HA), and gelatin] and the hydrogel network properties (e.g., factors affecting hydrogel crosslinking and degradation) are also reviewed. In the final part of the review, we summarize the applications of thiol–norbornene hydrogels in tissue engineering, including drug delivery, two-dimensional (2D) cell cultures, and 3D cell encapsulation.

THIOL–NORBORNENE PHOTOPOLYMERIZATION MECHANISMS

The gelation and *in situ* cell encapsulation of thiol–norbornene hydrogels were first reported with long-wave UV light irradiation[33] and a type I (cleavage-type) photoinitiator, such as Irgacure-2959 or lithium arylphosphinate [Figure 1(A)].[35] Mechanistically, UV light decomposes cleave-type photoinitiators into radicals. The later abstracts protons from sulfhydryl groups to form thiyl radicals, which react with strained vinyl bonds in the norbornene moiety on functionalized poly(ethylene glycol)–tetra-norbornene (PEG4NB). This reaction creates a thiol–ether bond and a carbon-centered radical capable of generating another thiyl radical. The alternate thiol–norbornene coupling and thiyl radical generation proceeds in a stoichiometric ratio until the limited moiety (thiol or norbornene) is depleted [Figure 1(B)]. Unlike the crosslinking of acrylate-based hydrogels, there is no homopolymerization between norbornene groups. Hence, the thiol–norbornene hydrogel network possesses only orthogonal crosslinks and an idealized structure.[33] Although network defects (e.g., unreacted norbornene/thiol, intramolecular cycles that do not contribute to crosslinking density) do exist in thiol–norbornene hydrogels, the degree of network heterogeneity is at a minimum compared to random chain-growth (i.e., acrylate-based) polymerization.[36] Furthermore, the thiol–norbornene reaction is not oxygen-inhibited and can be initiated several orders of magnitude faster than acrylate-based chain-growth photopolymerization.[37] As a result, thiol–norbornene gelation can be achieved within seconds in aqueous solutions without the use of high concentrations of macromer or photoinitiator.[34] Because of the orthogonal reactivity between norbornene and thiol and the lack of homopolymerization between strained norbornene groups, additional thiol–norbornene photoconjugation can be used to modify network properties.[33] Cysteine (Cys)-containing peptides or thiolated proteins can be conjugated in thiol–norbornene hydrogels formed with an off-stoichiometric ratio (i.e., the thiol-to-norbornene molar ratio less than 1 but higher than the critical ratio for gelation). This additional light-mediated thiol–norbornene conjugation can be performed in the presence of encapsulated cells; this allows one to dynamically control the biochemical microenvironment of the cell-laden hydrogels.

As reported in our recent publications, visible light can also be used to initiate thiol–norbornene photopolymerization and form hydrogels with different architecture, including bulk gels, microgels, and multilayer gels.[38–40] The mechanism of the visible-light-initiated thiol–norbornene reaction is similar to that of the UV-based system, except that a type II (noncleavage-type) photoinitiator (e.g., eosin-Y, rose bengal) is used. Upon visible light ($400\ \text{nm} < \lambda < 700\ \text{nm}$) exposure, eosin-Y is excited; this causes the abstraction of hydrogen from a coinitiator and the generation of secondary radicals. In this visible-light-mediated gelation, multifunctional thiol-bearing molecules serve as both hydrogel crosslinkers and coinitiators in the initiation step. Although the initiation efficiency of visible-light-based thiol–norbornene photopolymerization is not as high as its UV-based counterpart, visible-light-based thiol–norbornene photopolymerization may be more appropriate for certain applications where the use of UV light is undesirable.

MACROMERS DEVELOPED FOR THE FORMATION OF THIOL–NORBORNENE HYDROGELS

Synthetic Macromer-Based Thiol–Norbornene Hydrogels

The majority of radical-mediated thiol–norbornene hydrogels are prepared by PEG-based macromers. PEG is a hydrophilic and nonfouling macromer commonly used in hydrogel fabrication. To form PEG-based thiol–norbornene hydrogels, one can modify the hydroxyl end groups of PEG with norbornene or thiol. Anseth et al.[33] functionalized multi-arm PEG with 5-

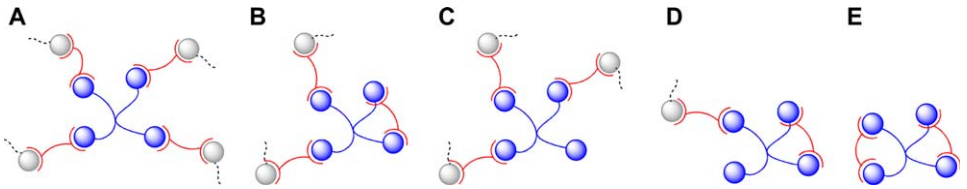

Figure 2. Schematics of the potential products after step-growth thiol–norbornene reactions between tetrafunctional PEGNB and bifunctional DTT: (A) defect-free, fully crosslinked thiol–norbornene network; (B) fully reacted thiol–ene network with a primary cycle; (C) partially crosslinked thiol–ene network with an unreacted, dangling PEGNB arm; (D) dangling polymer with a primary cycle and an unreacted PEGNB arm; and (E) soluble polymer with fully reacted functional groups. D and E did not contribute to network crosslinking. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

norbornene-2-carboxylic acid with dicyclohexylcarbodiimide as the coupling reagent and 4-dimethylaminopyridine as the catalyst. This chemistry yields hydrolytically labile ester linkages between the PEG backbone and norbornene. Therefore, the thiol–norbornene hydrogels formed with this particular chemistry may be hydrolytically degraded when it is in contact with an aqueous solution.[36] To increase the hydrolytic stability of the PEGNB macromer, we reported the preparation of amide-tethered poly(ethylene glycol)–norbornene (i.e., PEGaNB) by reacting amine-terminated PEG with norbornene acid.[41] Because the amide bond is more resistant to hydrolysis than ester bond, thiol–ene hydrogels prepared from PEGaNB are more resistant to hydrolytic degradation than those prepared from ester-based poly(ethylene glycol)–norbornene (i.e., PEGeNB), Cui and coworkers[42,43] combined linear PEGNB with poly(dimethyl siloxane)–norbornene and used tetrafunctional PEG-thiol (PEGSH) as a crosslinker for UV-light-mediated thiol–norbornene photopolymerization. The resulting hydrogels were highly elastic and strong and should be useful for applications requiring high resilience and strength.

HA-Based Thiol–Norbornene Hydrogels

HA is a nonsulfated glycosaminoglycan found in the extracellular matrix. HA is a natural ligand for CD44 and has been modified with various functional groups suitable for preparing hydrogels with inherent bioactivity, biocompatibility, and biodegradability (by hyaluronidase). For example, HA–methacrylate can be homopolymerized by means of chain-growth photopolymerization.[44] HA–methacrylate can also be crosslinked with bifunctional thiols (e.g., dithiothreitol (DTT) or biscysteine peptides) to form Michael-type hydrogels[45] or can be processed to become macroporous hydrogels.[46] The Burdick group has recently prepared norbornene-functionalized hyaluronic acid (NorHA), which was used to prepare photopatternable HA-based thiol–norbornene hydrogels.[47] To prepare NorHA, HA was converted to tetrabutyl ammonium salt; this was followed by a reaction with 5-norbornene-2-carboxylic acid in anhydrous dimethyl sulfoxide for 20 h at 45°C in the presence of 4-dimethylaminopyridine and di-*tert*-butyl dicarbonate (Boc2O). The synthesized NorHA could be crosslinked by DTT via the same step-growth radical-mediated photopolymerization. Similar to the PEG-based thiol–ene hydrogels, NorHA–DTT hydrogels were cytocompatible and photopatternable. Cell-adhesive ligands (e.g., Cys–Arg–Gly–Asp–Ser or CRGDS) were required in this system to support cell adhesion.[47]

Gelatin-Based Thiol–Norbornene Hydrogels

Gelatin is a form of denatured collagen that has gained increasing popularity in hydrogel fabrication because of its inherent cell affinity and protease degradability. At a low concentration or high temperature, gelatin can be dissolved easily in aqueous solution. Sol–gel transition occurs when the temperature of the gelatin solution is below a critical value (gelatin-concentration-dependent) at physiological pH. Chemical modification or crosslinking is required to prepare covalently crosslinked gelatin hydrogels. The most commonly used chemistry for crosslinking gelatin is the reaction of primary amines of gelatin with glutaraldehyde. However, glutaraldehyde is highly cytotoxic and cannot be used in the presence of cells. To improve the cytocompatibility of gelatin crosslinking, various chemistries have been explored. Similar to HA-based hydrogels, gelatin can also be methacrylated (GelMA) and homopolymerized via chain-growth photopolymerization or crosslinked by multifunctional thiols through Michael-type addition.[48] Gelatin can also be thiolated and crosslinked with PEG-diacrylate (PEGDA) to

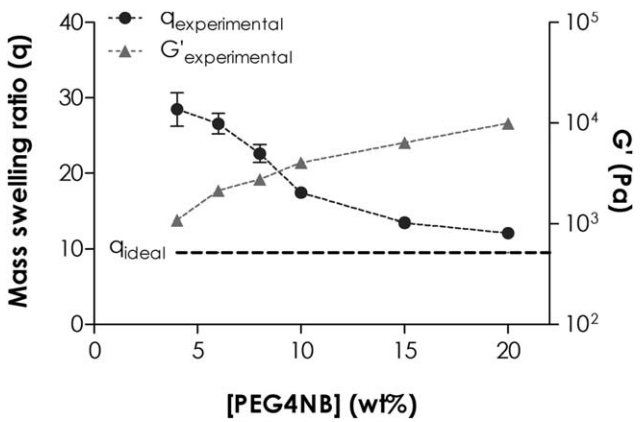

Figure 3. Effect of the PEG4NB macromer concentration on thiol–ene hydrogel equilibrium swelling (left *y* axis) and elastic modulus (right *y* axis). The swelling ratio of an ideal network was calculated on the basis of the molecular weight between crosslinks of given macromer molecular weights (molecular weight of PEG4NB = 20 kDa, molecular weight of DTT = 154 Da) and functionalities (functionality of PEG4NB = 4, functionality of DTT = 2). The ideal swelling ratio (q_{ideal}) was calculated on the basis of the known macromer structure and Flory–Rehner theory (q: swelling ratio; G′: shear modulus). Reprinted with permission from 36. Copyright 2012 American Chemical Society.

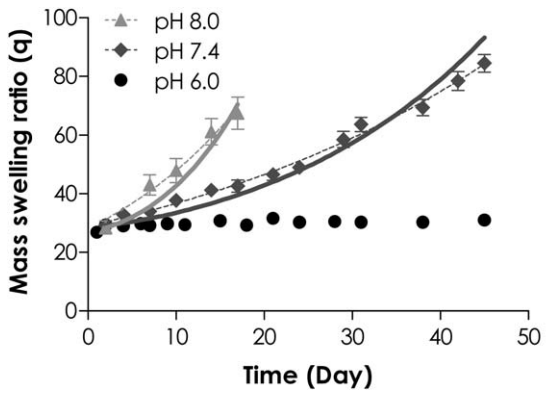

Figure 4. Effect of the buffer pH on the mass swelling ratio of the PEG4NB–DTT hydrogels (4 wt %). The symbols represent the experimental data, whereas the dashed curves represent the exponential curve fitting to the experimental data. The apparent degradation rate constants for the gels degraded at pH 7.4 and 8.0 were 0.024 ± 0.001 and 0.057 ± 0.002, respectively, on day 1. The solid curves represent model predictions with best fit kinetic rate constants: k' of pH 7.4 = 0.011 on day 1 and k' of pH 8.0 = 0.027 on day 1. No curve fitting or model prediction was made for the gels degraded at pH 6.0 due to the stability of the gels under acidic conditions. Reprinted with permission from 36. Copyright 2012 American Chemical Society.

form covalent hydrogels.[49] Our group recently developed a norbornene-functionalized gelatin (GelNB) for fabricating gelatin-based thiol–norbornene hydrogels.[50] Norbornene was functionalized on gelatin through a reaction with carbic anhydride in aqueous buffer with basic pH. The reaction proceeded for 3 days; this was followed by another 3 days of dialysis to obtain a moderate degree of functionalization (~50%). GelNB can be crosslinked with bifunctional DTT or tetrafunctional PEG4SH. The cytocompatibility of GelNB hydrogels was verified with the *in situ* encapsulation of human mesenchymal stem cells (hMSCs). Compared with the chain-growth GelMA hydrogels, the step-growth GelNB hydrogels afforded a higher degree of intercellular connectivity and supported faster cell spreading.

NETWORK PROPERTIES OF THIOL-NORBORNENE HYDROGELS

Crosslinking, Swelling, and Network Ideality of Thiol–Norbornene Hydrogels

In contrast to a chain-growth hydrogel network (e.g., PEGDA or GelMA hydrogel) in which the crosslinks are composed of a random number of homopolymerized (meth)acrylates, all crosslinks in a step-growth hydrogel only contain a single covalent bond. In the case of a radical-mediated thiol–norbornene hydrogel, the crosslinks are thioether bonds [Figure 1(B)]. Unlike the chain-growth polymerization of acrylate-based macromers, the initiation of radical-mediated thiol–norbornene photopolymerization is not inhibited by oxygen.[33] Therefore, the gel point of this step-growth gelation in an aqueous solution is several orders of magnitude faster than the chain-growth photopolymerization at equivalent functional group concentrations.[37] When compared with other step-growth hydrogels formed by Michael-type conjugation addition at identical macromer compositions, radical-mediated thiol–norbornene gelation also exhibits faster gelation.[36] After reaching complete functional group conversion, the step-growth thiol–norbornene hydrogels should contain a high degree of network ideality because of the orthogonal thiol–norbornene reaction [Figure 2(A)]. The network ideality here refers to a fully crosslinked polymer network without structural defects, such as intramolecular primary cycles [Figure 2(B)], unreacted functional groups [Figure 2(C)], dangling polymers [Figure 2(D)], or complete soluble polymers [Figure 2(E)]. In an ideal and fully crosslinked step-growth network with fixed macromer functionality and molecular weight, the hydrogel crosslinking density and its equilibrium swelling should be a fixed value regardless of macromer concentration.[36] Practically, however, network defects do occur that can affect the structural ideality of the hydrogels. The existence of unreacted functionality [Figure 2(B)], primary cycles [Figure 2(C)], and/or dangling polymers [Figure 2(D)] reduces the overall crosslinking density of thiol–norbornene hydrogels. These phenomena are more likely to occur at lower macromer concentrations.[51] Therefore, macromer concentration-dependent hydrogel swelling is observed experimentally (Figure 3).[36] Thiol–norbornene hydrogels with lower swelling ratios have higher elastic moduli, and this inverse relationship is also observed in chain-growth polymerized hydrogel networks.[52]

Degradation of Thiol–Norbornene Hydrogels Induced by Hydrolysis

The reaction of hydroxyl-terminated PEG with norbornene acid yields a macromer PEGeNB that is hydrolytically labile.[33,36,41] The hydrolysis of ester bonds eventually leads to the hydrolytic degradation of step-growth thiol–norbornene hydrogels, which can be described with the following pseudo-first-order degradation kinetics:[36,51]

$$[\text{Ester}] = [\text{Ester}]_0 e^{-k'[OH^-]t}$$

where [Ester] and $[\text{Ester}]_0$ are the concentrations of ester bonds on the PEG backbone at any time during degradation and at time zero before degradation, respectively; k' is the degradation rate constant, $[OH^-]$ is the concentration of hydroxyl ions in the degradation medium, and t is the time of degradation. The hydrolytic degradation of thiol–norbornene hydrogels depends largely on the pH value of the surrounding solution. For example, PEG4NB–DTT hydrogels incubated under acidic conditions (pH 6.0) had an almost constant swelling ratio over the course of a 45-day incubation, whereas hydrogels with the same compositions exhibited increasing swelling over time under slightly basic conditions (pH 7.4 and 8.0; Figure 4).[36] Figure 4 also shows that the degradation profiles agreed with the prediction with the pseudo-first-order degradation kinetics described previously.

In addition to the influence of pH, thiol–norbornene hydrogel degradation is also affected by the macromer concentration. At a higher macromer concentration (i.e., in a hydrogel with a lower swelling ratio), thiol–norbornene hydrogels degraded much more slowly compared to hydrogels crosslinked at a lower macromer concentration.[36] This might have been a result of the decreased accessibility of water molecules to the crosslinks. The hydrolytic degradation rate of thiol–norbornene hydrogels is

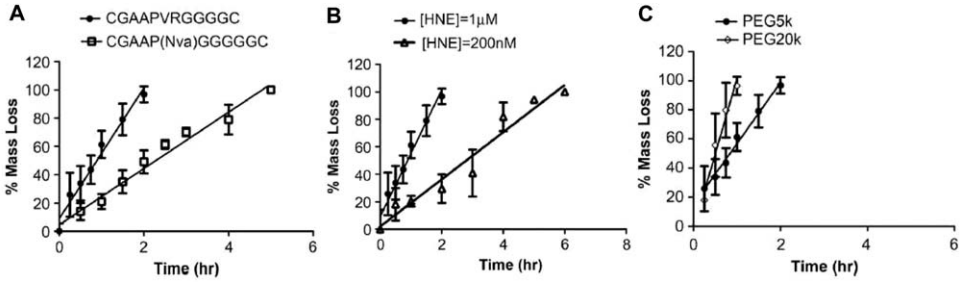

Figure 5. Profiles of the thiol–norbornene hydrogel mass loss upon exposure to HNE. The (A) influence of the HNE-sensitive peptide substrate (CGAAPVRGGGGC: peptide with fast cleavage rate; CGAAP(Nva)GGGGGC: peptide with slow cleavage rate. Nva is a non-natural amino acid residue.), (B) concentration of HNE, and (C) molecular weight of PEGNB were experimentally studied (PEG5k: PEGNB 5kDa; PEG20k: PEGNB 20kDa). All of gels were made of 10 wt % PEG macromer and were exposed to 1 μM HNE (unless otherwise noted). Reprinted with permission from 37. Copyright 2009 Elsevier.

further affected by the sequence of biscysteine-bearing peptide linkers. For example, hydrogels crosslinked by peptides containing aromatic [e.g., Cys–Gly–Gly–Tyr–Cys or CGGYC] or hydrophobic (e.g., Cys–Gly–Gly–Leu–Cys or CGGLC) residues yielded slower degradation rates compared with gels crosslinked by peptide linkers without side groups (e.g., Cys–Gly–Gly–Gly–Cys or CGGGC). The steric hindrance and hydrophobic effect of amino acid side groups might retard the degradation rate.[36]

Degradation Induced by Linker Cleavage: Acts of Exogenously Added Stimuli

Thiol–norbornene hydrogels can be degraded by exogenously applied stimuli, including enzyme and additional light exposure. If protease-sensitive peptides are used as part of the hydrogel crosslinkers, gels can be degraded enzymatically when they are placed in a solution containing the protease of interest. The termini of the peptide crosslinkers are usually Cys residues that provide sulfhydryl groups for the orthogonal thiol–ene photoclick reactions. The protease sensitivity of thiol–norbornene hydrogels can be tailored by the alteration of the sequence of protease-sensitive peptide crosslinkers. Theoretically, if protease diffusion is faster than its enzymatic reaction, hydrogel degradation will proceed in a bulk degradation fashion when the protease is added exogenously to the solution. On the other hand, if the rate of peptide linker cleavage is faster than the rate of protease infiltration into the hydrogels, the hydrogels are eroded from the surface. Because most proteases are proteins with molecular weights on the order of a few tens of kilodaltons, rarely will the timescale for protease diffusion into hydrogels be faster than the timescale of substrate cleavage. Hence, when a protease is added exogenously to induce hydrogel degradation, surface erosion will most likely be the mode of gel degradation.[53] Because the erosion of the hydrogel starts from the surface, protease-induced surface erosion causes the loss of hydrogel mass linearly as a function of time. Aimetti et al.[53] exploited this feature to fabricate human neutrophil elastase (HNE)-responsive thiol–norbornene hydrogels [PEG4NB crosslinked by HNE-sensitive peptide CGAAPV↓RGGGGC (arrow indicates protease cleavage site)] for delivering protein therapeutics. The rate of protease-induced hydrogel mass loss could be tailored by the substitution of a nonnatural amino acid residue in the peptide sequence [Figure 5(A)], by the supplementation of protease at difference concentrations [Figure 5(B)], or by changes in the molecular weight of the PEG4NB macromer [Figure 5(C)]. As the hydrogel network was degraded in a surface erosion manner, the otherwise entrapped proteins were released only in the presence of HNE. Our laboratory also used a similar strategy to liberate pancreatic β-cell aggregates generated in thiol–norbornene hydrogels.[37] Specifically, a chymotrypsin cleavable peptide (i.e., CGGY↓C) was used as the gel crosslinker. When the cell-laden hydrogels were placed in a buffer containing chymotrypsin, the erosion of gel led to liberation of cell clusters that could be used in biological analyses or applications.

There is a growing interest in the development of biomimetic materials with dynamically adaptable gel properties postgelation.[8,9,15] For example, macromers with photolabile groups can be used to fabricate hydrogels with dynamically tunable crosslinking densities. To introduce tunable gel biomechanical (i.e., the reduction in the gel crosslinking density) and/or biochemical properties (e.g., the removal of pendent bioactive motifs) through additional light-mediated bond cleavage, we recently reported the synthesis of photodegradable step-growth thiol–norbornene hydrogels by visible-light-initiated thiol–ene photoclick reactions.[54] A visible light source (wavelength = 400−700 nm) was used to excite photosensitizer eosin-Y, which generated thiyl radicals from the biscysteine peptide linker incorporated with a photolabile amino acid, L-2-nitrophenylalanine. Upon exposure to UV light (302 or 365 nm), hydrogels were degraded because of the photolysis of the peptide linker. The rate of hydrogel degradation was easily manipulated by the wavelength and UV light intensity. This new step-growth hydrogel system preserved the favorable properties offered by photochemistry, including photopolymerization and photodegradation.

Although radical-mediated thiol–ene hydrogels can be designed to degrade via hydrolysis, proteolysis, or photolysis, it is also possible to combine several degradation mechanisms into one hydrogel matrix. For instance, a simple dual-mode enzymatic and hydrolytic degradable hydrogel was created without the alteration of the hydrogel molecular structure or hydrophilicity.[36] Through a variation in the ratio between the noncleavable crosslinker CGGGC and a chymotrypsin-sensitive peptide crosslinker CGGY↓C at different compositions, the degradation of these thiol–norbornene hydrogels could be tuned from purely surface erosion to bulk degradation upon exposure to the same concentration of a chymotrypsin-containing solution. Hydrogels containing a high percentage of CGGYC crosslinker (75−100%)

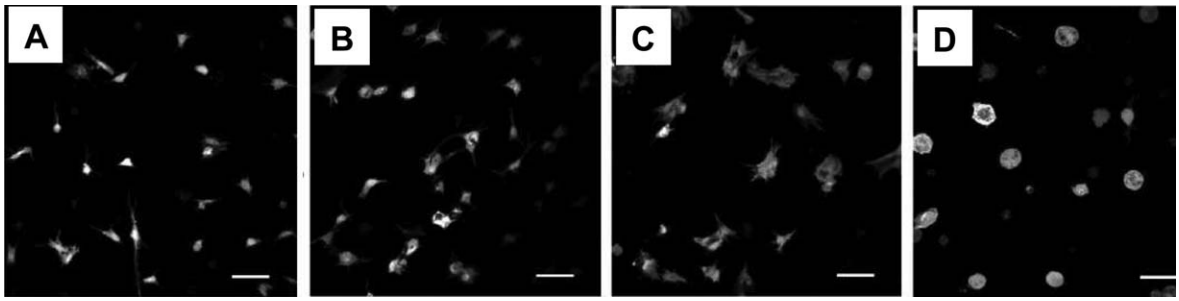

Figure 6. Morphology of the hMSCs encapsulated in MMP-degradable thiol–norbornene hydrogels. The encapsulated hMSCs were cultured in (A) growth, (B) osteogenic, (C) chondrogenic, and (D) adipogenic media and were stained with Calcein AM after 14 days of culturing (imaged by confocal microscopy; the scale bar represents 50 μm). Reprinted with permission from 55. Copyright 2011 Elsevier.

were eroded in a surface erosion mechanism. When the percentage of CGGYC decreased, the degradation mode transitioned to a bulk degradation mechanism. These diverse degradation behaviors may be useful for the dynamic control of growth factor or drug delivery.

Degradation Induced by Linker Cleavage: Acts of Local Cellular Activity

Thiol–norbornene hydrogels can be used to study the influence of the local matrix environment on the cell fate processes (e.g., growth, differentiation, migration, invasion) because many of these processes require a protease-induced matrix cleavage. One important class of protease relevant to these cell fate processes is matrix metalloproteinase (MMP). MMPs are not only essential in normal cell development but are also responsible for many malignant cell behaviors, including inflammation and tumor progression. The imbalance between the activities of MMPs and their inhibitors (e.g., tissue inhibitors of metalloproteinases) are the main cause of many diseases. To construct a relevant cellular microenvironment for studying the critical influence of MMPs on cell fate processes, thiol–norbornene hydrogels are often prepared by the crosslinking of a multi-arm PEGNB and MMP-sensitive peptide linker.[33,56] A typical example is the use of a peptide crosslinker containing the sequence CGPQG↓IWGQC. When cells are encapsulated within hydrogels containing an MMP-sensitive linker, they can remodel their local microenvironment to accommodate cellular activities, such as migration, proliferation, matrix deposition, or other MMP-related intracellular signaling events. For example, Anseth et al.[55] developed a synthetic hydrogel niche for understanding hMSC proliferation, morphogenesis, and differentiation in thiol–norbornene hydrogels containing different compositions of MMP-sensitive peptide crosslinkers (Figure 6). We also reported a similar hydrogel platform for studying the influence of the local matrix conditions on the growth, morphogenesis, invasion, and drug responsiveness in pancreatic cancer cells.[57–59] Unlike the release of proteins and drugs via a surface erosion mechanism caused by exogenously added proteases, the degradation of thiol–norbornene hydrogels caused by cell-secreted proteases is mostly a local event because of the short-range action of proteases. However, these short-range protease activities do cause a reduction in the hydrogel crosslinking density[57] and, hence, bulk gel mechanical properties, given that sufficient amount of peptide linkers is cleaved.

THIOL–NORBORNENE HYDROGELS IN TISSUE ENGINEERING APPLICATIONS

Controlled Delivery

In the design of hydrogels for protein delivery, two issues are at the forefront of design criteria, namely, protein bioactivity and bioavailability.[4,5] Thiol–norbornene hydrogels are ideal for controlled protein delivery because of their mild and diverse gel crosslinking process, hydrophilic network structure, and tunable permeability. In regard to the preservation of protein bioactivity, McCall and Anseth[34] compared the stability and release of encapsulated proteins [i.e., lysozyme and transforming growth factor β (TGFβ)] within and from chain-growth PEGDA hydrogels and step-growth PEG-based thiol–norbornene hydrogels under the same UV light exposure conditions. They found that step-growth thiol–norbornene hydrogels delivered proteins in their bioactive form. On the other hand, a significant bioactivity loss was observed in chain-growth PEGDA hydrogels. The authors attributed the enhanced protein recovery from the thiol–norbornene hydrogels to the fact that thiol–norbornene photopolymerization was not susceptible to oxygen inhibition. Therefore, the hydrogel formed more rapidly under a lower initiator concentration. Full protein bioactivity was observed when TGFβ was photoencapsulated and delivered from thiol–norbornene hydrogels.

In addition to the preservation of protein bioactivity, PEG-based thiol–norbornene hydrogels can also be designed to exhibit an affinity for therapeutically relevant growth factors. For example, Murphy and coworkers[60–65] prepared thiol–norbornene microgels with multi-arm PEGNB macromers and dithiol-containing linkers from an aqueous two-phase separation system. These microgels also were immobilized with affinity peptides capable of sequestering vascular endothelial growth factors (VEGFs). With increasing affinity peptide concentration, VEGF was sequestered in the microgels for a prolonged period of time and was delivered slowly from the microgels. Although this affinity strategy has been demonstrated in chain-growth PEGDA hydrogels for protein sequestration and sustained release, thiol–norbornene hydrogels afford an idealized hydrogel network that may present less nonspecific interactions/reactions between the encapsulated proteins and polymer network and may yield more predictable protein–ligand affinity binding and release results.

PEG-based thiol–norbornene hydrogels have been used as protease-responsive drug-delivery matrices. As described previously,

Aimetti et al.[53] constructed an HNE-sensitive hydrogel using PEG4NB and an HNE cleavable peptide crosslinker. They encapsulate bovine serum albumin as a model drug and found that the zero-order release of bovine serum albumin was enabled by HNE-mediated hydrogel erosion. This system might serve as a depot for delivering anti-inflammatory proteins at an injury site. Yang et al.[66] developed an alternative strategy for MMP-responsive dexamethasone (Dex) delivery. They conjugated Dex to an MMP-sensitive peptide (i.e., Dex–KGPQG↓IAGQCK) containing an additional Cys for thiol–norbornene mediated peptide immobilization. The hydrogel was formed by the reaction of PEG4NB and an MMP-cleavable peptide (KCGPQG↓IAGQCK). The release of Dex was controlled via the local cleavage of the peptide by MMP secreted from the encapsulated hMSCs. Benoit et al.[67] prepared thiol–norbornene hydrogels crosslinked by an elegantly designed protease-sensitive peptide linker containing therapeutic peptide sequences. Peptide drugs could only be liberated in the presence of nearby cellular activity. This strategy could potentially provide a new delivery mechanism for the localization of peptide drugs and to decrease the clearance rate of small therapeutics.

2D Cell Culture Substrate

Hydrogels are ideal substrates for studying the influence of the matrix stiffness on the cell fate because their crosslinking density, and hence, the stiffness of hydrogels can be easily controlled by the adjustment of the polymerization conditions.[3] For hydrogels that do not present a cell binding motif, cell-adhesive ligands (e.g., fibronectin, laminin, or RGDS peptide) are often immobilized during network crosslinking or postgelation modification. Thiol–norbornene hydrogels are particularly useful in this endeavor because their modular crosslinking nature allows independent control over the substrate stiffness (by the alteration of PEGNB formulations) and biochemical properties (with different dithiol crosslinkers).[59,68] For example, Gould et al.[69] used thiol–norbornene hydrogels crosslinked by a multi-arm PEGNB and biscysteine-containing peptide linkers (MMP-sensitive or insensitive) to evaluate the influence of the matrix stiffness and integrin binding on the matrix production from valvular interstitial cells (VICs). They further explored the correlations between paracrine signaling and substrate stiffness using cocultured valvular endothelial cells and VICs.[70] Similar thiol–norbornene hydrogels were used to study the dependency of integrin signaling on the substrate elasticity in the contexts of the osteogenic differentiation of hMSCs,[71] drug responsiveness in melanoma cells,[72] and phenotypic variations of tumorigenic versus nontumorigenic human mesenchymal cells.[73]

3D Cell Encapsulation

The crosslinking process of thiol–norbornene hydrogels (either initiated by long-wave UV or visible light exposure) is mild and can be performed in the presence of mammalian cells. This cytocompatible nature, together with its diversity in gel crosslinking, affords a material platform for 3D cell cultures. Furthermore, because norbornene is not reactive without the presence of active radical species, thiol–norbornene hydrogels with excess norbornene moieties can be used as dynamic culture platforms where thiolated bioactive cues can be added postgelation to alter cell fate behaviors.[33] Similar to the 2D studies described previously, modularly crosslinked thiol–norbornene hydrogels are ideal for studying the effects of an individual parameter on specific cell behaviors. Earlier work by Benton et al.[56] demonstrated the cytocompatibility of thiol–norbornene hydrogels in the 3D culturing of VICs. They used an MMP-sensitive peptide crosslinker (GPQG↓IWGQ) to permit the cell-mediated remodeling of extracellular microenvironment. Gould and Anseth[74] further compared the variation of VIC phenotypes in the presence of different integrin-binding peptides immobilized within the hydrogel network. Anderson et al.[55] encapsulated hMSCs in thiol–norbornene hydrogels with different degrees of MMP sensitivity and studied the impact of matrix compositions on osteogenic, adipogenic, and chondrogenic differentiation of 3D hMSCs. Kyburz and Anseth[75] examined the motility of hMSC in an MMP-degradable PEGNB hydrogel with various crosslinking densities and showed that a lower crosslinking density and a higher RGD concentration facilitated 3D hMSC migration and spreading.[75] Mariner et al.[76] studied the influence of microRNA on the osteogenic differentiation of 3D hMSCs. Focused on cartilage tissue engineering, Bryant et al.[77] recently reported the improved retention of chondrocyte-secreted proteoglycans within thiol–norbornene hydrogels prepared from PEGNB, PEG–dithiol, and a pendant HA-binding peptide. Thiol–norbornene hydrogels have also been exploited as a material platform for understanding 3D protease activity. For example, Leight et al.[78] developed a material tool to measure MMP activity in encapsulated hMSCs. An otherwise quenched fluorescent MMP-sensitive peptide was used as a gel crosslinker to reveal the local protease activity as cells underwent 3D morphogenesis. Schultz and Anseth[79] used a multiple-particle tracking technique to investigate the sol–gel transition and protease-mediated degradation of 3D matrices.

Thiol–norbornene hydrogels are highly cytocompatible because the orthogonal crosslinking reactions are mild and require only a fraction of radicals of those used in the chain-growth polymerized hydrogels (e.g., PEGDA hydrogels). Our group evaluated the cytocompatibility of a PEG-based thiol–norbornene hydrogel using a radical-sensitive pancreatic β-cell line, MIN6, and concluded that the orthogonal thiol–ene photoclick reaction was more cytocompatible than chain-growth PEGDA hydrogels at equivalent reactive macromer functionality.[37,80] The inherent degradability of the PEGNB macromer (because of the ester bond hydrolysis) also has been shown to increase the proliferation and spreading of hMSCs.[41] Roberts and Bryant[81] encapsulated bovine chondrocytes in chain-growth PEGDA and step-growth thiol–norbornene hydrogels and showed that the latter promoted hyaline-like cartilage production with positive staining for aggrecan and collagen II, especially when the cell-laden hydrogels were cultured under mechanical loading. Sridhar et al.[82] immobilized TGF-β in a thiol–norbornene hydrogel network to enhance glycosaminoglycan secretion from the encapsulated chondrocytes. In other studies, embryonic stem cell-derived motor neurons were encapsulated in MMP-sensitive thiol–norbornene hydrogels, and their axon extension improved.[83] Nguyen et al.[84] evaluated the effects of various bioactive cues on the capillary network formation of human umbilical vein endothelial cells using thiol–norbornene hydrogel arrays. Additionally, Mariner et al.[85]

prepared recombinant human bone morphogenic protein 2 (rhBMP-2)-loaded thiol–norbornene hydrogels and observed improved bone fracture healing in a rat critical size bone defect animal model.

The 3D culturing of cancer cells is an emerging research area in tissue engineering. Cancer cells cultured in 3D resemble tumor tissues better than those cultured on 2D tissue culture plastic. Various cancer cells have been encapsulated and cultured in 3D matrices, including thiol–norbornene hydrogel. For example, Schwartz and coworkers[86,87] and Singh et al.[88] cultured human fibrosarcoma cells (HT-1080) in an MMP-degradable thiol–norbornene hydrogel and studied the 3D HT-1080 cell migration mechanisms. Wang et al.[89] established a brain tumor model by encapsulating glioblastoma cells (U87) in an HA-entrapped eight-armed PEGNB hydrogel crosslinked with MMP-sensitive crosslinkers. Our recent studies showed that hepatoma cells (i.e., Huh7, HepG2) encapsulated in thiol–norbornene hydrogels exhibited a drastic improvement in hepatocyte-specific functions and gene expression.[90] Furthermore, we also studied the growth and morphogenesis of pancreatic cancer cells (i.e., PANC-1) in thiol–norbornene hydrogels with independently adjusted matrix compositions, including the matrix stiffness, protease-sensitive peptide linkers, and integrin-binding ligands.[59] The effect of the epidermal growth factor receptor inhibition on the PANC-1 cell drug resistance was also examined with MMP-sensitive PEGNB hydrogels with different stiffnesses.[58] With a similar material design principle, the effects of cytokines [TGFβ and epidermal growth factor (EGF)] and collagen 1 on pancreatic cancer cells (i.e., COLO-357) fate, including proliferation, invasion, and drug resistance, were evaluated in an orthogonally controlled manner. This study also revealed that certain cancer stem cell markers (e.g., sonic hedgehog, CD24, and VEGF) were upregulated simply because the cancer cells were cultured in a 3D matrix.[57]

CONCLUSIONS AND OUTLOOK

Because its invention in 2009,[33] radical-mediated thiol–norbornene hydrogels have emerged as an attractive class of biomaterial for tissue engineering and regenerative medicine applications. Either long-wave UV light or visible light could be used to initiate the photocrosslinking of thiol–norbornene hydrogels.[33,38] These hydrogels have been prepared as tunable substrates for 2D cell cultures, as microgels or bulk gels for affinity-controlled or protease-responsive drug delivery, and as scaffolds for *in situ* cell encapsulation and for 3D cell cultures. In terms of applications, thiol–norbornene hydrogels have been exploited for valvular and vascular tissue engineering, musculoskeletal tissue regeneration, and cancer cell biology. It is expected that new applications will continue to emerge in the near future for thiol–norbornene hydrogels as these gels are diverse in terms of gelation mechanisms and have excellent cytocompatibility.

One area that can be further developed is the postgelation modification capability of the thiol–norbornene reaction. Fairbanks al.[33] first demonstrated this unique feature through the preparation of thiol–norbornene hydrogels with an off-stoichiometric thiol-to-norbornene ratio (i.e., with excess norbornene groups during photopolymerization). They photopatterned a CRGDS peptide in the cell-laden hydrogel by incubating the gel in a medium containing a soluble peptide and additional photoinitiator, followed by a secondary light-mediated thiol–norbornene conjugation. The secondary thiol–norbornene photoclick reaction imparted cell adhesiveness in the otherwise inert PEG-based hydrogels. Theoretically, it is also possible to perform the postgelation conjugation of whole proteins within cell-laden hydrogels, as long as the protein of interest contains free sulfhydryl groups for a secondary thiol–norbornene reaction. However, the slow diffusion of macromolecular proteins, disulfide bond formation, and the binding of infiltrated proteins to cell surface receptors before light-mediated protein immobilization may affect the efficacy of this approach. The slow diffusion of macromolecular proteins within crosslinked hydrogels can be resolved by the introduction of a macroporous structure in bulk thiol–ene hydrogels. The selective caging chemistry could be used to prevent the undesired reactions of thiol groups. Photolabile chemical bonds sensitive to light with orthogonal wavelengths could be designed for the light-mediated uncaging of thiol-protected groups and for secondary thiol–norbornene photoclick reactions. Cell-laden thiol–norbornene hydrogels can also be designed to stiffen overtime in a user-defined manner. Matrix stiffening is induced through additional thiol–norbornene crosslinking with a similar experimental process as described previously for postgelation peptide conjugation. This will be highly valuable in the study of matrix mechanics on cellular fate, such as tissue fibrosis or tumor progression.

Another unique feature of thiol–norbornene hydrogels that can be further explored is the visible-light-mediated gelation with noncleavage-type photoinitiators, such as eosin-Y and rose bengal.[38] The use of visible light to crosslink hydrogels is arguably more cytocompatible than the use of long-wave UV light. However, conventional visible-light-mediated gelation is often based on the chain-growth polymerization of PEGDA, which requires the use of high concentrations of coinitiator (e.g., triethanolamine) and comonomer (i.e., N–vinylpyrrolidone, NVP).[91–93] These additional components, together with the high radical concentration required for the initiation of chain-growth polymerization, limit the applicability of this system in tissue engineering applications. Visible-light-initiated thiol–norbornene gelation should be more cytocompatible than conventional visible-light-initiated chain-growth gelation as coinitiator or comonomer is not required. However, one hurdle to overcome for this new gelation chemistry is that the gelation efficiency is not as high as using cleavage-type initiator and long-wave UV light based initiation.[38] This disadvantage can be addressed with higher macromer concentrations and/or multifunctional crosslinkers. Visible-light-mediated thiol–norbornene hydrogels should appeal to researchers interested in clinically relevant studies.

In summary, thiol–norbornene photoclick hydrogels have emerged as a versatile biomaterial platform. As demonstrated by research results from multiple laboratories over the past 5 years, the biophysical and biochemical properties of thiol–norbornene hydrogels (prepared from pure synthetic, natural, or hybrid

materials) can be tailored easily and independently. Because the hydrogels formed via this chemistry exhibit high cytocompatibility for various cell types, this class of hydrogels should be highly valuable in future regenerative medicine and tissue engineering applications.

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