RSC Advances

RSC Advances

www.rsc.org/advances

ROYAL SOCIETY OF CHEMISTRY

www.rsc.org/advances

Recent advances in crosslinking chemistry of biomimetic poly(ethylene glycol) hydrogels

Chien-Chi Lin

Department of Biomedical Engineering, Purdue School of Engineering and Technology, Indiana University-Purdue University Indianapolis, Indianapolis, IN. 46202, USA

To whom correspondence should be addressed:

Chien-Chi Lin, Ph.D.
Assistant Professor
Department of Biomedical Engineering
Indiana University-Purdue University Indianapolis
Indianapolis, IN 46202
Phone: 317-274-0760
Fax: 317-278-2455
Email: lincc@iupui.edu

Abstract

The designs and applications of biomimetic hydrogels have become an important and integral part in modern tissue engineering and regenerative medicine. Many of these hydrogels are prepared from synthetic macromers (e.g., poly(ethylene glycol) or PEG) as these macromers provide high degree of tunability in matrix crosslinking, degradation, and modification. For a hydrogel to be considered biomimetic, it has to recapitulate key features found in the native extracellular matrix, such as appropriate matrix mechanics and permeability, the ability to sequester and to deliver drugs, proteins, and or nucleic acids, as well as the ability to provide receptor-mediated cell-matrix interactions and protease-mediated matrix cleavage. A variety of chemistries have been employed to impart these biomimetic features into hydrogel crosslinking. These chemistries, such as radical-mediated polymerizations, enzyme-mediated crosslinking, bio-orthogonal click reactions, and supramolecular assembly, may be different in crosslinking mechanisms but they are required to be efficient in gel crosslinking and ligand bioconjugation under aqueous reaction conditions. The biomimetic hydrogels prepared should display a diverse array of functionality and should also be cytocompatible for *in vitro* cell culture and/or *in situ* cell encapsulation. The focus of this article is to review recent progress in the crosslinking chemistries of biomimetic hydrogels with a special emphasis on hydrogels crosslinked from poly(ethylene glycol)-based macromers.

Introduction

Biomimetic hydrogels are a class of water-imbibing but insoluble polymer networks that present aspects of native extracellular matrix (ECM) to the surrounding or encapsulated cells, including the ability to emulate native matrix mechanics, to sequester and deliver growth factors, as well as to provide cell-matrix interactions, which include ligand-receptor binding and protease-medicated matrix cleavage.[1-4] To mimic matrix mechanics, one can simply adjust the degree of network crosslinking of a hydrogel.[5-11] The growth factor sequestration is often achieved by covalent immobilization of 'affinity ligands' (e.g., heparin, affinity peptides, small molecular weight ligands, and aptamers, etc.) in the network.[2, 12-17] The presentation of receptor-binding ligands (e.g., Arg-Gly-Asp peptide, often as network-immobilized pendent motifs) induces receptor-mediated intracellular signaling important in maintaining or guiding cell viability and function.[3, 18, 19] Furthermore, the presence of protease-sensitive substrates (e.g. matrix metalloproteinases (MMPs) cleavable peptides, often serve as gel crosslinker) permits cell-mediated local matrix cleavage and subsequent cell fate processes including migration, extension of cellular processes, and proliferation.[4, 20, 21] It is known that matrix mechanics affects profoundly cell fate processes through regulating intracellular tensions.[22-24] Emerging work has also demonstrated that, together with matrix degradation, mechanics of the matrix influences cell spreading and cell fate determination.[25-27] Hydrogels are ideal matrices for this type of study as the mechanical properties of these water-swollen matrices can be easily and sometimes independently tuned to mimic native tissue elasticity and bio-functionality.[28] The overarching goal of creating biomimetic hydrogels is to recapitulate local cell-matrix interactions for improving the outcome of global tissue regeneration and/or to understand fundamental mechanisms by which specific extracellular signals influence cell fate determination.

In the past few decades, significant efforts are dedicated to the design and synthesis of biomimetic hydrogels for tissue engineering and regeneration medicine applications.[1, 3, 4, 29] Many of these hydrogels are fabricated to present soluble or immobilized proteins/peptides, as well as controllable matrix elasticity to the cells encapsulated within or adhere onto the biomimetic hydrogels. Biomimetic hydrogels can be tailored to allow both two-dimensional (2D) and three-dimensional (3D)

3

cell cultures.[30] Compared with flat, rigid, and 2D tissue culture polystyrene (TCPS) or animal-based 3D matrices (e.g., Matrigel or collagen gel), biomimetic hydrogels composed of synthetic polymers, such as poly(ethylene glycol) or PEG, are more flexible and tunable in material properties. Notable applications of biomimetic hydrogels include preservation and differentiation of stem/progenitor cell; exploration of tumor cell migration, invasion, drug responsiveness, and epithelial-mesenchymal transition (EMT); as well as tissue/organ regeneration. The goal of this article is to review some important hydrogel crosslinking chemistries and to provide an update on recent advances in the fabrication of PEG-based biomimetic hydrogels.

Functionalization of PEG-based biomimetic hydrogels using photopolymerizations

Chain-growth photopolymerizations

PEG-based hydrogels possess tissue-like elasticity, high water content and solute permeability, as well as cyto- and biocompatibility.[1, 31] These preferential properties have rendered PEG-based hydrogels highly useful in a variety of biomedical applications, including controlled release of therapeutically relevant agents and encapsulation and delivery of cells for immuno-isolation or cell-based therapy.[32] Since the chemical makeup of PEG ($-(CH_2CH_2O)_n-$) contains no biological recognition site, modification of PEG-based hydrogels are required to render the otherwise inert network biomimetic and cell-responsive.[33] One classic example of functionalizing PEG hydrogel is through co-polymerization of functionalized (e.g., acrylated or methacrylated) peptides during network cross-linking.[18, 34, 35] Propagation of radicals (generated from cleavage type photoinitiator) on vinyl group results in simultaneous network crosslinking and peptides immobilization (**Figure 1A**). Acrylated or methacrylate peptides (**Figure 1B**) can be easily co-polymerized within PEG-diacrylate (PEGDA) or PEG-dimethacrylate (PEGDMA) hydrogels through chain-growth homo-polymerization. Hern and Hubbell used N-hydroxysuccinimidyl (NHS)-activated esters (either directly to acrylic acid or with a PEG spacer) to introduce an acrylate moiety to the N-terminal α-amine of the peptide.[18] These acrylated peptides can be co-polymerized with PEGDA to form cell-adhesive or bioactive PEG hydrogels. Due to its simplicity in material preparation, this approach will continue to be an important method of

functionalizing PEG hydrogels. However, two caveats of this approach are that: (1) the pendent peptides are co-polymerized within hydrophobic poly(acrylate) or poly(methacrylate) kinetic chains and may have decreased accessibility to the co-encapsulated cells; and (2) the immobilization efficiencies of (meth)acrylate-based pendent peptides are generally low (~60%)[19, 36] and the sol fraction of the bioactive peptides may cause undesired biological response in the co-encapsulated cells.

The co-polymerization of pendent peptide within PEG hydrogels provides binding sites in the gels for cell surface receptor activation. To render PEG-based hydrogel truly cell responsive, protease sensitivity must also be integrated in biomimetic hydrogel design. PEG hydrogels crosslinked from PEGDA or PEGDMA are hydrolytically and proteolytically stable in therapeutically relevant time scales. To render the covalently crosslinked PEG hydrogels degradable, segments of degradable motifs can be incorporated in the macromer backbone.[32, 37] For example, polymerization of acrylated PLA-PEG-PLA macromers yields hydrolytically degradable hydrogels with predictable gel degradation rate.[32, 38, 39] Protease responsiveness can be integrated in PEG-based hydrogels through crosslinking with peptide substrates for selective proteases. For example, hetero-bifunctional PEG macromers, such as acrylate-PEG-NHS, were used to react with MMP-sensitive peptides (e.g., NH_2-GPQG↓IWGQK) through nucleophilic addition to primary amines, thus producing a homopolymerizable PEG macromer with protease sensitivity (**Figure 1C**).[40] Cells encapsulated within this type of hydrogel networks were able to remodel their local matrix through protease secretion. While protease sensitivity of this type of chain-growth PEG hydrogel can be adjusted by the content of protease sensitive macromer (i.e., acryl-peptide-PEG-peptide-acryl) added during network crosslinking, the accessibility of protease sensitive sections of the macromer to the co-encapsulated cells might be limited because of the presence of poly(acrylate) kinetic chains following crosslinking. Another limitation of this system is that the NHS on acrylate-PEG-NHS might react with bioactive lysine residues presenting within the protease sensitive sequence.
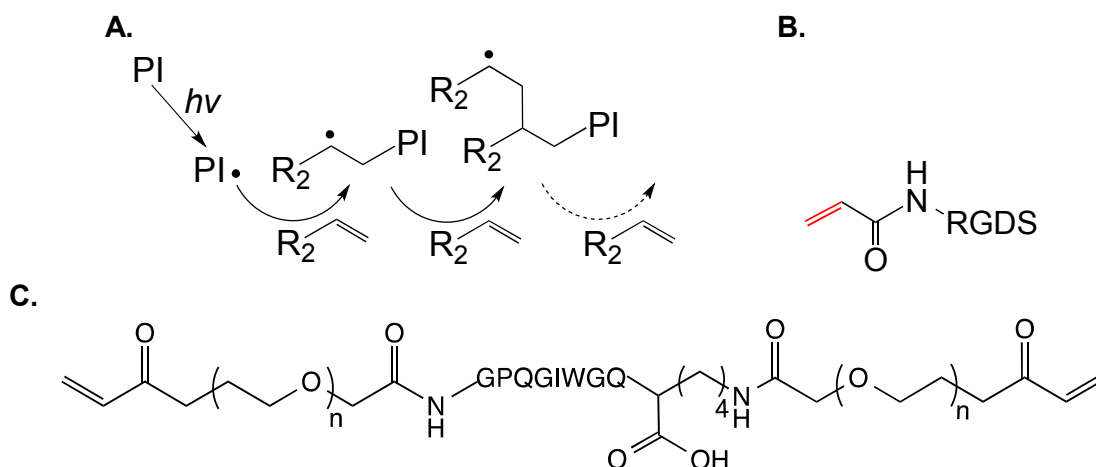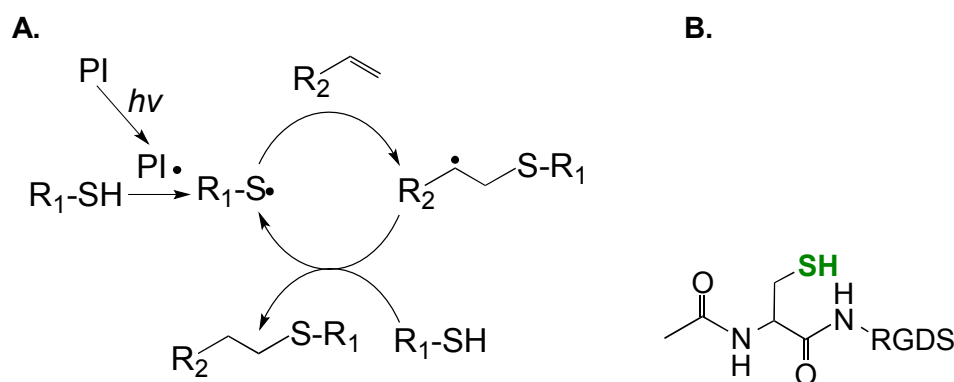
Figure 1. (A) Schematic of chain-growth photopolymerization for forming peptide-immobilized hydrogel (PI: photopolymerization, *hv*: light source). (B) Acrylated RGDS peptide for co-polymerization into PEGDA hydrogel. (C) Schematic of Acrylate-PEG-GPQGIWGQK-PEG-Acrylate crosslinker.

Step-growth photopolymerizations

PEG-based hydrogels can be prepared from step-growth photopolymerization (**Figure 2A**), which yields gels with more homogeneous network structure and with better mechanical properties when compared to chain-growth polymerized gels with similar macromer content.[41, 42] The use of photopolymerization permits not only rapid gelation (gel points within seconds), but also spatial-temporal control over gelation kinetics. The crosslinking of PEG-based step-growth hydrogels requires the use of macromers with an average functionality of greater than two.[43, 44] Anseth and colleagues developed a radical-mediated, step-growth photopolymerization scheme, termed thiol-norbornene photopolymerization, for forming biomimetic step-growth PEG hydrogels.[45] A multi-arm PEG macromer functionalized with norbornene was used to react orthogonally with thiol-containing bi-functional peptides via long wavelength ultraviolet (UV) light mediated step-growth photopolymerization and to form hydrogels. The peptide crosslinker contains a sequence that can be cleaved by MMPs and is flanked by terminal cysteine residues (e.g., CGPQG↓IWGQC). The additional cysteines permit radical-mediated thiol-norbornene 'photo-click' reaction while the presence of MMP-sensitive sequence allows cell-mediated network cleavage to accommodate cellular processes, such as migration, proliferation,

and differentiation.[46-48] Other bioactive motifs (e.g., cell adhesive ligands RGDS) can be incorporated within these hydrogels through conjugating mono-cysteine peptides during network crosslinking. The incorporation of pendent bioactive motifs can be easily achieved by using peptide bearing a cysteine residue (**Figure 2B**). When compared to chain-growth acrylate homopolymerizations, step-growth thiol-norbornene gelation has been shown to exhibit better cytocompatibility for radical sensitive cells, including pancreatic β-cells[41] and chondrocytes.[49] In addition to UV light mediated cross-linking, our own group has developed a visible light (400-700nm) mediated thiol-norbornene photo-crosslinking aided by a non-cleavage type photosensitizer eosin-Y.[50-52] Upon visible light exposure, excited eosin-Y molecules deprotonate thiols to give thiyl radicals that subsequently initiate thiol-norbornene reactions to produce step-growth hydrogels with protease sensitive and cell adhesive properties. The use of visible light eliminates the concerns that UV light, even at long wavelength and low intensity, could induce cellular damages of biological complications. Readers are directed to a recent review that summarizes the progress of thiol-norbornene hydrogels to-date.[53]


Figure 2. Schematic of step-growth photopolymerization for forming hydrogel (PI: photopolymerization, hv: light source). (B) Cysteine-containing RGDS peptide for co-polymerization into the step-growth hydrogel (N-terminal acetylated peptide).

Mixed-mode photopolymerizations

Acrylated PEGs (i.e., linear PEGDA or multi-arm PEG-acrylates) are a unique class of macromer as they can be crosslinked into hydrogels with chain-growth (through homopolymerization of PEG-acrylates),[18] step-growth hydrogels (by reacting with thiol-containing crosslinkers via nucleophilic conjugation addition reaction),[44] or mixed-mode network (through radical mediated thiol-acrylate

polymerization).[54] Peptides or proteins with one or more sulfhydryl groups can also be covalently incorporated in PEG hydrogels through a mixed-mode photopolymerization.[54] **Figure 3** shows the mechanism of mixed-mode thiol-acrylate polymerization and incorporation of biomolecules, such as peptides. When higher concentration of thiol is used, the polymerization shifts toward step-growth polymerization.[55] Bioactive peptides, either incorporated as pendant ligands or as part of the crosslinkers, can be easily incorporated as long as cysteine residues are incorporated into the peptide sequences during peptide synthesis. Peptides with additional spacers inserted between the cysteine and bioactive motifs can be used to increase the accessibility of immobilized peptides to soluble proteins [12, 16] or to enhance mesenchymal stem cell viability in PEG hydrogels.[54] This class of hydrogels can be prepared via either long-wave UV light (365nm)[56] or visible light (400-700nm)[57] initiation as long as an appropriate initiator is used (e.g., type-1 initiator for UV-mediated crosslinking and type-2 initiator for visible light-mediated crosslinking). It is worth noting that mixed-mode hydrogels polymerized from acrylated/methacrylated macromers and thiol-containing crosslinkers contain hydrolytically labile thioether ester bonds.[36, 58] Hydrolysis of these ester bonds may facilitate cell spreading and viability but may also lead to early disintegration of the gel network. Also, the immobilization efficiency of acrylated peptides (~60%) has been shown to be lower than that of thiolated peptide (~80-90%).[58]
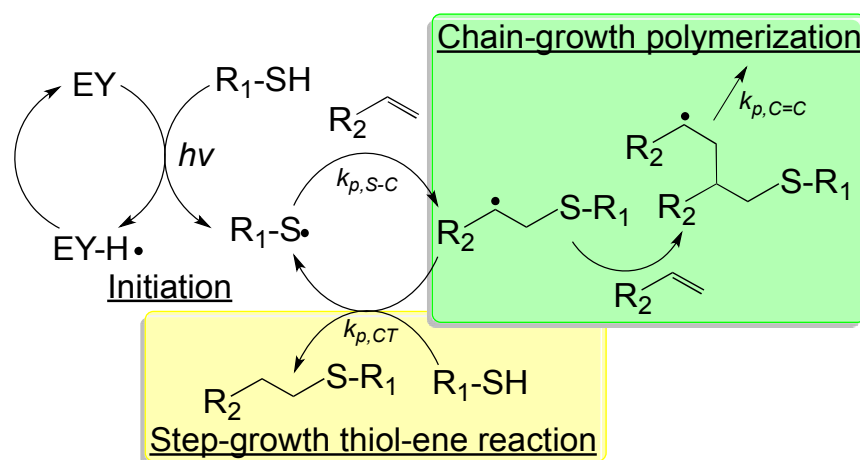

Figure 3. Schematic of a visible light initiated mixed-mode photopolymerization for forming hydrogel (EY: eosin-Y; *hv*: visible light source; $k_{p,S-C}$, $k_{p,C=C}$, $k_{p,CT}$: kinetic constants for thiol-carbon radical propagation, carbon-carbon radical propagation, and chain-transfer, respectively.).[36]

Enzyme-mediated crosslinking of biomimetic hydrogels

Several enzymes have been employed for fabricating hydrogels in biomedical applications. For example, horseradish peroxidase (HRP) catalyzes the formation of carbon-carbon bond or carbon-oxygen bond between phenols or anilines in the presence of hydrogen peroxide (H_2O_2). Similarly, glucose oxidase (GOX), when mixed with glucose and dissolved oxygen, generates gluconolactone and H_2O_2 that is further reduced into hydroxyl ion (OH^-) and hydroxyl radical ($\bullet OH$) in the presence of ferrous ion (Fe^{2+}). When sufficient vinyl monomers are present in the solution, hydroxyl radicals initiate chain-growth polymerization to form a covalently crosslinked hydrogel.[59-61] Except for a few examples,[62, 63] the cyto- and biocompatibility of hydrogel crosslinked by HRP or GOX is adversely affected due to the requirement (in the case of HRP) or generation (in the case of GOX) of H_2O_2. While past efforts have demonstrated unique biomedical applications of PEG-based hydrogels crosslinked by enzymatic activity of HRP or GOX, the use of these enzymes to prepare biomimetic hydrogels that have both cell adhesiveness and protease sensitivity has attracted less attention.

Thrombin is a critical enzyme in the coagulation cascade. In the presence of factor XIIIa, an activated transglutaminase, thrombin converts soluble fibrinogen into insoluble fibrin clot. Factor XIIIa catalyzes an acyl-transfer reaction between the γ-carboxamide group of protein bound glutaminyl residues and the amino group of lysine residues to form covalent isopeptide bridges. Lutolf *et al.* harnessed this efficient reaction and developed biomimetic hydrogels formed and capable of being degraded by enzymatic reactions (**Figure 4**).[64] Specifically, factor XIIIa was utilized to simultaneously cross-link peptide-functionalized PEG and incorporate bioactive peptides. The fusion peptides used contained substrates for factor XIIIa and MMP (e.g., Ac-FKGG-GPQGIWGQ-ERCG-NH$_2$ and H-NQEQVSPL-ERCG-NH$_2$). Some sequences also contained the cell adhesive ligand, such as H-NQEQVSPL-RGDSPG-NH$_2$. Among these peptides, the sequence NQEQVSPL was derived from the N-terminus of α_2-plasmin inhibitor (α_2PI$_{1-8}$), whereas the sequence Ac-FKGG was optimized for rapid transglutaminase reaction.[65] Upon the addition of factor XIIIa and Ca^{2+}, the two segments containing Lys and Gln residues (i.e., Ac-FKGG and NQEQVSPL, respectively) were catalyzed into covalent

linkage that either has MMP sensitivity (from sequence GPQGIWGQ) or cell adhesiveness (from sequence RGDS). Depending on the gel formulations and enzyme concentrations, the gelation could occur within several minutes and the resulting gels supported spreading, proliferation, and migration of human dermal fibroblasts. Hydrogels crosslinked by factor XIIIa-mediated enzymatic reactions have been used in a variety of functional tissue engineering applications. For instance, diverse 3D peptide (e.g., RGD) or protein (e.g., fibrin, VEGF, or PDGF) patterns could be created within PEG-based hydrogels through selective light-activated enzymatic reactions.[66] hMSCs encapsulated within this dynamically patterned hydrogels showed pattern shape-guided invasion[66] and pattern gradient-induced morphogenesis.[67]
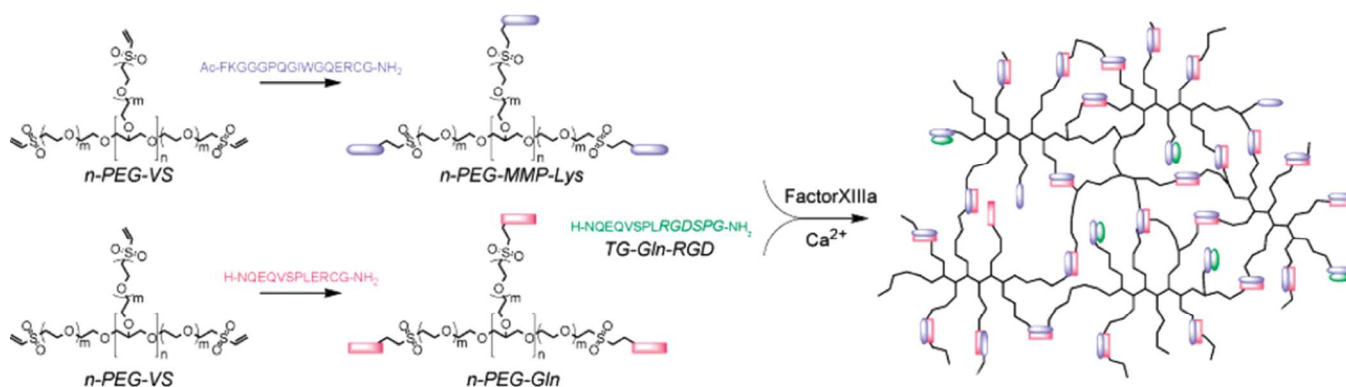
Figure 4. Schematic of Factor XIIIa-catalyzed formation of PEG-peptide biomimetic hydrogel. Factor XIIIa was used to cross-link two PEG-peptide conjugates (n-PEG-MMP-Lys and n-PEG-Gln) in combination with a cell adhesion peptide (TG-Gln-RGD) to form multifunctional biomimetic hydrogels. Reprinted with permission from [64]. Copyright 2007, American Chemical Society.

Tyrosinase, an enzyme that oxidizes phenols, is another enzyme useful in crosslinking of hydrogels and underwater bioadhesives.[68] For example, Messersmith and colleagues synthesized 3,4-Dihydroxyphenylalanine (DOPA) modified PEGs,[69] which were cross-linked into hydrogels in the presence of tyrosinase (**Figure 5**). In another example, Park *et al.* prepared tyramine-functionalized Pluronic F-127 tri-block copolymers, which were utilized to form self-assembled micelles.[70] The tyramine-conjugated micelles were converted to highly reactive catechol conjugated micelles by tyrosinase. Stable hydrogels were formed due to cross-linking of Pluronic copolymer micelles. Although these hydrogels did not contain peptide linkers sensitive to cell-secreted proteases or cell adhesion

ligands, it will be possible to create such biomimetic matrices using macromers pre-conjugated with cell adhesive and/or protease sensitive peptides.
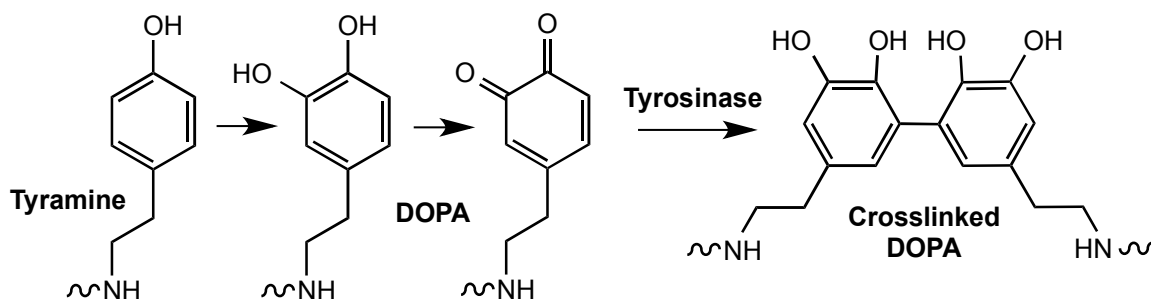

Figure 5. Oxidative conversion of tyramine to catechol and crosslinking by tyrosinase.

Click hydrogels as biomimetic matrices

'Click' chemistry is used to describe highly efficient, quantitative, and orthogonal reactions between mutually reactive functional groups and it has been used to create functional polymers and network hydrogels for biomedical applications.[71, 72] For example, Hubbell and colleagues pioneered the development of PEG-based click hydrogels. They incorporated elegant MMP-sensitive peptide sequence in the hydrogels by using nucleophilic Michael-type addition reactions between multi-arm PEG-vinylsulfone and MMP-sensitive peptide crosslinker with terminal cysteines.[20] Cell adhesive ligands could be easily conjugated using the same Michael-type addition chemistry. In addition to vinylsulfone, methacrylate, acrylate, and maleimide can also be used to react with bis-cysteine peptide (or multifunctional thiol macromers) for forming cell responsive hydrogels. The major benefit of biomimetic hydrogels formed by nucleophilic conjugation addition reaction is that it does not involve the generation of radicals, which poses major cytocompatibility concerns for radical-sensitive cells. Some of the other notable click chemistries useful in creating biomimetic hydrogels include native chemical ligation,[69, 73] oxime-ligand,[74] azide-alkyne addition,[75-78] Diels-Alder reaction[79] and tetrazine chemistry.[80, 81] Similar to the Michael-type conjugation reaction, these chemoselective chemistries are not light dependent and do not require initiators for initiating gel crosslinking. In general, this gelation chemistry lacks spatial-temporal control in gelation kinetic. Furthermore, the reaction rates may be slow at neutral pH value.

The cross-linking chemistries discussed above have been highly useful in creating biomimetic hydrogels for 3D cell studies. However, most of these chemistries do not permit dynamic modification of biophysical or biochemical gel properties. The ability to dynamically control gel properties is especially important if one considers that, stem and progenitor cells receive complex and dynamic extracellular signals during morphogenesis. Pathological processes in many diseases are also induced by the deregulation of biological signals. It has become increasingly evident that biomaterials capable of mimicking dynamic changes of biological cues are powerful tools for studying tissue regeneration. Significant efforts have been dedicated to formulating such dynamic matrices. For example, Shoichet *et al.* incorporated a nitrobenzyl-protected cysteine in agarose hydrogels to guide 3D cell growth and migration.[82, 83] Upon UV exposure, the nitrobenzyl group is removed, revealing the free sulfhydryl group for additional thiol-maleimide conjugation. Biomolecules can be patterned in 3D to guide cell migration in a spatial-temporally regulated manner.

It will be beneficial if the modification of hydrogel properties can be performed in the presence of cells. For example, Fairbanks *et al.* developed step-growth thiol-norbornene hydrogels that were crosslinked with an excess amount of norbornene functionality during network crosslinking.[45] Due to the lack of homopolymerization between norbornene groups and the step-growth nature of thiol-norbornene reaction, additional thiol-bearing molecules can be patterned within the gel network in the presence of cells. In addition to immobilizing pendant ligand in the presence of cells, one may wish to 'exchange' the ligands to truly recapitulate a dynamic developmental process during tissue morphogenesis. In this regard, an addition-fragmentation-chain transfer reaction was developed to allow controlled and reversible exchange of biochemical ligands within an allyl sulfide functionalized PEG hydrogel (**Figure 6**).[84]
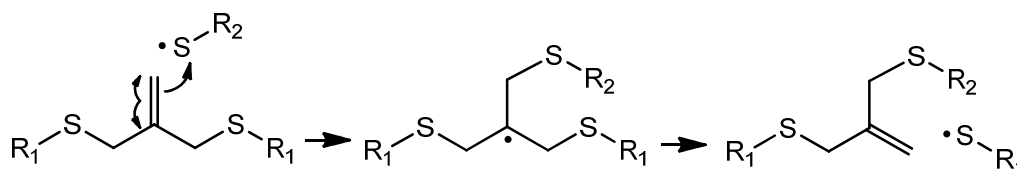

Figure 6. Mechanism of addition fragmentation chain transfer of an allyl sulfide functional group upon attack by a thiyl radical. Redraw with permission from [84]. Copyright 2014, John Wiley and Sons.

Significant achievements have been made in using orthogonal 'click' chemistry to synthesized biomimetic and dynamically tunable hydrogels. Anseth and colleagues created photolabile hydrogels by incorporating nitrobenzyl groups to the PEG or peptide crosslinkers.[85-87] Hydrogels were firstly formed via redox crosslinking and gel crosslinking density was decreased in a spatial-temporally controlled manner by adjusting the dosage and location of UV light exposure. When the photolabile group was incorporated on pendant peptides, UV light exposure caused the liberation of these peptides. The system is cytocompatible and the modification of hydrogel properties could be performed in the presence of cells.

Dynamically tunable hydrogels can be prepared by copper-catalyzed azide-alkyne Huisgen cycloaddition reactions (CuAAC). Anseth *et al.* used this chemistry to fabricate PEG-based hydrogels with patternability.[88] Unfortunately, this reaction is not suitable for *in situ* cell encapsulation due to the cytotoxicity of copper ion. Later, they developed an alternative approach where cyclooctyne, a macromer synthesized originally by the Berttozi group,[89] was used to react with azide free of cytotoxic metal ions.[76] The metal-free and orthogonal reactivity between a strained cyclooctyne and an azide has allowed researchers to design multifunctional macromers that can be cleaved by cell-secreted proteases and for spatial-temporally controlled conjugation of bioactive motifs. For example, DeForest and Anseth designed a 'sequential-click' approach in a step-growth network to allow for forming the hydrogel network and modifying the properties through orthogonal conjugation and cleavage of biomimetic peptides.[76-78, 90] To expand the utility of dynamic patterning of bioactive motifs in 3D, DeForest and Tirrell recently reported the conjugation and removal of whole protein within the orthogonally crosslinked network.[91] Two bioorthogonal photochemistries, oxime ligation (**Figure 7A**) and ortho-nitrobenzyl esterphotocission (**Figure 7B**), were employed to permit user-defined spatial-temporal photo-patterning and removal of whole proteins (**Figure 7C**). The Anseth group has expanded the toolkits of bio-orthogonal click hydrogels to include: (1) tetrazine-norbornene click reaction (**Figure 8A**)[80] and (2) ligation between aliphatic hydrazine with a benzaldehyde- or an aliphatic aldehyde (**Figure 8B**).[92] The hydrazine-aldehyde click reaction is particularly intriguing as it results a covalently

adaptable network that can respond to cell-induced stress through breaking/reforming elastically active crosslinks while maintaining a macroscopically stable material.

A.

B.

C.
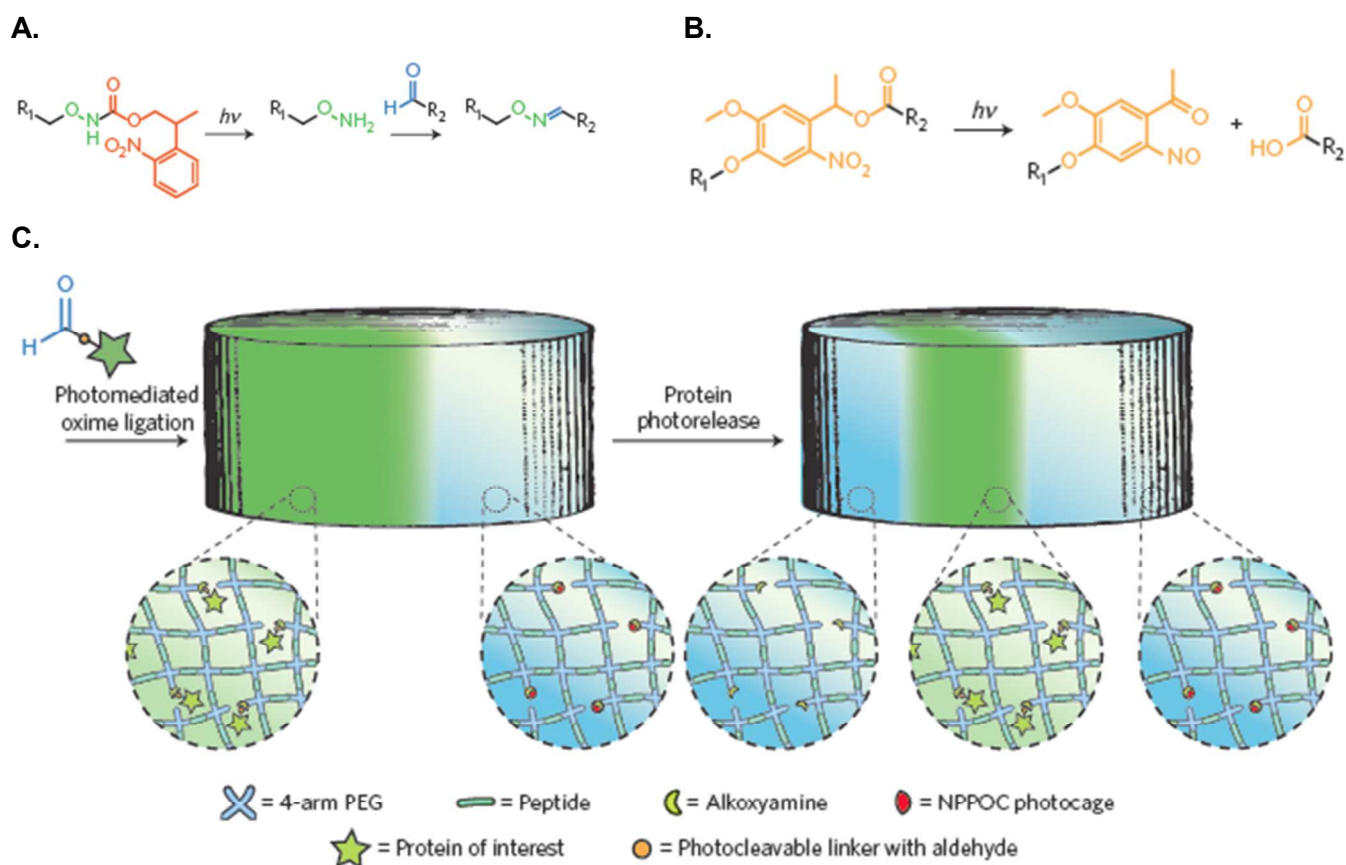
Figure 7. (A) Caged alkoxyamines undergo irreversible β-elimination upon exposure to 365 or 740nm light. The liberated alkoxyamines react with aldehyde-functionalized proteins (R**2**) to form oxime linkages. (B) **o**-nitrobenzyl ester (**o**NB) moieties linking the protein of interest (R**1**) and the hydrogel (R**2**) undergo photocleavage upon exposure to 365nm or 740nm light. (C) Schematic representation of the photo-reversible patterning strategy. NHS-**o**NB-CHO-functionalized proteins are first tethered to the gel through photo-mediated oxime ligation and subsequently removed on secondary light exposure. Reprinted with permission from [91]. Copyright 2015, Nature Publishing Group.

A.

B.
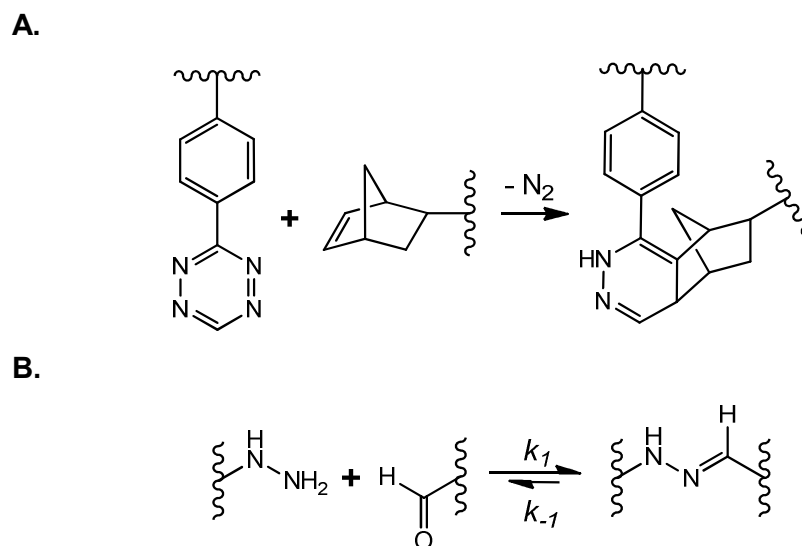
Figure 8. (A) Tetrazine-norbornene click reaction; (B) Covalently adaptable network formed by N-methyl hydrazine-butyraldehyde ligation.

**Hydrogels formed from supramolecular assembly**

In addition to the aforementioned covalent crosslinking strategies, PEG-based biomimetic hydrogels can be prepared through macromolecular or supramolecular self-assembly. Macromolecular/supramolecular assembled hydrogels are favorable in many applications because the gelation was induced by a purely physical process, which does not rely on radical species.[93] However, when compared with covalently crosslinked hydrogels, these gels can have weaker mechanical properties due to the instability of physical interactions between macromolecules. The most commonly used macromolecules suitable for forming self-assembled hydrogels are amphiphilic cyclodextrins (CD), including α-CD, β-CD, and γ-CD that composes 6, 7, and 8 cyclic saccharides.[94, 95] The inner hydrophobic cavity of CDs affords physical interactions with hydrophobic molecules while the hydrophilic outer surface facilitates the dissolution of the molecules in aqueous environment. The hydroxyl groups on CDs provide handles for facile chemical modifications, which expand the utility of CDs in biomaterials, drug delivery, and tissue engineering applications.

Supramolecular polymers are increasingly being used as 'building blocks' to fabricate diverse 3D crosslinked polymer networks. For example, macrocyclic CD and cucurbit[n]uril are routinely used

with specific 'guest' molecules to form poly-rotaxane or catenane. In particular, CD forms inclusion complexes with hydrophobic guest molecules, such as adamantane, azobenzene, ferrocene, and stilbene.[93] In one example, Burdick and colleagues harnessed the inclusion complex formation between CD and adamantane (Ad) that are separately conjugated to hyaluronic acid (HA) to prepare shear-thinning hydrogels for tissue engineering applications.[96-98] The inclusion complexes formed between CD and Ad led to HA hydrogel formation but the complexes disassembled under shear force, leading to gel-sol transition. Upon the removal of shear force, the host-guest complexes, and hence the crosslinked hydrogel, re-forms. This new class of supramolecular polymer offers high injectability for implantation biomaterials in a minimally invasive manner. Similar to CDs that bind to hydrophobic molecules, curbit[n]uril (CB) binds strongly to cationic guest molecules, such as naphthalene and viologen. The complexation between CB and naphthyl-/viologen-functionalized polymers has been used to prepare protein-loaded hydrogels for controlled release applications.[99-101] The aforementioned examples have demonstrated the great utilities of supramolecular assembly in hydrogel formation for biomedical applications.

Supramolecular assembly strategies have also been integrated in the design of PEG-based biomimetic hydrogels. For example, Elisseeff and colleagues have developed a αCD-threaded PEGDA hydrogels for tuning biophysical and biochemical properties of chain-growth PEG-based hydrogels.[102] **Figure 9** illustrates how supramolecular polymers can serve as 'carriers' to impart multiple and orthogonal functionalities to a hydrogel network. The PEG 'necklaces' are decorated/threaded with functionalized αCD to produce hydrogels with tunable mechanics (by changing PEGDA concentration), adhesion (by adjusting concentration of functionalized αCD but not PEGDA), or chemistry (by introducing αCD with different functional groups, e.g., $-CH_3$ or $-PO^{4-}$). By manipulating the compositions of these supramolecules, multifunctional hydrogels were created for promoting adhesion, proliferation, and differentiation of hMSCs[102] or for studying the roles of matrix mechanics and functions on cancer cell invasion.[103] While not demonstrated in this publication, protease-sensitivity can be integrated into the PEG-CD hydrogels by replacing PEGDA with diacrylated PEG-peptide macromers (**Figure 1C**).
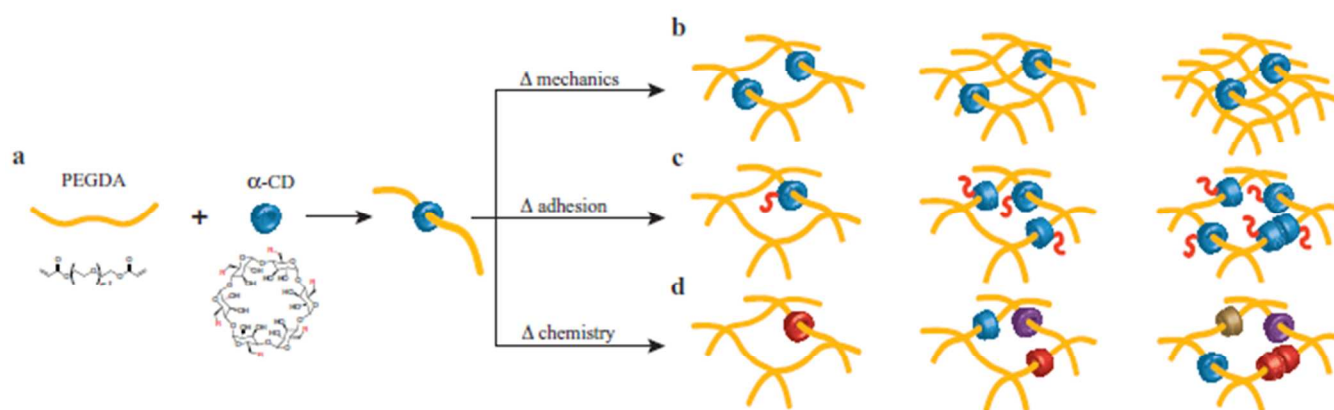
Figure 9. Schematic of a molecular-necklace system to create multifunctional hydrogels with independent control of gel mechanics, cell adhesiveness, and chemical functionality. a) Inclusion complex between PEGDA and α-CD (R = hydroxyl or other functional groups). b) Tuning the mechanical properties of α-CD-PEG hydrogel independent of α-CD concentration. c) Tuning the concentration of cell adhesive peptide through threading different amount of functionalized α-CD independent of gel cross-linking density. d) Tuning the chemical functionality of hydrogel through threading αCD with different functional groups (i.e., hydrophobic, hydrophilic or charged groups). Reprinted with permission from [102]. Copyright 2013, John Wiley and Sons.

The example shown in **Figure 9** exploited modified CD as carriers of functional or bioactive motifs. The self-assembly between CD and PEG has also been utilized to prepare 'pseudopolyrotaxane' hydrogels. For instance, Cooper-White *et al.* explored the gelation kinetics and viscoelastic properties of such hydrogels formed by assembly of αCD and pluronic polymers (F68 and F127) either without or with micelle formation.[104] The formation of 'poly-CDs' at a concentration of over 40mM of αCD (25°C) is critical in this physical gelation/assembly system. The threading of pluronic polymers into pre-assembled poly-CDs leads to gelation (**Figure 10A**). In the presence of pluronic micelles, however, the threading of poly-CDs to pluronic polymer becomes more difficult due to the presence of steric hindrance (**Figure 10B**). The gelation was slower and the gels were weaker in the presence of pluronic micelles. Although these hydrogels could be formed with strong mechanical properties (G' ~ 10^6 Pa), they were not stable and dissociate rapidly when immersing in a liquid containing no pseudopolyrotaxane molecules. Cooper-White and colleagues recently reported an improved design of pseudopolyrotaxane hydrogels in which pluronic/CD assembly was combined with enzyme-mediated crosslinking of tyramine-modified PEG.[105] The gelation was facilitated through a horseradish peroxidase (HRP) and hydrogen peroxide (H_2O_2) mediated tyramine oxidation (similar to

Figure 5). However, careful optimization of parameters in enzymatic crosslinking is critical in maintaining acceptable cell viability since H_2O_2 is toxic to cells.
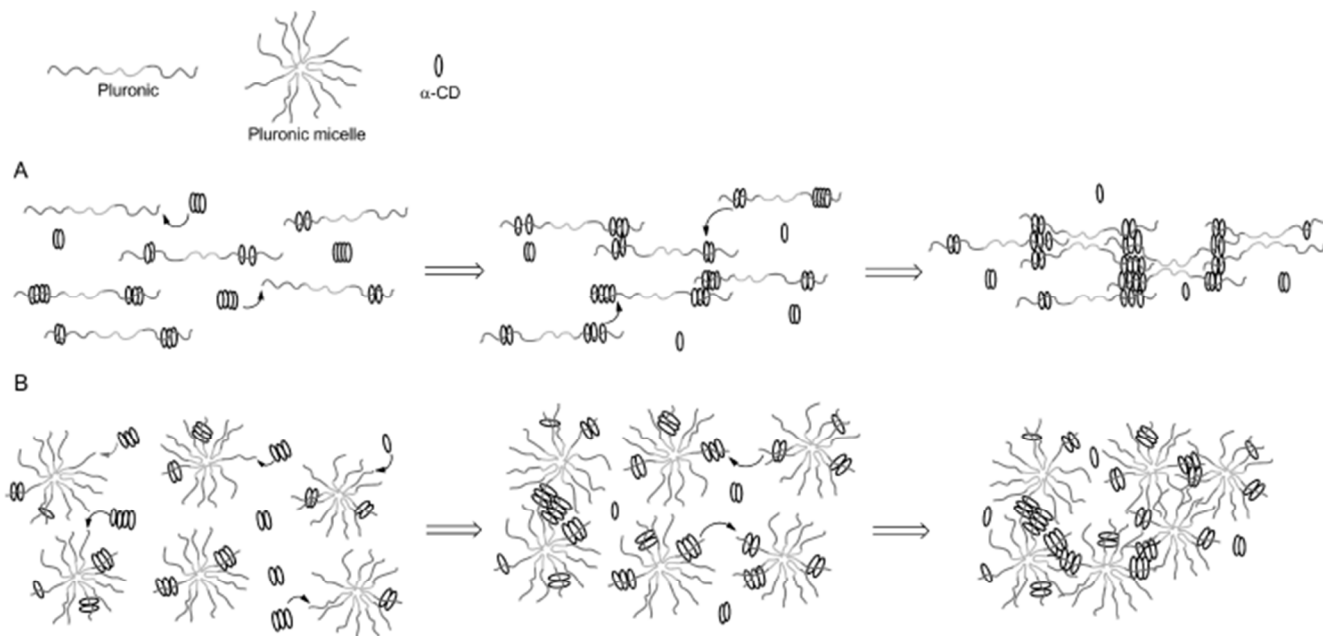
Figure 10. Temporal gelation mechanism of linear Pluronic (e.g.: F6810 and F6820) or Pluronic micelle (e.g.: F12710) and αCD. (A) Gelation without micelle formation. (B) Gelation in the presence of micelles. Reprinted with permission from [104]. Copyright 2013, American Chemical Society.

Conclusions

The diversity of biomimetic PEG-based hydrogels has been expanded greatly in recent years owing to the discovery/adaptation of bioconjugation chemistries suitable for biomedical applications. Past efforts have also demonstrated that a hydrogel matrix with multiple functionalities outperforms a single-purpose one for most tissue regeneration applications. While no single chemistry is perfect for all applications, one can certainly adopt and integrate the available chemistries to create suitable biomimetic matrices for a particular tissue regeneration need. It is expected that, due to its simplicity, diversity, and proven cytocompatibility in many cell types, radical-mediated hydrogel crosslinking (whether initiated by UV or visible light) will continue to serve as an indispensable chemistry for the design and synthesis of biomimetic hydrogels. The rise of bio-orthogonal chemistry, enzyme-mediated

crosslinking, and supramolecular chemistries offer great opportunities for scientists to create complex multifunctional hydrogel matrices for addressing biological questions that are otherwise difficult to answer. Lastly, the combination of two or more of these diverse chemistries is anticipated to significantly increase the applicability of biomimetic hydrogels in tissue engineering and regenerative medicine applications.

Acknowledgement

Reference:

1.   N. A. Peppas, J. Z. Hilt, A. Khademhosseini, and R. Langer, *Adv. Mater.* **2006**, *18*, 1345-1360.
2.   C. C. Lin and K. S. Anseth, *Pharma. Res.* **2009**, *26*, 631-643.
3.   M. W. Tibbitt and K. S. Anseth, *Biotechnol. Bioeng.* **2009**, *103*, 655-663.
4.   M. P. Lutolf and J. A. Hubbell, *Nature Biotechnology* **2005**, *23*, 47-55.
5.   D. A. Wang, C. G. Williams, Q. A. Li, B. Sharma, and J. H. Elisseeff, *Biomaterials* **2003**, *24*, 3969-3980.
6.   S. J. Bryant, K. S. Anseth, D. A. Lee, and D. L. Bader, *Journal of Orthopaedic Research* **2004**, *22*, 1143-1149.
7.   S. J. Bryant, R. J. Bender, K. L. Durand, and K. S. Anseth, *Biotechnology and Bioengineering* **2004**, *86*, 747-755.
8.   S. J. Bryant, T. T. Chowdhury, D. A. Lee, D. L. Bader, and K. S. Anseth, *Annals of Biomedical Engineering* **2004**, *32*, 407-417.
9.   X. Z. Shu, Y. C. Liu, F. S. Palumbo, Y. Lu, and G. D. Prestwich, *Biomaterials* **2004**, *25*, 1339-1348.
10. S. R. Peyton, C. B. Raub, V. P. Keschrumrus, and A. J. Putnam, *Biomaterials* **2006**, *27*, 4881-4893.
11. L. M. Weber, C. G. Lopez, and K. S. Anseth, *Journal of Biomedical Materials Research Part A* **2009**, *90A*, 720-729.
12. C.-C. Lin and K. S. Anseth, *Advanced Functional Materials* **2009**, *19*, 2325-2331.
13. C.-C. Lin and A. T. Metters, *Journal of Biomedical Materials Research Part A* **2007**, *83A*, 954-964.
14. C.-C. Lin and A. T. Metters, *Biomacromolecules* **2008**, *9*, 789-795.
15. C.-C. Lin, A. T. Metters, and K. S. Anseth, *Biomaterials* **2009**, *30*, 4907-4914.
16. C.-C. Lin, P. D. Boyer, A. A. Aimetti, and K. S. Anseth, *Journal of Controlled Release* **2010**, *142*, 384-391.
17. S. M. Willerth, P. J. Johnson, D. J. Maxwell, S. R. Parsons, M. E. Doukas, and S. E. Sakiyama-Elbert, *Journal of Biomedical Materials Research Part A* **2007**, *80A*, 13-23.
18. D. L. Hern and J. A. Hubbell, *Journal of Biomedical Materials Research* **1998**, *39*, 266-276.
19. D. L. Elbert and J. A. Hubbell, *Biomacromolecules* **2001**, *2*, 430-441.
20. M. P. Lutolf, J. L. Lauer-Fields, H. G. Schmoekel, A. T. Metters, F. E. Weber, G. B. Fields, and J. A. Hubbell, *Proc. Natl. Acad. Sci. USA.* **2003**, *100*, 5413-5418.
21. A. B. Pratt, F. E. Weber, H. G. Schmoekel, R. Muller, and J. A. Hubbell, *Biotechnol. Bioeng.* **2004**, *86*, 27-36.
22. S. R. Peyton, C. M. Ghajar, C. B. Khatiwala, and A. J. Putnam, *Cell Biochemistry and Biophysics* **2007**, *47*, 300-320.
23. A. M. Kloxin, C. J. Kloxin, C. N. Bowman, and K. S. Anseth, *Advanced Materials* **2010**, *22*, 3484-3494.
24. K. A. Mosiewicz, L. Kolb, A. J. van der Vlies, and M. P. Lutolf, *Biomaterials Science* **2014**, *2*, 1640-1651.
25. N. Huebsch, P. R. Arany, A. S. Mao, D. Shvartsman, O. A. Ali, S. A. Bencherif, J. Rivera-Feliciano, and D. J. Mooney, *Nature Materials* **2010**, *9*, 518-526.
26. S. Khetan, M. Guvendiren, W. R. Legant, D. M. Cohen, C. S. Chen, and J. A. Burdick, *Nature Materials* **2013**, *12*, 458-465.
27. C. Yang, M. W. Tibbitt, L. Basta, and K. S. Anseth, *Nature Materials* **2014**, *13*, 645-652.
28. X. Tong and F. Yang, *Biomaterials* **2014**, *35*, 1807-1815.
29. M. C. Cushing and K. S. Anseth, *Science* **2007**, *316*, 1133-1134.
30. C. A. DeForest and K. S. Anseth, *Annu. Rev. Chem. Biomol. Eng.* **2012**, *3*, 421-444.
31. B. V. Slaughter, S. S. Khurshid, O. Z. Fisher, A. Khademhosseini, and N. A. Peppas, *Adv. Mater.* **2009**, *21*, 3307-3329.
32. K. T. Nguyen and J. L. West, *Biomaterials* **2002**, *23*, 4307-4314.

RSC Advances Accepted Manuscript

33. C. R. Nuttelman, M. A. Rice, A. E. Rydholm, C. N. Salinas, D. N. Shah, and K. S. Anseth, *Prog. Polym. Sci.* **2008**, *33*, 167-179.
34. J. L. West and J. A. Hubbell, *Reactive Polymers* **1995**, *25*, 139-147.
35. J. L. West and J. A. Hubbell, *Macromolecules* **1999**, *32*, 241-244.
36. Y. T. Hao, H. Shih, Z. Munoz, A. Kemp, and C. C. Lin, *Acta Biomaterialia* **2014**, *10*, 104-114.
37. S.-H. Lee, J. J. Moon, J. S. Miller, and J. L. West, *Biomaterials* **2007**, *28*, 3163-3170.
38. M. N. Mason, A. T. Metters, C. N. Bowman, and K. S. Anseth, *Macromolecules* **2001**, *34*, 4630-4635.
39. A. T. Metters, K. S. Anseth, and C. N. Bowman, *Polymer* **2000**, *41*, 3993-4004.
40. B. K. Mann, A. S. Gobin, A. T. Tsai, R. H. Schmedlen, and J. L. West, *Biomaterials* **2001**, *22*, 3045-3051.
41. C. C. Lin, A. Raza, and H. Shih, *Biomaterials* **2011**, *32*, 9685-9695.
42. B. D. Fairbanks, M. P. Schwartz, A. E. Halevi, C. R. Nuttelman, C. N. Bowman, and K. S. Anseth, *Adv Mater.* **2009**, *21*, 5005-5010.
43. H. Shih and C. C. Lin, *Biomacromolecules* **2012**, *13*, 2003-2012.
44. A. Metters and J. Hubbell, *Biomacromolecules* **2005**, *6*, 290-301.
45. B. D. Fairbanks, M. P. Schwartz, A. E. Halevi, C. R. Nuttelman, C. N. Bowman, and K. S. Anseth, *Adv. Mater.* **2009**, *21*, 5005-+.
46. C. S. Ki, T.-Y. Lin, M. Korc, and C.-C. Lin, *Biomaterials* **2014**, *35*, 9668-9677.
47. T.-Y. Lin, C. S. Ki, and C.-C. Lin, *Biomaterials* **2014**, *35*, 6898-6906.
48. C. Wang, X. Tong, and F. Yang, *Molecular Pharmaceutics* **2014**, *11*, 2115-2125.
49. J. J. Roberts and S. J. Bryant, *Biomaterials* **2013**, *34*, 9969-9979.
50. H. Shih and C. C. Lin, *Macromol. Rapid Commun.* **2013**, *34*, 269-273.
51. H. Shih, A. K. Fraser, and C. C. Lin, *ACS Appl. Mater. Interfaces.* **2013**, *5*, 1673-1680.
52. H. Shih, R. G. Mirmira, and C.-C. Lin, *Journal of Materials Chemistry B* **2015**, *3*, 170-175.
53. C.-C. Lin, C. S. Ki, and H. Shih, *Journal of Applied Polymer Science* **2015**, *132*,
54. C. N. Salinas and K. S. Anseth, *Macromolecules* **2008**, *41*, 6019-6026.
55. A. E. Rydholm, C. N. Bowman, and K. S. Anseth, *Biomaterials* **2005**, *26*, 4495-4506.
56. S. J. Bryant, C. R. Nuttelman, and K. S. Anseth, *Journal of Biomaterials Science-Polymer Edition* **2000**, *11*, 439-457.
57. G. M. Cruise, O. D. Hegre, D. S. Scharp, and J. A. Hubbell, *Biotechnol. Bioeng.* **1998**, *57*, 655-665.
58. Y. Hao and C.-C. Lin, *Journal of Biomedical Materials Research Part A* **2014**, *102*, 3813-3827.
59. L. M. Johnson, C. A. DeForest, A. Pendurti, K. S. Anseth, and C. N. Bowman, *Acs Applied Materials & Interfaces* **2010**, *2*, 1963-1972.
60. P. S. Hume, C. N. Bowman, and K. S. Anseth, *Biomaterials* **2011**, *32*, 6204-6212.
61. R. Shenoy, M. W. Tibbitt, K. S. Anseth, and C. N. Bowman, *Chemistry of Materials* **2013**, *25*, 761-767.
62. L.-S. Wang, J. E. Chung, P. P.-Y. Chan, and M. Kurisawa, *Biomaterials* **2010**, *31*, 1148-1157.
63. D. J. Menzies, A. Cameron, T. Munro, E. Wolvetang, L. Grondahl, and J. J. Cooper-White, *Biomacromolecules* **2013**, *14*, 413-423.
64. M. Ehrbar, S. C. Rizzi, R. G. Schoenmakers, B. San Miguel, J. A. Hubbell, F. E. Weber, and M. P. Lutolf, *Biomacromolecules* **2007**, *8*, 3000-3007.
65. B. H. Hu and P. B. Messersmith, *Journal of the American Chemical Society* **2003**, *125*, 14298-14299.
66. K. A. Mosiewicz, L. Kolb, A. J. van der Vlies, M. M. Martino, P. S. Lienemann, J. A. Hubbell, M. Ehrbar, and M. P. Lutolf, *Nature materials* **2013**, *12*, 1072-8.
67. P. S. Lienemann, Y. R. Devaud, R. Reuten, B. R. Simona, M. Karlsson, W. Weber, M. Koch, M. P. Lutolf, V. Milleret, and M. Ehrbar, *Integrative Biology* **2015**, *7*, 101-111.
68. M. Cencer, Y. Liu, A. Winter, M. Murley, H. Meng, and B. P. Lee, *Biomacromolecules* **2014**, *15*, 2861-2869.
69. B. P. Lee, J. L. Dalsin, and P. B. Messersmith, *Biomacromolecules* **2002**, *3*, 1038-1047.
70. S. H. Lee, Y. Lee, S.-W. Lee, H.-Y. Ji, J.-H. Lee, D. S. Lee, and T. G. Park, *Acta Biomaterialia* **2011**, *7*, 1468-1476.

71. Y. Jiang, J. Chen, C. Deng, E. J. Suuronen, and Z. Zhong, *Biomaterials* **2014**, *35*, 4969-4985.
72. S. Liu, K. T. Dicker, and X. Jia, *Chemical communications (Cambridge, England)* **2015**, *51*, 5218-37.
73. B.-H. Hu, J. Su, and P. B. Messersmith, *Biomacromolecules* **2009**, *10*, 2194-2200.
74. G. N. Grover, J. Lam, T. H. Nguyen, T. Segura, and H. D. Maynard, *Biomacromolecules* **2012**, *13*, 3013-3017.
75. D. D. McKinnon, T. E. Brown, K. A. Kyburz, E. Kiyotake, and K. S. Anseth, *Biomacromolecules* **2014**, *15*, 2808-2816.
76. C. A. DeForest, B. D. Polizzotti, and K. S. Anseth, *Nat. Mater.* **2009**, *8*, 659-664.
77. C. A. DeForest, E. A. Sims, and K. S. Anseth, *Chem. Mater.* **2010**, *22*, 4783-4790.
78. C. A. DeForest and K. S. Anseth, *Nat. Chem.* **2011**, *3*, 925-931.
79. K. C. Koehler, D. L. Alge, K. S. Anseth, and C. N. Bowman, *Biomaterials* **2013**, *34*, 4150-4158.
80. D. L. Alge, M. A. Azagarsamy, D. F. Donohue, and K. S. Anseth, *Biomacromolecules* **2013**, *14*, 949-953.
81. H. Zhang, K. T. Dicker, X. Xu, X. Jia, and J. M. Fox, *Acs Macro Letters* **2014**, *3*, 727-731.
82. Y. Luo and M. S. Shoichet, *Biomacromolecules* **2004**, *5*, 2315-2323.
83. Y. Luo and M. S. Shoichet, *Nature Materials* **2004**, *3*, 249-253.
84. N. R. Gandavarapu, M. A. Azagarsamy, and K. S. Anseth, *Advanced Materials* **2014**, *26*, 2521-2526.
85. A. M. Kloxin, A. M. Kasko, C. N. Salinas, and K. S. Anseth, *Science* **2009**, *324*, 59-63.
86. A. M. Kloxin, J. A. Benton, and K. S. Anseth, *Biomaterials* **2010**, *31*, 1-8.
87. A. M. Kloxin, M. W. Tibbitt, A. M. Kasko, J. A. Fairbairn, and K. S. Anseth, *Advanced Materials* **2010**, *22*, 61-+.
88. B. D. Polizzotti, B. D. Fairbanks, and K. S. Anseth, *Biomacromolecules* **2008**, *9*, 1084-1087.
89. N. J. Agard, J. A. Prescher, and C. R. Bertozzi, *Journal of the American Chemical Society* **2004**, *126*, 15046-15047.
90. C. A. DeForest and K. S. Anseth, *Angew. Chem. Int. Ed.* **2012**, *51*, 1816-1819.
91. C. A. DeForest and D. A. Tirrell, *Nat Mater* **2015**,
92. D. D. McKinnon, D. W. Domaille, J. N. Cha, and K. S. Anseth, *Advanced Materials* **2014**, *26*, 865-872.
93. R. Dong, Y. Zhou, X. Huang, X. Zhu, Y. Lu, and J. Shen, *Advanced Materials* **2015**, *27*, 498-526.
94. J. Zhang and P. X. Ma, *Advanced Drug Delivery Reviews* **2013**, *65*, 1215-1233.
95. B. V. K. J. Schmidt, M. Hetzer, H. Ritter, and C. Barner-Kowollik, *Progress in Polymer Science* **2014**, *39*, 235-249.
96. M. Guvendiren, H. D. Lu, and J. A. Burdick, *Soft Matter* **2012**, *8*, 260-272.
97. C. B. Rodell, A. L. Kaminski, and J. A. Burdick, *Biomacromolecules* **2013**, *14*, 4125-4134.
98. C. B. Rodell, J. W. MacArthur, Jr., S. M. Dorsey, R. J. Wade, L. L. Wang, Y. J. Woo, and J. A. Burdick, *Advanced Functional Materials* **2015**, *25*, 636-644.
99. E. A. Appel, F. Biedermann, U. Rauwald, S. T. Jones, J. M. Zayed, and O. A. Scherman, *Journal of the American Chemical Society* **2010**, *132*, 14251-14260.
100. E. A. Appel, X. J. Loh, S. T. Jones, F. Biedermann, C. A. Dreiss, and O. A. Scherman, *Journal of the American Chemical Society* **2012**, *134*, 11767-11773.
101. E. A. Appel, X. J. Loh, S. T. Jones, C. A. Dreiss, and O. A. Scherman, *Biomaterials* **2012**, *33*, 4646-4652.
102. A. Singh, J. Zhan, Z. Ye, and J. H. Elisseeff, *Advanced Functional Materials* **2013**, *23*, 575-582.
103. J. N. Beck, A. Singh, A. R. Rothenberg, J. H. Elisseeff, and A. J. Ewald, *Biomaterials* **2013**, *34*, 9486-9495.
104. C. Pradal, K. S. Jack, L. Grondahl, and J. J. Cooper-White, *Biomacromolecules* **2013**, *14*, 3780-3792.
105. C. Pradal, L. Grondahl, and J. J. Cooper-White, *Biomacromolecules* **2015**, *16*, 389-403.