Thiol-norbornene photo-click hydrogels for tissue engineering applications

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Abstract

Thiol-norbornene (thiol-ene) photo-click hydrogels have emerged as a diverse material system for tissue engineering applications. These hydrogels are cross-linked through light mediated orthogonal reactions between multi-functional norbornene-modified macromers (e.g., poly(ethylene glycol), hyaluronic acid, gelatin) and sulfhydryl-containing linkers (e.g., dithiothreitol, PEG-dithiol, bis-cysteine peptides) using low concentration of photoinitiator. The gelation of thiol-norbornene hydrogels can be initiated by long-wave UV light or visible light without additional co-initiator or co-monomer. The cross-linking 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 owing to the orthogonal reactivity between norbornene and thiol moieties. Uniquely, the cross-linking of step-growth thiol-norbornene hydrogels is not oxygen-inhibited, therefore the gelation is much faster and highly cytocompatible compared with chain-growth polymerized hydrogels using similar gelation conditions. These hydrogels have been prepared as tunable substrates for 2D cell culture, as microgels or bulk gels for affinity-based or protease-sensitive drug delivery, and as scaffolds for 3D cell culture. 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, as well as cancer cell biology. This article provides an up-to-date overview on thiol-norbornene hydrogel cross-linking and degradation mechanisms, tunable material properties, as well as the use of thiol-norbornene hydrogels in drug delivery and tissue engineering applications.

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1. Introduction

Hydrogels are hydrophilic polymeric networks capable of imbibing large quantity of water without dissolving. A typical hydrogel can swell and hold up water to more than 90% to 99% of its mass. Owing to this high degree of swelling, hydrogels are ideal for a variety of biomedical applications.[1] Recent efforts have focused on using hydrogels as material platforms for three-dimensional (3D) tissue culture and for repairing damaged tissues.[2-3] Additionally, hydrogels can serve as carriers for delivering 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 promoting/maintaining 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 fabricating matrices with desired functions and properties.[6] Taking the advantages from both classes of materials, recent work has focused on synthesizing hybrid hydrogels with both natural and synthetic components.[7-8]

In addition to material selection, the method by which the initially viscous precursor solution cross-links 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 temperature of the precursor solution, while 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), PEO-PPO-PEO) 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 high degree of cyto- and biocompatibility. However, these purely physical hydrogels can be mechanically weak and may not be ideal for applications where high mechanical strength is needed. Alternatively, hydrogels can be formed by cross-linking soluble polymer chains covalently 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, which are generated from initiators excited/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 cross-links. Radical mediated polymerizations are typically fast and in some cases the reaction kinetics can be controlled spatial-temporally. Alternatively, covalent hydrogels can also be formed through cross-linking of mutually reactive macromer species, such as copper-catalyzed azide-alkyne cycloaddition (CuAAC)[12] and 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

cross-links, the cross-linking reaction starts soon after the mutually reactive components are mixed together. Therefore, the cross-linking reactions cannot be controlled spatial-temporally.

Among the commonly used covalent cross-linking methods, photopolymerization is one that permits facile control over polymerization kinetics because the initiation and termination of cross-linking 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 the random chain polymerization while retaining the benefits of photochemistry, Anseth and colleagues developed a light and radical-mediated step-growth polymerization scheme based on orthogonal reaction between thiol and norbornene.[33] Thiol-norbornene (or thiol-ene) photo-click reaction is one unique polymerization mechanism that combines advantages from both radical-mediated polymerization and bio-orthogonal click reaction. Thiol-norbornene hydrogels are diverse in gelation and are highly cyto- and biocompatible for biomedical applications. The step-growth thiol-norbornene reaction occurs in stoichiometric ratio.[33] It is usually performed in physiologically relevant conditions with a lower radical concentration in the initiation step compared with chain-growth polymerization process.[34] It minimizes chemical toxicity during the reaction that may cause undesired damage to the biological components present in the reaction mixture. Furthermore, the biophysical (e.g., cross-linking density) or biochemical (e.g., integrin-binding motifs) properties in the thiol-norbornene hydrogel can be modified using additional light exposure in the presence of cells.[33] This unique light-dependent feature permits 'dynamic' modification of material properties for guiding cell fate process in a spatial-temporal 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 hydrogel network properties (e.g., factors affecting hydrogel cross-linking and degradation) are also reviewed. The final part of the review summarizes the applications of thiol-norbornene hydrogels in tissue engineering, including drug delivery, 2D cell culture, and 3D cell encapsulation.

2. Thiol-norbornene photopolymerization mechanism

The gelation and *in situ* cell encapsulation of thiol-norbornene hydrogels were first reported using long-wave UV light irradiation[33] and a type I (cleavage-type) photoinitiator, such as Irgacure-2959 or lithium arylphosphinate (LAP, Fig. 1A).[35] Mechanistically, UV light decomposes cleave-type photoinitiators into radicals. The later abstracts proton from sulfhydryl groups to form thiyl radicals that react with strained vinyl bonds in the norbornene moiety on functionalized PEG-norbornene (PEGNB). This reaction creates 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 stoichiometric ratio until the limited moiety (thiol or norbornene) is depleted (Fig. 1B). Unlike the cross-linking of acrylate-based hydrogels, there is no homopolymerization between norbornene groups. Hence, the thiol-norbornene hydrogel network possesses only

orthogonal cross-links and an idealized structure.[33] Although network defects (e.g., unreacted norbornene/thiol, intra-molecular cycles that do not contribute to crosslinking density) do exist in thiol-norbornene hydrogels, the degree of network heterogeneity is minimum compared to random chain-growth (i.e., acrylate-based) polymerization.[36] Furthermore, 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 using 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 photo-conjugation can be utilized to modify network properties.[33] Cysteine-containing peptides or thiolated proteins can be conjugated in thiol-norbornene hydrogels formed with off stoichiometric ratio (i.e., 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, thereby allowing 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 visible light initiated thiol-norbornene reaction is similar to that of the UV-based system, except that a type II (non-cleavage type) photoinitiator (e.g., eosin-Y, rose bengal) is used. Upon visible light (400 nm $< \lambda < 700$ nm) exposure, eosin-Y is excited, causing the abstraction of hydrogen from a co-initiator and the generation of secondary radicals. In this visible light mediated gelation, multifunctional thiol-bearing molecules serve as both hydrogel cross-linkers and co-initiators in the initiation step. While the initiation efficiency of visible light based thiol-norbornene photopolymerization is not as efficient 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 un-desirable.

3. Macromers developed for forming thiol-norbornene hydrogels

3.1 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 non-fouling 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 and colleagues functionalized multi-arm PEG with 5-norbornene-2-carboxylic acid using dicyclohexylcarbodiimide (DCC) as the coupling reagent and 4-dimethylaminopyridine (DMAP) as the catalyst.[33] This chemistry yields hydrolytically labile ester linkages between PEG backbone and norbornene. Therefore, the thiol-norbornene hydrogels formed with this particular chemistry may be hydrolytically degraded when in contact with an aqueous solution.[36] To increase the hydrolytic stability of the PEG-norbornene (PEGNB) macromer, we reported the preparation of amide-tethered PEG-norbornene (i.e., PEGaNB) by reacting amine-terminated PEG with norbornene acid.[41] Since 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 PEG-norbornene (i.e., PEGeNB). Cui *et al.* combined linear PEG-norbornene with poly(dimethylsiloxane)-norbornene (PDMS-norbornene) and used tetra-functional PEGSH as cross-linker for UV light-mediated thiol-norbornene photopolymerization.[42-43] The resulting hydrogels are highly elastic and strong and should be useful for applications requiring high resilience and strength.

3.2 Hyaluronan-based thiol-norbornene hydrogels

Hyaluronan (HA) is a non-sulfated glycosaminoglycan (GAG) found in extracellular matrix (ECM). 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 homo-polymerized by means of chain-growth photopolymerization.[44] HA-methacrylate can also be cross-linked with bi-functional thiols (e.g. DTT or bis-cysteine peptides) to form Michael-type hydrogels[45] or be processed to become macro-porous hydrogels.[46] The Burdick group has recently prepared norbornene-functionalized HA (NorHA), which was used to prepare photo-patternable HA-based thiol-norbornene hydrogels.[47] To prepare NorHA, HA was converted to tetrabutylammonium salt (HA-TBA), followed by reacting with 5-norbornene-2-carboxylic acid in anhydrous DMSO for 20 hr at 45°C in the presence of DMAP and di-tert-butyl dicarbonate (Boc2O). The synthesized NorHA could be cross-linked by DTT via the same step-growth radical-mediated photopolymerization. Similar to the PEG-based thiol-ene hydrogels, NorHA-DTT hydrogels were cytocompatible and photo-patternable. Cell-adhesive ligands (e.g., CRGDS) were required in this system to support cell adhesion.[47]

3.3 Gelatin-based thiol-norbornene hydrogels

Gelatin is a form of denatured collagen that has gained increasing popularity in hydrogel fabrication due to 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 a physiological pH. Chemical modification or cross-linking is required for preparing covalently cross-linked gelatin hydrogels. The most commonly used chemistry to cross-link gelatin is by reacting primary amines of gelatin with glutaraldehyde (GTA). However, GTA is highly cytotoxic and cannot be used in the presence of cells. To improve the cytocompatibility of gelatin cross-linking, various chemistries have been explored. Similar to HA-based hydrogels, gelatin can also be methacrylated (i.e., GelMA) and homo-polymerized via chain-growth photopolymerization or cross-linked by multi-functional thiols through Michael-type addition.[48] Gelatin can also be thiolated and cross-linked with PEGDA to form covalent hydrogels.[49] Our group has recently developed a norbornene-functionalized gelatin (GelNB) for fabricating gelatin-based thiol-norbornene hydrogels.[50] Norbornene was functionalized on gelatin through reaction with carbic anhydride in aqueous buffer with basic pH. The reaction proceeded for three days, followed by another three days of dialysis to obtain a moderate degree of functionalization (~50%). GelNB can be cross-linked with bi-functional DTT or tetra-functional PEG4SH. The cytocompatibility of GelNB hydrogels was verified with *in situ* encapsulation of human

mesenchymal stem cells (hMSCs). Compared with chain-growth GelMA hydrogels, step-growth GelNB hydrogels afforded higher degree of intercellular connectivity and supported faster cell spreading.

4. Network properties of thiol-norbornene hydrogels

4.1 Cross-linking, swelling, and network ideality of thiol-norbornene hydrogels

Differing from a chain-growth hydrogel network (e.g., PEGDA or GelMA hydrogel) in which the cross-links are composed of random number of homopolymerized (meth)acrylates, all cross-links in a step-growth hydrogel only contain a single covalent bond. In the case of radical mediated thiol-norbornene hydrogel, the cross-links are thioether bonds (Fig. 1B). 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 high degree of network 'ideality' due to the orthogonal thiol-norbornene reaction (Fig. 2A). The network ideality here refers to a fully cross-linked polymer network without structural defects such as intra-molecular primary cycles (Fig. 2B), unreacted functional groups (Fig. 2C), dangling polymer (Fig. 2D), or complete soluble polymer (Fig. 2E). In an ideal and fully cross-linked step-growth network with fixed macromer functionality and molecular weight, hydrogel cross-linking 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 (Fig. 2B), primary cycle (Fig. 2C), and/or dangling polymer (Fig. 2D) reduces the overall cross-linking 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 (Fig. 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]

4.2 Degradation of thiol-norbornene hydrogels induced by hydrolysis

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

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

Here, [Ester] and $[Ester]_0$ are the concentrations of ester bond on PEG backbone at any time during degradation and at time zero prior to degradation, respectively. k' is the degradation

rate constant, [OH⁻] is the concentration of hydroxyl ion 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 in acidic condition (pH 6.0) had an almost constant swelling ratio over the course of 45-day incubation, whereas hydrogels with the same compositions exhibited increasing swelling over time in slightly basic conditions (pH 7.4 and pH 8.0) (Fig. 4).[36] Fig. 4 also shows that the degradation profiles agree with the prediction using the pseudo-first order degradation kinetics described above.

In addition to the influence of pH, thiol-norbornene hydrogel degradation was also affected by macromer concentration. It appears that, at a higher macromer concentration (i.e., hydrogel with lower swelling ratio), thiol-norbornene hydrogels degraded much slower compared to hydrogels crosslinked at a lower macromer concentration.[36] This might be a result of decreased accessibility of water molecules to the cross-links. Hydrolytic degradation rate of thiol-norbornene hydrogel can further be affected by the sequence of bis-cysteine-bearing peptide linker. 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 linker without side group (i.e, Cys-Gly-Gly-Gly-Cys or CGGGC). The steric hindrance and hydrophobic effect of amino acid side groups might retard the degradation rate.[36]

4.3 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 cross-linkers, gels can be degraded enzymatically when they are placed in a solution containing the protease of interest. The termini of the peptide cross-linkers are usually cysteine residues that provide sulfhydryl groups for the orthogonal thiol-ene photo-click reactions. Protease sensitivity of thiol-norbornene hydrogels can be tailored by altering the sequence of protease-sensitive peptide cross-linkers. 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. Since most proteases are proteins with molecular weights on the order of a few tens of kDa, rarely will the time scale for protease diffusion into hydrogels be faster than the time scale 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] Since 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.* exploited this feature to fabricate human neutrophil elastase (HNE) responsive thiol-norbornene hydrogels (PEG4NB cross-linked by HNE-sensitive peptide CGAAPV↓RGGGGC) for delivering protein therapeutics.[53] The rate of protease-induced hydrogel mass loss could be tailored by substituting a non-natural amino acid residue in the peptide sequence (Fig. 5A), by supplementing protease at difference concentrations (Fig. 5B), or by changing the molecular weight of PEG4NB macromer (Fig.

5C). As the hydrogel network was degraded in a surface erosion manner, the otherwise entrapped proteins were released only in the presence of HNE. Our lab also used a similar strategy to liberate pancreatic β-cell aggregates generated in the thiol-norbornene hydrogels.[37] Specifically, a chymotrypsin cleavable peptide (i.e., CGGY↓C) was used as the gel cross-linker. When the cell-laden hydrogels were placed in buffer containing chymotrypsin, the erosion of gel led to liberation of cell clustered that could be used in biological analyses or applications.

There is a growing interest in developing biomimetic materials with dynamically adaptable gel properties post gelation.[8-9, 15] For example, macromers with photolabile groups can be used to fabricate hydrogels with dynamically tunable cross-linking density. To introduce tunable gel biomechanical (i.e., reduction of gel cross-linking density) and/or biochemical properties (e.g., 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 photo-click reactions.[54] A visible light source (wavelength: 400–700nm) was utilized to excite photosensitizer eosin-Y that generated thiyl radicals from the bis-cysteine peptide linker incorporating with a photo-labile amino acid L-2-nitrophenylalanine (NPA). Upon exposure to UV-light (302nm or 365nm), hydrogels were degraded due to photolysis of the peptide linker. The rate of hydrogel degradation was easily manipulated by wavelength and UV light intensity. This new step-growth hydrogel system preserves the favorable properties offered by photochemistry, including photopolymerization and photodegradation.

While the 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 altering hydrogel molecular structure or hydrophilicity.[36] By varying the ratio between non-cleavable cross-linker CGGGC and a chymotrypsin-sensitive peptide cross-linker 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 chymotrypsin-containing solution. Hydrogels containing high percentage of CGGYC cross-linker (75–100%) 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 dynamically controlling growth factor or drug delivery.

4.4 Degradation induced by linker cleavage – Acts of local cellular activity

Thiol-norbornene hydrogels can be used to study the influence of local matrix environment on cell fate processes (e.g., growth, differentiation, migration, and invasion) because many of these processes require 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 also responsible for many malignant cell behaviors, including inflammation and tumor progression. Imbalance between activities of MMPs and their inhibitors (e.g., tissue inhibitor of metalloproteinases or TIMPs) 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 cross-linking multi-arm PEGNB and MMP-sensitive peptide linker.[33, 55-56] A typical example is the use of peptide cross-linker containing sequence of CGPQG ↓ IWGQC. When cells are encapsulated within hydrogels containing 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 and colleagues developed a synthetic hydrogel niche for understanding human mesenchymal stem cells (hMSCs) proliferation, morphogenesis, and differentiation in thiol-norbornene hydrogels containing different compositions of MMP-sensitive peptide cross-linkers (Fig. 6).[55] We have also reported a similar hydrogel platform to study the influence of local matrix conditions on the growth, morphogenesis, invasion, and drug responsiveness in pancreatic cancer cells.[57-59] Unlike the release of proteins/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 due to the short-range action of proteases. However, these short-range protease activities do cause reduction of hydrogel cross-linking density,[57] and hence bulk gel mechanical property, given that sufficient amount of peptide linkers are cleaved.

5. Thiol-norbornene hydrogels in tissue engineering applications

5.1 Controlled delivery

When designing 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 due to their mild and diverse gel cross-linking process, hydrophilic network structure, and tunable permeability. In regard to preserving protein bioactivity, McCall *et al*. compared the stability and the release of encapsulated protein (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.[34] They found that step-growth thiol-norbornene hydrogels delivered proteins in their bioactive form. On the other hand, 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 is not susceptible to oxygen inhibition. Therefore, hydrogel was 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 preserving protein bioactivity, PEG-based thiol-norbornene hydrogels can also be designed to exhibit affinity for therapeutically relevant growth factors. For example, Murphy and his colleagues prepared thiol-norbornene microgels using multi-arm PEGNB macromers and di-thiol containing linkers from an aqueous two-phase separation system.[60-65] These microgels also were immobilized with affinity peptides capable of sequestering vascular endothelial growth factor (VEGF). By 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 non-specific 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 also utilized as a protease-responsive drug delivery matrix. As described above, Aimetti *et al*. constructed HNE sensitive hydrogel using PEG4NB and HNE cleavable peptide cross-linker.[53] They encapsulate bovine serum albumin (BSA) as a model drug and found that zero-order release of BSA 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*. developed an alternative strategy for MMP-responsive dexamethasone (Dex) delivery.[66] They conjugated Dex to an MMP sensitive peptide (i.e., Dex-KGPQG↓IAGQCK) containing an additional cysteine for thiol-norbornene mediated peptide immobilization. The hydrogel was formed by reacting PEG4NB and MMP-cleavable peptide (KCGPQG↓IAGQCK). The release of Dex was controlled via local cleavage of peptide by MMP secreted from encapsulated hMSCs. Benoit and colleagues prepared thiol-norbornene hydrogels cross-linked by elegantly designed protease sensitive peptide linker containing therapeutic peptide sequences.[67] Peptide drugs could only be liberated in the presence of nearby cellular activity. This strategy potentially could provide a new delivery mechanism for localization of peptide drug and for decreasing the clearance rate of the small therapeutics.

5.2. 2D cell culture substrate

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

5.3. 3D cell encapsulation

The cross-linking 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 cross-linking, affords a material platform for cell culture in 3D. Furthermore, since norbornene is not reactive

without the presence of active radical species, thiol-norbornene hydrogels with excess norbornene moieties can be used as a 'dynamic' culture platform where thiolated bioactive cues can be added post-gelation to alter cell fate behaviors.[33] Similar to the 2D studies described above, modularly cross-linked thiol-norbornene hydrogels are ideal for studying the effect of an individual parameter on specific cell behaviors. Earlier work by Benton *et al.* demonstrated the cytocompatibility of thiol-norbornene hydrogels in 3D culture of VICs.[56] They utilized MMP sensitive peptide cross-linker (GPQG↓IWGQ) to permit cell-mediated remodeling of extracellular microenvironment. Gould *et al.* further compared the variation of VIC phenotypes in the presence of different integrin-binding peptides immobilized within the hydrogel network.[74] Anderson *et al.* encapsulated human mesenchymal stem cells (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 hMSCs in 3D.[55] Kyburz *et al.* examined the motility of hMSC in MMP degradable PEGNB hydrogel with varying cross-linking densities and showed that a lower cross-linking density and a higher RGD concentration facilitate hMSC migration and spreading in 3D.[75] Mariner *et al.* studied the influence of microRNA on osteogenic differentiation of hMSCs in 3D.[76] Focused on cartilage tissue engineering, Bryant and colleagues recently reported the improved retention of chondrocyte-secreted proteoglycans within thiol-norbornene hydrogels prepared from PEG-norbornene, PEG-dithiol, and pendant hyaluronic acid binding peptide.[77] Thiol-norbornene hydrogels have also been exploited as a material platform for understanding protease activity in 3D. For example, Leight *et al.* developed a material tool to measure MMP activity in the encapsulated hMSCs.[78] An otherwise quenched fluorescent MMP-sensitive peptide was used as gel cross-linker to reveal local protease activity as cells undergo morphogenesis in 3D. Schultz and Anseth utilized multiple particle tracking (MPT) technique to investigate the sol-gel transition and protease-mediated degradation of 3D matrices.[79]

Thiol-norbornene hydrogels are highly cytocompatible because the orthogonal cross-linking reactions are mild and require only a fraction of radicals as that used in chain-growth polymerized hydrogels (e.g., PEGDA hydrogels). Our group has evaluated the cytocompatibility of PEG-based thiol-norbornene hydrogel using a radical-sensitive pancreatic β-cell line, MIN6, and concluded that the orthogonal thiol-ene 'photo-click' reaction is more cytocompatible than chain-growth PEGDA hydrogels at equivalent reactive macromer functionality.[37, 80] The inherent degradability of PEG-norbornene macromer (due to ester bond hydrolysis) also has been shown to increase proliferation and spreading of hMSCs.[41] Roberts and Bryant encapsulated bovine chondrocytes in chain-growth PEGDA and step-growth thiol-norbornene hydrogels and showed that the later promoted hyaline-like cartilage production with positive staining for aggrecan and collagen II, especially when the cell-laden hydrogels were cultured under mechanical loading.[81] Sridhar *et al.* immobilized TGF-β1 in thiol-norbornene hydrogel network to enhance glycosaminoglycans (GAGs) secretion from the encapsulated chondrocytes.[82] 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.* evaluated effects of various bioactive cues on capillary network formation of human umbilical vein endothelial cells (HUVECs) using thiol-norbornene hydrogel arrays.[84] Additionally, Mariner *et al.* prepared rhBMP-2 loaded

thiol-norbornene hydrogels and observed improved bone fracture healing in a rat critical size bone defect animal model.[85]

3D culture of cancer cells is an emerging research area in tissue engineering. Cancer cells grown in 3D resemble tumor tissues better than cultured on 2D tissue culture plastic. Various cancer cells have been encapsulated and cultured in 3D matrices, including thiol-norbornene hydrogel. For example, Schwartz *et al.* and Singh *et al.* cultured human fibrosarcoma cells (HT-1080) in an MMP-degradable thiol-norbornene hydrogel and studied HT-1080 cell migration mechanism in 3D.[86-88] Wang *et al.* established a brain tumor model by encapsulating glioblastoma (GBM) cells (U87) in HA entrapped 8-arm PEG-norbornene hydrogel cross-linked with MMP-sensitive cross-linkers.[89] Our recent studies showed that hepatoma cells (i.e., Huh7, HepG2) encapsulated in thiol-norbornene hydrogels exhibited 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 matrix stiffness, protease-sensitive peptide linkers, and integrin-binding ligands.[59] The effect of epidermal growth factor receptor (EGFR) inhibition on PANC-1 cell drug resistance was also examined using MMP-sensitive PEG-norbornene hydrogels with different stiffness.[58] Using a similar material design principle, the effects of cytokines (TGFβ-1 and EGF) and collagen 1 on pancreatic cancer cell (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 up-regulated simply because the cancer cells were cultured in 3D matrix.[57]

6. Conclusions and Outlook

Since 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 photo-crosslinking of thiol-norbornene hydrogels.[33, 38] These hydrogels have been prepared as tunable substrates for 2D cell culture, 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 culture. 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 post-gelation modification capability of thiol-norbornene reaction. Fairbanks and Anseth *et al.* first demonstrated this unique feature through preparing thiol-norbornene hydrogels with off-stoichiometric thiol-to-norbornene ratio (i.e., with excess norbornene groups during photopolymerization).[33] They photo-patterned CRGDS peptide in the cell-laden hydrogel by incubating the gel in medium containing soluble peptide and additional photoinitiator, followed by a secondary light-mediated thiol-norbornene conjugation. The secondary thiol-norbornene photo-click

reaction imparted cell adhesiveness in the otherwise inert PEG-based hydrogels. Theoretically, it is also possible to perform post-gelation conjugation of whole proteins within the cell-laden hydrogels, as long as the protein of interest contains free sulfhydryl groups for secondary thiol-norbornene reaction. However, slow diffusion of macromolecular proteins, disulfide bond formation, and binding of infiltrated proteins to cell surface receptors prior to light-mediated protein immobilization may affect the efficacy of this approach. Slow diffusion of macromolecular proteins within cross-linked hydrogels can be resolved by introducing macro-porous structure in bulk thiol-ene hydrogels. Selective 'caging' chemistry could be used to prevent undesired reactions of thiol groups. Photo-labile chemical bonds sensitive to lights with orthogonal wavelengths could be designed for light mediated 'un-caging' of thiol-protected groups and for secondary thiol-norbornene photo-click reaction. 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 cross-linking using a similar experimental process as described above for post-gelation 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 using non-cleavage type photoinitiators, such as eosin-Y and rose bengal.[38] The use of visible light to cross-link hydrogel is arguably more cytocompatible than using long-wave UV light. However, conventional visible light mediated gelation is often based on chain-growth polymerization of PEGDA, which requires using high concentrations of co-initiator (e.g., triethanolamine, TEA) and co-monomer (i.e., NVP).[91-93] These additional components, together with the high radical concentration required for initiating 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 co-initiator or co-monomer 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 by using higher macromer concentrations and/or multi-functional cross-linkers. Visible light mediated thiol-norbornene hydrogels should have appeal to researchers interested in clinically relevant studies.

In summary, thiol-norbornene photo-click hydrogels have emerged as a versatile biomaterial platform. As demonstrated by research results from multiple laboratories in the past five 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.

Acknowledgments

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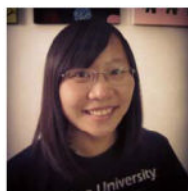
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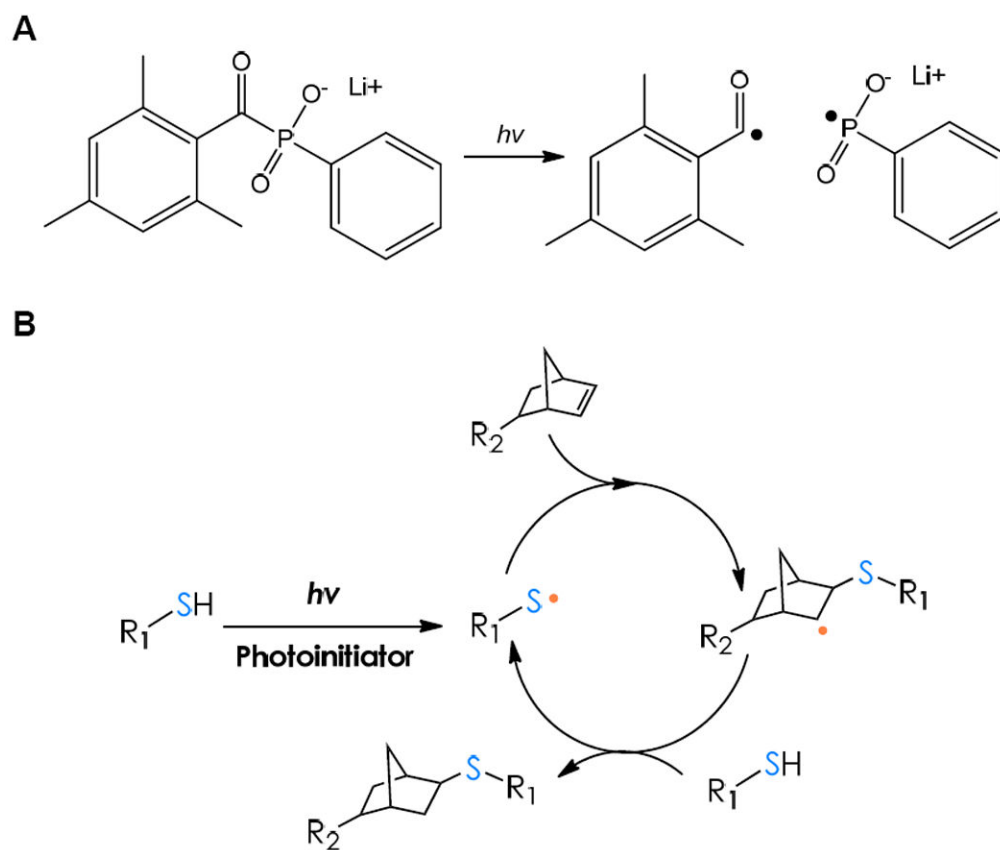
Figure 1.
(A) Photo-cleavage of type I photoinitiator lithium arylphosphinate (LAP) into radicals. (B) Schematics of radical-mediated step-growth thiol-norbornene photo-click reaction using thiol-containing molecule (R1-SH) and norbornene-functionalized macromer (R2-norbornene).
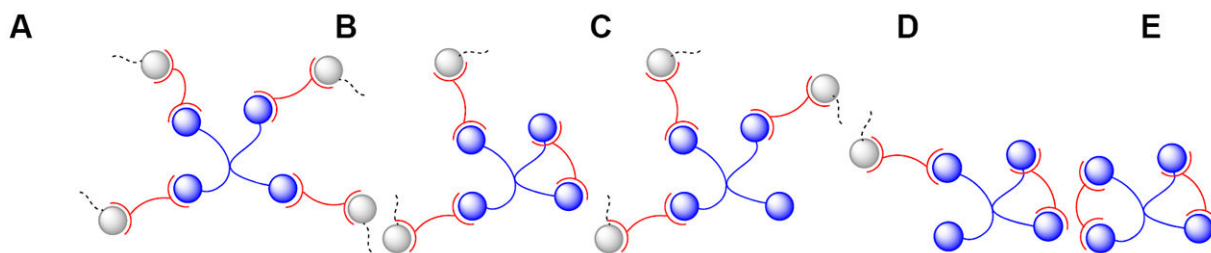
Figure 2.
Schematics of potential products after step-growth thiol-norbornene reactions between tetra-functional PEGNB and bi-functional DTT: (A) Defect-free, fully cross-linked thiol-norbornene network. (B) Fully reacted thiol-ene network with a primary cycle. (C) Partially cross-linked thiol-ene network with an un-reacted, dangling PEG-norbornene arm. (D) Dangling polymer with a primary cycle and an un-reacted PEG-norbornene arm. (E) Soluble polymer with fully reacted functional groups. Note: D and E do not contribute to network cross-linking.
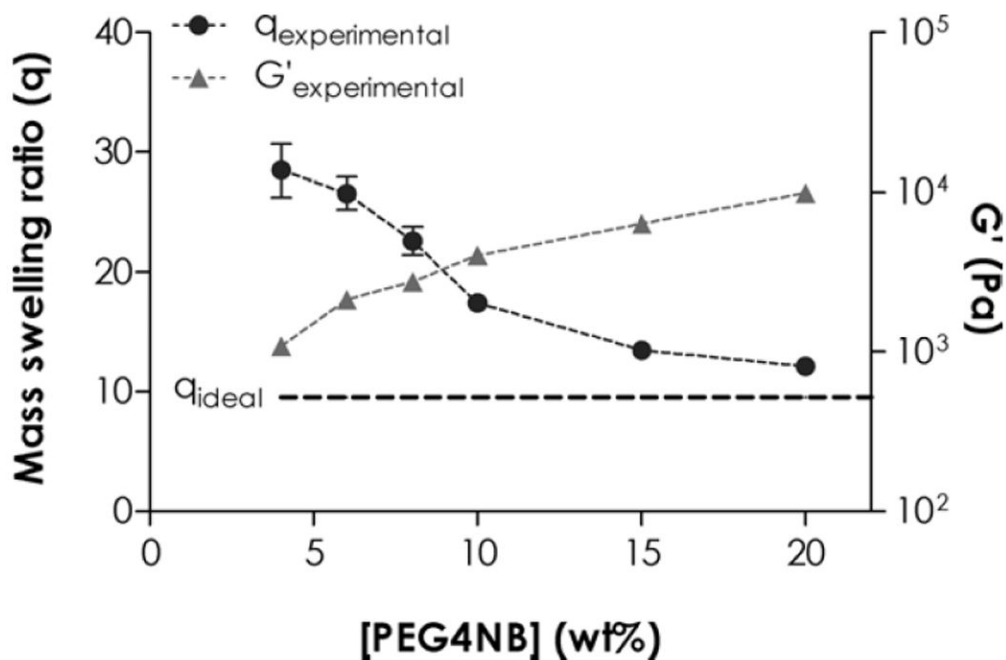
Figure 3.
Effect of PEG4NB macromer concentration on thiol-ene hydrogel equilibrium swelling (left y-axis) and elastic modulus (right y-axis). Swelling ratio of an ideal network was calculated based on the molecular weight between crosslinks ((M_c)$^-$) of given macromer molecular weights (MWPEG4NB = 20 kDa, MWDTT = 154 Da) and functionalities (fPEG4NB = 4, fDTT = 2). Ideal swelling ratio (qideal) was calculated based on the known macromer structure and the Flory-Rehner theory. Reprinted with permission from 36. Copyright 2012 American Chemical Society.
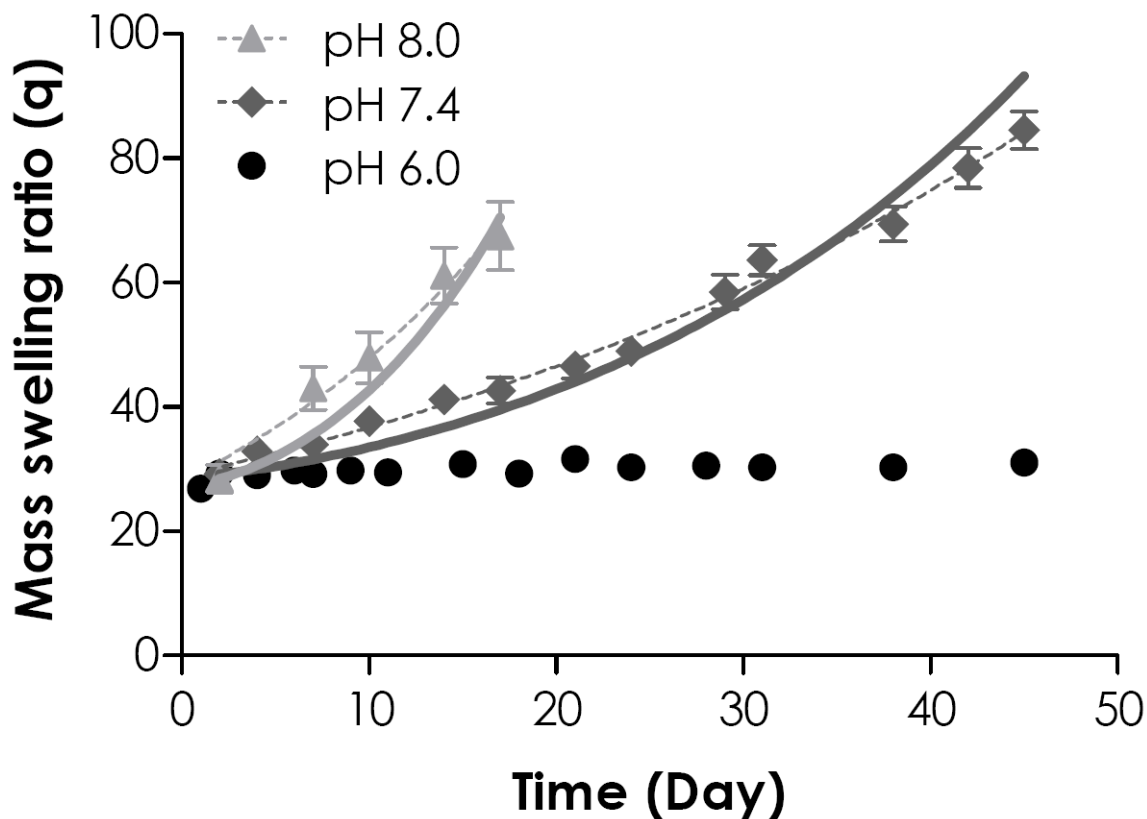
Figure 4.
Effect of buffer pH on mass swelling ratio of PEG4NB-DTT hydrogels (4wt%). Symbols represent experimental data while dashed curves represent exponential curve fitting to the experimental data. The apparent degradation rate constants (khyd) for gels degraded in pH 7.4 and pH 8.0 were 0.024 ± 0.001 and 0.057 ± 0.002 day-1, respectively. Solid curves represent model predictions with best-fit kinetic rate constants: k'pH 7.4 = 0.011 day-1 and k'pH 8.0 = 0.027 day-1. No curve fitting or model prediction was made for gels degraded in pH 6.0 due to the stability of gels in acidic conditions. Reprinted with permission from 36. Copyright 2012 American Chemical Society.
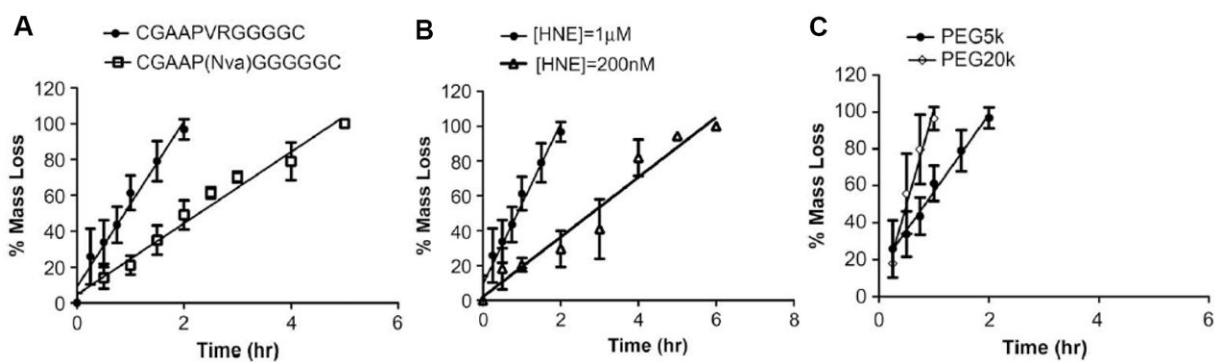
Figure 5.
Profiles of thiol-norbornene hydrogel mass loss upon exposure to HNE. Influence of HNE-sensitive peptide substrate (A), concentration of HNE (B), and molecular weight of PEGNB (C) were experimentally studied. All gels were made of 10 wt% PEG macromer and exposed to 1 µM HNE (unless otherwise noted). Reprinted with permission from 37. Copyright 2009 Elsevier.
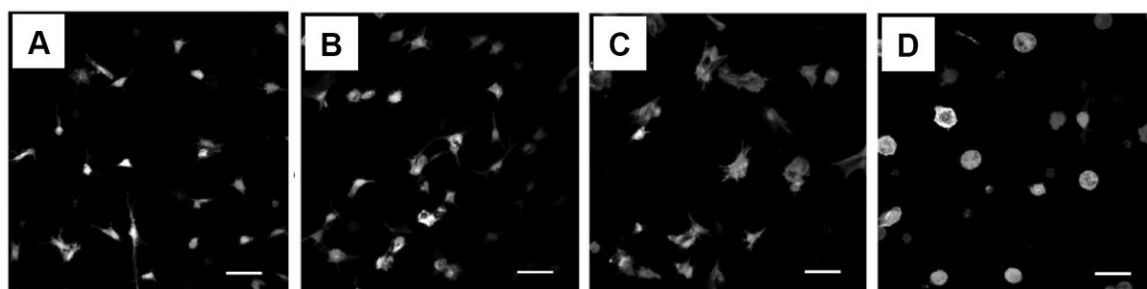
Figure 6.
Morphology of hMSCs encapsulated in MMP-degradable thiol-norbornene hydrogels. Encapsulated hMSCs were cultured in (A) growth, (B) osteogenic, (C) chondrogenic, or (D) adipogenic media and were stained with Calcein AM after 14 days of culture (imaged by confocal microscopy. Scale bar represents 50 μm). Reprinted with permission from 55. Copyright 2011 Elsevier.