Bioconjugate Chemistry

Review

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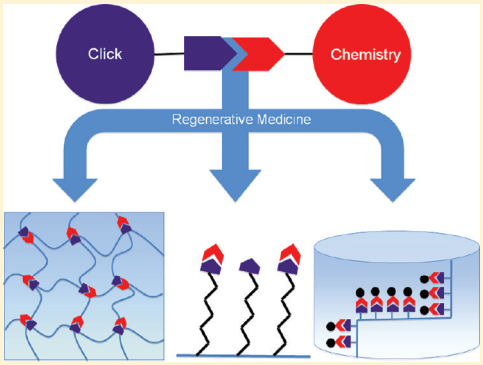
Regenerative Biomaterials that "Click": Simple, Aqueous-Based Protocols for Hydrogel Synthesis, Surface Immobilization, and 3D Patterning

Chelsea M. Nimmo[†,‡] and Molly S. Shoichet*[,†,‡,§,∥]

[†]The Donnelly Centre for Cellular and Biomolecular Research, [‡]Department of Chemistry, [§]Department of Chemical Engineering and Applied Chemistry, and [∥]Institute of Biomaterials and Biomedical Engineering, University of Toronto, Toronto, Ontario, Canada

ABSTRACT: The click chemistry era has generated a library of versatile "spring-loaded" reactions that offer high yields, regio- and stereospecificity, and outstanding functional group tolerance. These powerful transformations are particularly advantageous for the design of sophisticated biomaterials that require high levels of precision and control, namely, materials that promote tissue regeneration such as hydrogels, 2D functionalized substrates, and 3D biomimetic scaffolds. In this review, the synthesis and application of regenerative biomaterials via click chemistry are summarized. Particular emphasis is placed on the copper(I)-catalyzed alkyne−azide cycloaddition, Diels−Alder cycloadditions, and thiol−click coupling.



INTRODUCTION

In the simplest terms, tissue engineering has been defined as the delivery of biomolecules, cells, and supporting structures to the body to promote self-healing.[1] To accomplish this, biocompatible materials are required to serve as either structural supports for tissue regeneration or delivery vehicles for cell and drug transplantation. Biomaterials can also represent investigative tools to elucidate mechanisms vital to regeneration. Common materials employed within regenerative medicine strategies include both two-dimensional (2D) substrates and three-dimensional (3D) biomaterials. 2D substrates are most often used as exploratory tools to study the presentation of specific bioactive factors on cell fate. 3D biomaterials are similarly exploited, but can also be manipulated to serve as a space filling agent, a delivery vehicle, or a tissue engineering scaffold.[2] These strategies require well-defined structural materials and surfaces that impose sophisticated function in order to advance the field of regenerative medicine. This has created a trend toward the convergence of synthetic organic techniques within regenerative biomaterials.

The exponential growth of click chemistry research within the past decade has greatly facilitated the development of chemoselective chemistries applicable within regenerative medicine. In 2001, Sharpless and co-workers introduced the term "click chemistry" to define a set of nearly perfect reactions that resemble natural biochemical ligations.[3] These "spring-loaded" reactions are orthogonal, regioselective, and highly efficient. Moreover, click reactions can be performed in aqueous solutions at room or physiological temperature, and display outstanding functional group tolerance, making them compelling reactions within the bioengineering toolkit for polymer synthesis and bioconjugation.[4]

Given that there has been a number of outstanding reviews written on polymer synthesis via click chemistry for biomedical applications,[4−7] this review will focus on the emerging trend of click chemistry within the field of regenerative medicine. A prime example is click cross-linked hydrogels. Click reactions have also gained popularity as bioconjugation techniques for decorating 2D cell substrates, and as elegant patterning chemistries for immobilizing bioactive factors within 3D scaffolds (Figure 1). The review begins with a brief overview of three common click reactions employed within regenerative biomaterials, and further highlights their use in tissue engineering and regenerative medicine.

COMMON CLICK REACTIONS IN REGENERATIVE BIOMATERIALS

The term click chemistry often refers to the common copper(I)-catalyzed alkyne−azide cycloaddition (CuAAC) (Scheme 1A). This reaction is very similar to the classic Huisgen cycloaddition[8] where an organic azide reacts with an alkyne to form a triazole ring. Through the addition of a Cu(I) catalyst, Meldal[9] and Sharpless[10] demonstrated that the Huisgen cycloaddition reaction can proceed at low temperatures with high rates, efficiency, and regiospecificity. Moreover, near-perfect conversion is obtainable in both aqueous and organic

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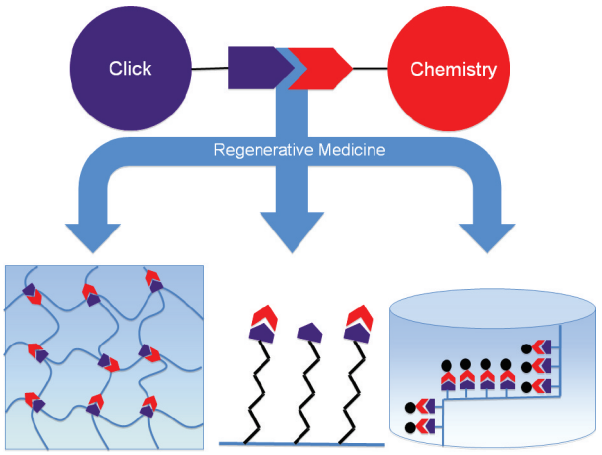
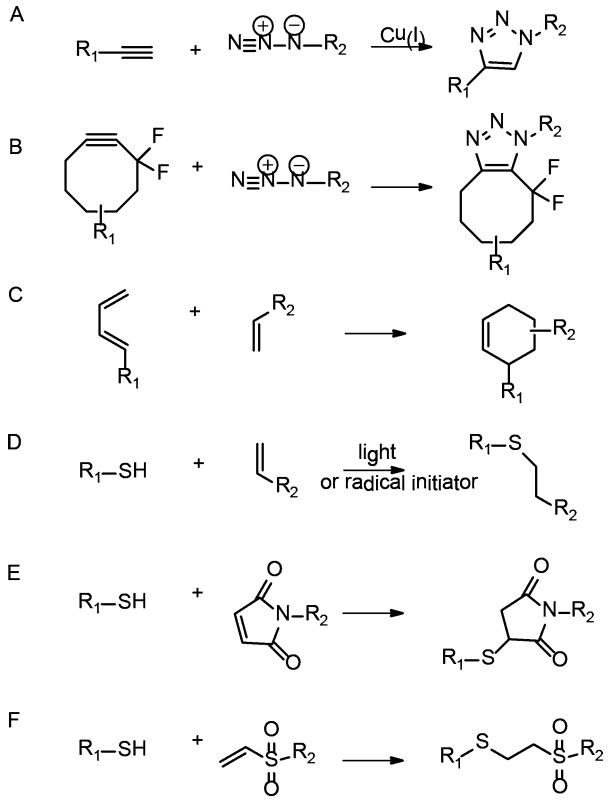


Figure 1. Schematic representation of click chemistry applied to regenerative biomaterials. Click chemistry has been employed as a cross-linking chemistry for hydrogel synthesis, and as bioconjugation techniques for decorating 2D cell culture.

Scheme 1. Common Click Reactions for Regenerative Biomatenals[a]



[a](A) Copper(I)-catalyzed alkyne–azide cycloaddition (CuAAC), (B) azide–alkyne coupling (SPAAC), (C) Diels–Alder (DA) cycloaddition, (D) Radical- mediated thiol–ene coupling, (E) Thiol-Michael addition between maleimides, and (F) between thiols and vinyl sulfones.

solvents. CuAAC has proved to be particularly advantageous within biomedical applications considering that the starting materials, azides and terminal alkynes, are remarkably stable within biological systems, enabling facile introduction of these reactive groups into a wide range of biomolecules. Moreover, alkynes and azides are not found in nature, thereby limiting side products from forming.

However, the term click chemistry is not limited to the CuAAC reaction, but embodies a synthetic philosophy of many reactions with distinct mechanisms and conditions. A comparison of click chemistries for regenerative biomaterials is summarized in Table 1. According to Sharpless, a reaction can achieve click status if it consists of readily available orthogonal reactants that combine under mild conditions to produce a single stereospecific product, with little or no isolation.[3] Within the past decade, there has been increasing investigation into reactions that meet this definition, yet do not require a metal catalyst.[11] The Bertozzi lab has developed a reaction of azides with cyclooctyne derivatives[12] referred to as strain-promoted azide–alkyne coupling (SPAAC) (Scheme 1B). These cyclooctyne derivatives greatly increase reactivity of azide–alkyne cycloadditions in the absence of copper, particularly when difluorinated.[13] Baskin et al. exploited this phenomenon to conjugate fluorophores to biological molecules by incorporating a *gem*-difluoro group next to a strained alkyne.[14] Reported reaction rates were 30–60 times faster compared to those with nonfluorinated cyclooctynes.

Another click cycloaddition is exemplified by the Diels–Alder (DA) reaction; a highly selective [4 + 2] cycloaddition between an electron-rich diene and an electron-poor dienophile (Scheme 1C). This reaction was first reported by Otto Diels and Kurt Alder in 1928,[15] making the DA cylocaddition the oldest known click reaction. DA chemistries offer high yields and minimal sides reactions, and require very little energy. Contrary to other click reactions, which commonly result in carbon–heteroatom bonds, carbon–carbon bonds are formed in DA cycloadditions. These bonds are thermally reversible at elevated temperatures. Water has been shown to accelerate DA reactions,[16,17] making the DA reaction particularly desirable for biomedical applications.

Beyond cycloadditions, other highly efficient reactions, such as nucleophilic substitutions, radical additions, and Michael additions, are also considered click reactions. In particular, thiol-click chemistries have recently gained merit within the field of regenerative biomaterials. Schlaad and co-workers were the first to identify the radical-mediated thiol–ene reaction as a click reaction.[80] In the mechanism, a radical abstracts a hydrogel from the thiol to form an active thiyl radical, allowing for an addition reaction between the thiyl radical and the carbon–carbon double bond or "ene". While both heat and light can be used to generate radicals to initiate the thiol–ene mechanism (Scheme 1D), photoinitiation of the radical addition reaction between thiols and alkenes allows for spatial and temporal control. Using light, the reaction can be easily manipulated by controlling the light intensity, the dose, or the duration.[79] This thiol-click reaction has found specific applications for biofunctionalization and surface modification.[18]

Thiol-Michael addition reactions represent another principle variation among the thiol–click chemistries applicable to regenerative biomaterials (Scheme 1E, F). The Michael addition of thiols to electron-deficient "enes" has been investigated since at least the 1940s,[81] and to this day, it continues to be a versatile tool within the field of bioconjugate chemistry. Thiol–click Michael additions typically involve α,β-unsaturated carbonyl compounds, and depend upon the nucleophilicity of the thiol component.[79] Thiol–maleimide click coupling (Scheme 1E) is a particularly relevant example for this review, as it is frequently exploited for protein conjugation.[71,72,75,76] The vinyl sulfone–thiol click reaction (Scheme 1F) has served as an important cross-linking mechanism for the synthesis of enzyme-degradable hydrogels.[44]

Table 1. Comparison of Common Click Reactions for Regenerative Biomaterials

click reaction	pH	reaction time	reactivity	specific advantages	specific disadvantages	biomaterial application
CuAAC	3–12	<1 h	increases at lower pH, or increased Cu(I)	azides/alkynes not found in nature	unstable and toxic copper catalyst required	hydrogel synthesis,[21−29] 2D protein immobilization[57−62]
SPAAC	7.4	<1 h	Increases with difluorination of cyclooctyne	no copper requirement	difficult synthesis of difluorinated cyclooctynes	PEG hydrogel synthesis[30,31]
DA	5.5–6.5	<8 h	accelerated by water	no catalyst required	longer reaction time	HA hydrogel synthesis,[42] 2D protein immobilization[63,64]
Thiol–ene	6–8	<2 h	radical-mediated	spatial/temporal control	cross-reactivity with thiols, photoinitiator often required	hydrogel synthesis,[48,49] 2D protein immobilization,[65,66] hydrogel patterning[30,31,74]
Thiol–Michael	6–8	<1 h	Depends on the nucleophilicity of the thiol component	facile introduction of proteins	cross-reactivity with thiols	hydrogel synthesis,[43,44,46,47] hydrogel patterning[71,72,75,76]

CLICK CROSS-LINKED HYDROGELS FOR TISSUE ENGINEERING AND DRUG DELIVERY

Hydrogels are water-swollen, cross-linked polymer networks capable of imitating the mechanical and architectural nature of the cellular microenvironement of soft tissue.[19] Due to their high water content, hydrogels permit facile transport of oxygen, nutrients, soluble factors, and waste. Moreover, many hydrogels are biocompatible, biodegradable, and can be synthesized and processed under relatively mild conditions. Accordingly, hydrogels represent an optimal platform for many regenerative medicine strategies. Often, hydrogel matrices are used to provide a blank canvas in which biomolecules are immobilized, thereby providing a defined chemical environment to guide cell fate. Synthetic poly(ethylene glycol) (PEG) and naturally derived agarose are two common examples of such hydrogels.[73,75]

The mechanical characteristics of hydrogels are dictated by the number of cross-links formed between the polymer chains via covalent bonds or noncovalent interactions. In the past, popular hydrogel cross-linking methodologies have included radical polymerization and high-energy irradiation.[20] The majority of these reactions require cytotoxic cross-linking agents or initiators, which can reduce biocompatibility of the material. Within the past decade, click chemistry has emerged as a viable alternative for both chemically cross-linked hydrogels and their biofunctionalization. While copper is also known to be cytotoxic, the strained reactions described above overcome this deficit of CuAAC chemistry.

Alkyne–Azide Click Cross-Linked Hydrogels. Ossipov et al. were the first to recognize the CuAAC reaction as an efficient chemoselective cross-linking method for hydrogel synthesis.[21] Poly(vinyl alcohol) (PVA) was functionalized with either acetylene or azide groups, and cross-linked by mixing their aqueous solutions together with copper sulfate ($CuSO_4$) and sodium ascorbate as the cycloaddition catalyst. In another example, alkyne-modified PVA was cross-linked with bifunctional PEG-diazide cross-linkers. Hydrogels prepared with polyfunctional PVA formed higher-modulus gels with reduced swelling compared to those synthesized with the bifunctional PEG cross-linker.

Hawker and co-workers[22] applied the CuAAC click cross-linking reaction to the synthesis of pure PEG hydrogels. In their approach, diacetylene- and tetraazide-functionalized PEG were reacted in a 2:1 ratio at room temperature under aqueous conditions in the presence of $CuSO_4$ and sodium ascorbate. By manipulating both the polymer and catalyst concentration, they were able to tune the cross-linking efficiency. Following hydrogel formation, both acetylene and azide functionalized chromophores were swollen into the hydrogel to visualize any unreacted azide/acetylene functional groups. This revealed less than 0.2% unreacted functional groups, confirming the efficient nature of the CuAAC reaction. The degree of swelling and stress/extension properties of the hydrogels were also examined by varying the length of the diacetylene PEG chain.

To illustrate the utility of the CuAAC cross-linked PEG hydrogels as tissue engineering and drug delivery scaffolds, various researchers have incorporated peptides and degradable linkers within their click cross-linked networks. In particular, inclusion of the fibronectin tripeptide sequence, arginine-glycine-aspartate (RGD), has been shown to be an essential additive in almost all "blank slate" hydrogel formulations for tissue culture. RGD is a prime cell adhesion site that is

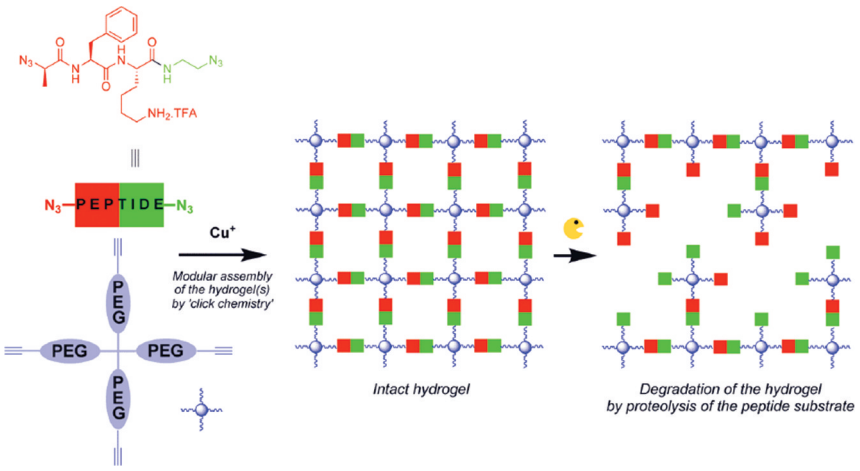


Figure 2. Schematic representation of the synthesis of CuAAC cross-linked PEG hydrogels.[25] Incorporation of enzymatically degradable peptides renders hydrogels susceptible to degradation by trypsin proteases. (Copyright American Chemical Society, reproduced with permission from ref 25.)

recognized by many integrin receptors;[23] therefore, incorporation of this peptide facilitates cell-matrix interactions. Liu et al. synthesized diazide-functionalized RGD peptides to cross-link tetraacetylene PEG under aqueous conditions with $CuSO_4$ and sodium ascorbate.[24] By varying the temperature, catalyst, and precursor concentrations, the gelation time was altered from 2 to 30 min. An increase in temperature or $CuSO_4$ resulted in a decreased gelation time. The storage modulus was also tailored by changing the azide linker length. These RGD peptide hydrogels were tested for fibroblast delivery to promote tissue repair. By increasing the concentration of RGD peptide, fibroblast adhesion and proliferation also increased.

In a similar approach, van Dijk et al. incorporated a protease-sensitive peptide within a CuAAC cross-linked PEG hydrogel (Figure 2).[25] Alkyne-functionalized star-shaped PEG molecules (either 4- or 8-armed with a MW of 10 and 20 kDa, respectively) were cross-linked with the protease-sensitive bis-azido peptide in aqueous solution in the presence of $CuSO_4$ and sodium ascorbate. Incubation of the hydrogels in trypsin leads to complete degradation of hydrogels after 40−80 h, depending on the cross-link density.

The CuAAC reaction has also been employed as a cross-linking method for natural polymers. While hydrogels formed from synthetic materials, such as PEG, offer a blank canvas permissive to cell function, materials native to the extracellular matrix (ECM) can promote cell function.[19] A prime example of a native material commonly employed in regenerative strategies is hyaluronic acid (HA)—a ubiquitous nonsulfated glycosaminoglycan, which impacts embryonic development, tissue organization, wound healing, and angiogenesis.[26] Crescenzi et al. used the CuAAC click reaction to cross-link HA.[27] Hyaluronic acid (HA) was modified with either azide or alkyne groups and cross-linked in water with Cu(I) at room temperature. Their hydrogel revealed intriguing characteristics for both drug release and tissue engineering applications. As a model for drug delivery, benzidamine and doxorubicin were encapsulated within the click hydrogels, which displayed release profiles ranging from hours to several weeks, depending on the cross-link density. To confirm the possibility that these hydrogels could serve as tissue engineering scaffolds, yeast cells were imbedded within the hydrogels, following removal of the copper catalyst through dialysis. Cells exhibited 80% proliferating activity after 24 h in culture. In a follow-up study, the influence of the dialkyne structure on the properties of these HA click cross-linked hydrogels was examined.[28] HA azido-derivatives cross-linked with shorter dialkynes experienced weaker storage moduli, corresponding to predicted cross-linking densities as determined by ^{1}H NMR.

Gao and co-workers also employed the CuAAC reaction to cross-link natural biopolymers.[29] Both HA and chondroitin sulfate (CS) were modified to contain azide functionalities, and cross-linked with gelatin that had been modified with an alkyne functionality. They argue that this triple copolymer system better mimics the natural components of ECM by incorporating proteoglycans, such as CS, and denatured collagen products, such as gelatin, to promote cell surface adhesion. Aqueous solutions of the polymers were combined and catalyzed by Cu(I) to form a hydrogel with the time to gelation varying as a function of catalyst concentration. Chondrocytes were cultured *in vitro* to assess the cytotoxicity of the click hydrogels. After 3 days in culture, a confluent layer of cells had formed, confirming the benefit of this click cross-linked hydrogel for chondrocyte adhesion and proliferation.

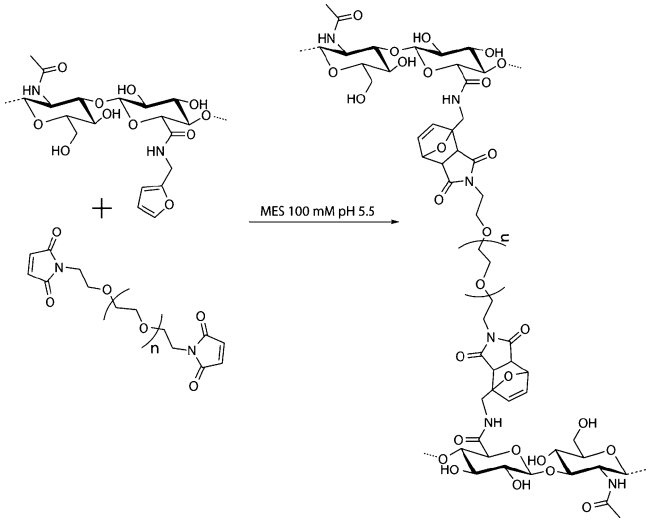
Anseth and co-workers have gone beyond traditional CuAAC cross-linking chemistry and synthesized PEG hydrogels via SPAAC, to bestow copper-free, physiological conditions within their networks.[30] In their approach, a four-arm PEG tetra-azide was reacted with difunctionalized difluorinated cyclooctyne polypeptide sequence, to incorporate enzymatically degradable cross-linker sequences throughout the material. Gelation occurred in less than 5 min, and both rheological and ^{1}H NMR data support the ideality of the network, similar to previous click-based networks.[22] This click strategy tolerates cell encapsulation with high viabilities (>90% at 24 h). As an extension of this study, De Forest et al. enabled control over cross-link density and shear moduli of these SPAAC cross-linked PEG hydrogels.[31] By altering either the azide to cyclooctyne ratio or the molecular weight of PEG, hydrogels were synthesized with tunable moduli ranging 1000−6000 Pa. These SPAAC cross-linked PEG hydrogels have also served as patterning platforms for the immobilization of biological functionalities using thiol−ene click photocoupling[30,31] (see Click Patterning of 3D Scaffolds section).

A drawback of CuAAC cross-linking is the lack of temporal and spatial control due to the generation of the catalytic Cu(I) species.[32] Spatial and temporal control of network formation is paramount in many tissue engineering applications. To overcome

this, Adzima et al. sought to catalyze the CuAAC cross-linking reaction via the photochemical reduction of Cu(II) to Cu(I).[32] Generating Cu(I) photochemically is analogous to the initiation of a radical or carbocation species in traditional photopolymerization processes, resulting in total spatial and temporal control of the CuAAC reaction. The authors developed this photoinducible azide−alkyne cycloaddtion (pCuAAC) reaction to synthesize hydrogels by irradiating multifunctional alkyne and azide functionalized PEG monomers in the presence of $CuSO_4$ and Irgacure 2959 photoinitiator. To extend this system to biological systems, the authors suggest modifications to mimic reverse-ATRP polymerizations that require significantly lower copper levels.[33]

Diels−Alder Click Cross-linked Hydrogels. Although the DA click reaction has long served as an exceptional cross-linking method for the synthesis of complex polymer networks,[34−37] the preparation of cross-linked hydrogels via DA chemistry remains largely unexplored. A few DA hydrogels have been synthesized with synthetic polymers;[38−41] however, these studies have mainly examined the effect of temperature on both gelation time and retro-DA reaction. Recently, Shoichet and co-workers reported a DA cross-linked hydrogel with a specific tissue engineering application in mind.[42] Furan-modified HA was synthesized and cross-linked via dimaleimide-PEG to form a stable hydrogel by mixing the two aqueous solutions at room temperature (Scheme 2). By controlling the furan to maleimide molar ratio, the mechanical and degradation properties of the DA hydrogels could be altered. The reported shear modulus of the hydrogels ranged from 275 to 680 Pa, similar to that of central nervous system tissue. After 14 days of culture *in vitro*, endothelial cells displayed high levels of cell survival (>98%), and appeared to interact with the HA matrix. The minimal swelling, complete degradation, and cell-interactive properties of these DA hydrogels make them promising materials for soft tissue engineering.

Scheme 2. Formation of Diels-Alder Hyaluronic Acid Click Hydrogels[42]



Thiol−Click Cross-Linked Hydrogels. Qiu et al. were the first to harness the thiol-Michael click reaction for hydrogel formation.[43] PEG-based copolymers containing multiple thiol functionalities were cross-linked via divinylsulfone-PEG in neutral phosphate buffer. This system proved to be bioorthogonal, as protein additives did not interfere with the click cross-linking reaction; however, these proteins did not contain any exposed thiols that could interfere with the reaction. A thiol-click cross-linked hydrogel may be inappropriate for protein delivery if any free thiol groups are present on the proteins. Notwithstanding this limitation, when proteins were incorporated into these gels, their release was sustained for 2−4 weeks.

Hubbell and co-workers took advantage of this Michael-type addition click reaction to synthesize hydrogels with characteristics similar to that of native ECM.[44] Their approach was to incorporate integrin-binding sites for cell adhesion and enzyme-degradable sites into the matrix such that cell-secreted matrix metalloproteinases (MMPs) would enable cell migration into and remodeling of the biomimetic ECM.[45] To achieve this, they cross-linked bis-cysteine MMP substrate peptides with vinyl sulfone-functionalized multiarm PEG. The resulting click hydrogel networks displayed a defined molecular architecture, and allowed for invasion by primary human fibroblasts. Cellular invasion was shown to be dependent on the proteolytic activity of the incorporated peptide. The hydrogels were also employed as a drug delivery vehicle for the recombinant human bone morphogenetic protein (BMP2) to rat cranium defects. Correlating with their work *in vitro*, bone regeneration *in vivo* depended on the enzymatic sensitivity of the incorporated substrate.

Chawla et al. recently developed a 3D cell culture platform for mesenchymal stem cells (MSCs) by cross-linking a saccharide-peptide copolymer via Michael-type conjugation addition between cysteine (Cys) and vinyl sulfone (VS).[46] By altering the pH of the cross-linking reaction, or the VS to Cys ratio, they were able to tune both the degradation and mechanical properties of the gel. Hydrogels that were cross-linked with an equimolar ratio of VS to Cys maintained their mechanically stability for longer than 21 days *in vitro*, similar to dextran hydrogels cross-linked by Michael addition.[47] These hydrogels also exhibited a rapid gelation time, suggesting utility for in situ surgical procedures, and displayed a microporous network when visualized under environmental scanning electron microscopy. Cell encapsulation was facilitated by the cross-linking reaction occurring in culture medium. MSCs remained viable after 14 days (>90%) in culture.

The radical-mediated thiol−ene click reaction has also been employed as a hydrogel cross-linking method. Anseth and co-workers developed a platform for hydrogel synthesis by a step-growth reaction mechanism via thiol and norbornene functionalities.[48] Not always achievable with simple Michael addition, thiol−ene photopolymerization offers spatial and temporal control of network formation. Hydrogels were synthesized by mixing norbornene-functionalized PEG with either chymotrypsin- or MMP-degradable linkers in a 1:1 stoichiometric ratio in PBS. The networks maintained high cell viability following encapsulation (>95% following 24 h). Anderson et al. utilized these thiol−ene photopolymerized PEG hydrogels to examine MSC behavior in response to network properties.[49] Both MMP cleavable peptide linkers and nondegradable PEG-dithiol linkers were incorporated into the hydrogel to monitor how MSC degradation of the matrix affects their differentiation behavior. Their findings suggest that directed chondrogenic and adipogenic differentiation of MSCs are facilitated by increased cell-mediated hydrogel degradation.

CLICK IMMOBILIZATION OF PEPTIDES ON 2D SURFACES

Well-defined chemically modified substrates, such as self-assembled monolayers (SAMs), serve as investigative tools to explore fundamental interactions applicable to regenerative strategies. Surface functionalization is particularly advantageous when engineering substrates for cell culture to harness control over cell-matrix interactions. Many have employed this strategy to immobilize ECM-derived biomolecules for ultimate characterization of their effects on cell adhesion.[50−53] Popular immobilization strategies in the past have been based on adsorption or covalent modification of a protein's functional group(s) to a chemically activated surface.[54,55] These methods can result in side reactions and are difficult to characterize both physically and in terms of cellular response. Accordingly, there has been a paradigm shift in the existing approaches for surface functionalization from unspecific and nonselective reactions toward highly specific orthogonal reactions that ensure bioactivity and facilitate characterization of engineered surfaces.[56]

CuAAC Click Immobilization. CuAAC is chemoselective against common functionalities in biomolecules, and thus, stable reactive species can be introduced within biomolecules with ease. Accordingly, CuAAC serves as an effective click reaction to chemoselectively immobilize peptides to otherwise bioinert SAMs. Becker and co-workers developed a universal technology for surface conjugation of any biomolecule containing accessible azide groups.[57] SAMs were subjected to oxidation by UV exposure, creating a monotonically increasing carboxyl density gradient. A bifunctional propargyl-functionalized linker was then attached to the carboxylic moieties of the gradient to yield an alkyne gradient, making it susceptible to modification with any azido-derivatized species via CuAAC chemistry. The authors tested their methodology by conjugating the RGD tripeptide sequence in a density gradient ranging from 0 to 140 pmol cm^{-2}. Smooth muscle cells cultured on the RGD gradient surfaces revealed enhanced cell attachment with increased RGD concentration. Alkyne-modified KGRGDS has also been successfully coupled by CuAAC chemistry to self-assembled polymeric micelles with azide-functional groups, thereby yielding RGD-functionalized polymeric nanoparticles that specifically bind to corneal epithelial cells.[58]

Becker and co-workers later exploited their CuAAC technology to couple osteogenic growth peptide (OGP) to SAMs in order to explore the effects of OGP density on preosteoblast cell adhesion, morphology, and proliferation.[59] OGP has been recognized as a promising agent for bone tissue engineering applications because it stimulates tissue regeneration of bone defects.[60] To create the peptide gradient, the carboxylic acid functionalities on the SAM layer were reacted with the amine terminal of a bifunctional amine-poly(ethylene oxide)-alkyne linker, resulting in an alkyne gradient. Azide-terminated OGP peptides were then conjugated to the alkyne gradient by immersing substrates in a solution of $CuSO_4$, sodium ascorbate, and peptide for 24 h at 40 °C. Preosteoblast cells were cultured on the OGP functionalized gradient surfaces in serum-free conditions for 7 days. Cell adhesion was highest at low OGP peptide concentrations. At day 3, cells experienced faster doubling rates compared to cells cultured on control surfaces, but this effect subsided by day 7. This is indicative of the natural transition made by osteoblasts from proliferative to maturation phases. Gene expression experiments also verified this phenomena with a 10-fold increase in collagen I expression between days 3 and 7, coinciding with the initial stages of bone mineralization.

Hudalla and Murphy fabricated SAMs expressing a variation of the adhesive RGD peptide, RGDSP, as a means to study stem cell adhesion.[50] This was achieved via CuAAC. SAMs were first prepared by immersing a gold substrate in an ethanolic solution of 80 mol % tri(ethylene glycol) alkanethiolate ($HS\text{-}EG_3$) and 20 mol % azide-terminated hexa(ethylene glycol) alkanethiolate ($HS\text{-}EG_6\text{-}N_3$). The resulting mixed SAMs contained approximately 10% $HS\text{-}EG_6\text{-}N_3$ and 90% $HS\text{-}EG_3$ (Figure 3A); however, this result could be

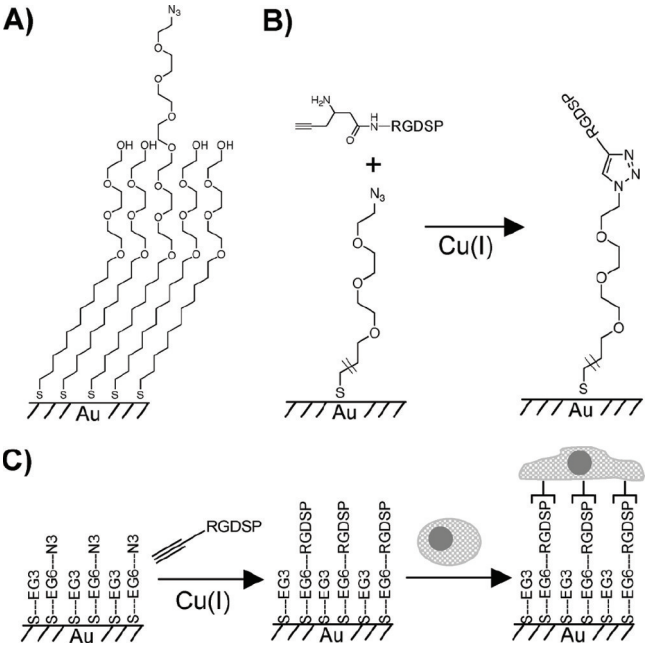


Figure 3. CuAAC click immobilization of peptides to SAM substrates to study stem cell adhesion.[50] (A) Mixed SAMs bearing azide groups; (B) reaction between azide-functionalized SAMs with acetylene-bearing RGDSP; (C) the surface density of RGDSP immobilized via CuAAC affects mesenchymal stem cell adhesion and spreading. (Copyright American Chemical Society, reproduced with permission[50].)

tailored by varying the ratio of $HS\text{-}EG_6\text{-}N_3$ and $HS\text{-}EG_3$. The SAMs were classified as bioinert, as they displayed minimal nonspecific protein adsorption. Acetylene-bearing RGDSP (Hex-RGDSP) was then conjugated to the SAMs via $HS\text{-}EG_6\text{-}N_3$ in the presence of a Cu(I) catalyst (Figure 3B). The CuAAC reaction efficiency illustrated near-quantitative conjugation upon the addition of a tertiary amine, which has been a proven method utilized by others to enhance CuAAC efficiency by binding the Cu(I) catalyst.[61] MSCs were cultured on top of the RGDSP-presenting SAMs. RDGSP surface density and intermolecular spacing regulated MSC morphology and attachment (Figure 3C).

In a followup study, Hudalla and Murphy demonstrated that CuAAC conjugation of biomolecules to SAM substrates can be conducted in parallel with other chemistries, namely, carbodiimide condensation.[62] Incorporation of carboxylate-terminated hexaethylene glycol alkanethiolate ($HS\text{-}EG_6\text{-}COOH$) with the original mixed SAM allowed for conjugation of two distinct peptides, RGDSP and TYRSRKY, a

proteoglycan-binding peptide. These experiments revealed that these distinct extracellular factors work synergistically to regulate MSC adhesion on 2D substrates. They also demonstrated that soluble biomolecules, such as heparin, can disrupt specific cell-material interactions, and in turn direct MSC adhesion.

Diels–Alder Click Immobilization. Yousaf and Mrksich were the first to exploit the DA reaction for protein immobilization on 2D surfaces.[63] In their approach, SAMs were modified with a hydroquinonequinone group, which upon oxidation provides a quinone, enabling a cycloaddition between the quinone and a cyclopentadiene (cp). To demonstrate this DA approach, they immobilized a biotin-cp conjugate, and tested the affinity of the immobilized biotin for streptavidin. This work demonstrated that the DA click reaction is an attractive bioconjugation technique for a wide variety of applications, which could be extended to regenerative therapies. The authors also state that this method would allow for controlled, sequential immobilization of several biomolecules.

Sun et al. demonstrated the use of sequential click reactions for protein immobilization on solid surfaces.[64] First, the DA click reaction was used to immobilize a heterobifunctional PEG linker carrying alkyne and cyclodiene terminal groups onto an N-(E-maleimidocaproyl)-functionalized glass slide. This resulted in an exposed alkyne-terminated PEGylated surface, vulnerable to conjugation with azide-containing biomolecules via CuAAC click chemistry. Again, biotin was chosen as a model protein for immobilization. Biotin-PEG-azide was added to a glass vial containing the alkyne-PEGylated glass slide, $CuSO_4$, and a tertiary amine ligand. The reaction was left at 4 °C for 12 h. Biotinylated glass slides were then immersed in a solution of dilute FITC-conjugated streptavidin at 4 °C for 2 h. Confocal fluorescence images verified the fidelity of the click protein immobilization. This technique is applicable to a wide range of functionally complex biomolecules for the design of biomimetic surfaces.

Thiol–Ene Click Immobilization. Waldmann and co-workers reported the use of the thiol–ene reaction to photochemically pattern proteins and other biomolecules onto solid surfaces.[65] Polyamidoamine dendrimers, containing an aminocaproic acid spacer, were covalently attached to a silicon oxide wafer surface. Cystamine was then conjugated to the spacer, which upon disulfide reduction, exposed free thiols on the surface. A solution of terminal-olefin-functionalized biomolecules were spread onto the surfaces, and covered with a photomask. Following irradiation at 365–405 nm, a covalently attached pattern of thioethers was obtained. As a test application, biotin was photochemically patterned onto a thiolated wafer, and subsequently incubated in a solution of Cy5-labeled streptavidin to render a fluorescent pattern surface. Immobilization was shown to be dependent on irradiation time and concentration of immobilized peptide. They further exemplified the broad applicability of their patterning method by immobilizing alkaline phosphatase, Ras, and phosphopeptide, all of which retained their bioactivity and binding affinities.

In an extension of this work, Waldmann and co-workers demonstrated fast, oriented covalent immobilization of proteins directly from lysates, eliminating any additional protein chemical modifications.[66] The authors took advantage of the fact that, in cells, many proteins are post-translationally S-farnesylated at a C-terminal "CAAX-box" by protein farnesyltransferase (FTase).[67] By genetically coding for the CAAX tag, the authors enabled farnesylation *in vitro* or *in vivo* with FTase, creating a facile method for "ene" incorporation into a protein of interest. Once farnesylated, these proteins were immobilized via the thiol–ene photochemical click reaction to surface-exposed thiols.

CLICK PATTERNING OF 3D SCAFFOLDS

Throughout the past decade, several scaffolds have emerged aiming to mimic the cellular microenvironment and ultimately control cell fate and guide tissue regeneration. These scaffolds can be fine-tuned to study a specific parameter of the microenvironment. Notably, the effect of peptide presentation on the cell has been examined by spatially immobilizing proteins and adhesion peptides in 3D patterns within scaffolds. Growth factors localized in 3D scaffolds remain bioactive[68−70] and have been shown to orient axonal growth,[71,72] guide cellular migration,[73] and cause morphological changes.[30] The stringent spatial resolution and controlled biochemical heterogeneity required for 3D patterning make simple bioorthogonal chemistries paramount. The use of click reactions for 3D patterning of scaffolds allows significant spatial control when combined with multiphoton processing.[75,76] Additionally, many of these hydrogel scaffolds are click cross-linked, and sequentially click patterned.[30,31,74]

Alkyne–Azide Click Patterning. Recently, Bowman and co-workers demonstrated 3D patterning of hydrogels via pCuAAC.[32] The transient generation of Cu(I) facilitates spatial and temporal control of the CuAAC reaction. PEG hydrogels were first synthesized by a thiol-yne reaction, ensuring a stoichiometric excess of alkynes. Postgelation, a solution of photoinitiator (Irgacure 2959), copper sulfate, and an azide-labeled fluorophore was swollen into the gel. Upon irradiation with a photomask, Cu(I) is generated within the irradiated areas, catalyzing the pCuAAC reaction between the azide-fluorophore and the pendant alkynes in the polymer network, ultimately producing a spatially defined fluorescent pattern within the hydrogel. The authors note that future work is required to translate this system to biological systems.

Thiol–Ene Click Patterned Scaffolds. Immobilization of bioactive growth factors via thiol conjugation has become increasingly popular given the ease of cysteine incorporation within a peptide, making the thiol–ene click reaction particularly relevant for 3D patterning. Anseth and co-workers developed a sequential click protocol relevant to both hydrogel synthesis and postgelation modification.[30,31,74] Click cross-linked PEG hydrogels were first formed via CuAAC, as an extension of the method taken by Malkock et al.[22] To enable photopatterning of their PEG hydrogels, multifunctional photoreactive polypeptide sequences were included within the network structure by incorporating the non-natural amino acid, Fmoc-Lys(alloc)-OH.[74] The allyloxycarbonyl (alloc) protecting group contains a vinyl functional group capable of reacting with any thiol-containing compound, such as cysteine. Upon exposure to UV light, thiyl radicals are generated via the photocleavage of a hydrogen-abstracting initiator, thereby using light to achieve spatial and temporal control of thiol–ene functionalization within the network. To illustrate this technique, a fluorescently labeled cysteine containing peptide was patterned within PEG hydrogels via transparency-based photolithographic patterning techniques.

This thiol−ene photopatterning method was later employed to immobilize peptides and proteins within PEG hydrogels cross-linked via SPAAC (Figure 4).[30] Spatial and temporal

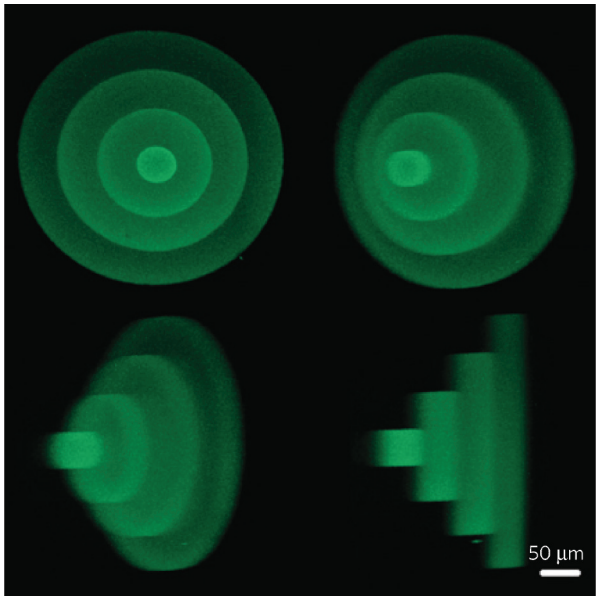


Figure 4. 3D biochemical patterning of PEG hydrogels via thiol−ene photocoupling.[30] Fluorescently tagged peptides were patterned within SPAAC cross-linked networks. (Copyright Macmillan Publishers Limited, reproduced with permission.[30])

control was validated by selectively exposing certain locations within the hydrogel matrix to light, and by controlling light intensity and exposure time. Furthermore, the thiol−ene reaction was confirmed to be cytocompatible, as 3T3 cells encapsulated within the hydrogel maintained high viability throughout patterning (>90% at 24 h post encapsulation), and thiol−ene immobilization of RGD within the network was shown to influence cell morphology. In a follow-up study, DeForest et al. verified that patterning concentration within the hydrogel is directly proportional to the dosage of light, as well as the photoinitiator concentration.[31] Using this system, they were able to construct well-defined 3D biochemical gradients of multiple peptides, offering potential promise to elucidate fundamental biological processes essential to regenerative medicine such as induced cell migration.

Thiol−Maleimide Click Patterning. The thiol−maleimide reaction exemplifies a variation of the thiol-Michael click reaction. Shoichet and co-workers have exploited this click reaction for 3D patterning of agarose hydrogels.[68,72,75,76] Covalent modification of agarose with S-2-nitrobenzyl-cysteine (S-NBC) renders a photolabile matrix, which upon UV irradiation releases free thiols capable of reacting with any thiol-reactive biomolecule through Michael addition. Thiol channels were created on exposure to a focused laser beam, and reacted with a maleimide-terminated RGD peptide. This immobilized RGD channel volume promoted neurite extension and cell migration.[72] This system was also applied to study the effect of immobilized platelet derived growth factor AA (PDGF-AA) on neural stem/progenitor cell (NSPC) differentiation.[68] Hydrogels with immobilized RGD and PDGF-AA supported NSPC adhesion and preferential differentiation to oligodendrocytes.

In order to advance this click technology toward more sophisticated architectures that better mimic the native extracellular matrix, agarose was later modified with a coumarin derivative, which upon exposure to a pulsed infrared laser yields free thiols.[75] The use of this photolabile group allows for intricate 3D control by way of multiphoton excitation. Aizawa et al. exploited this coumarin multiphoton patterning technique to immobilize a gradient of the angiogenic factor, VEGF165, within agarose hydrogels.[76] Primary endothelial cells responded to this immobilized gradient by displaying tip and stalk cell morphology, eventually forming tubule-like structures as they migrated in response to the VEGF gradient (Figure 5).

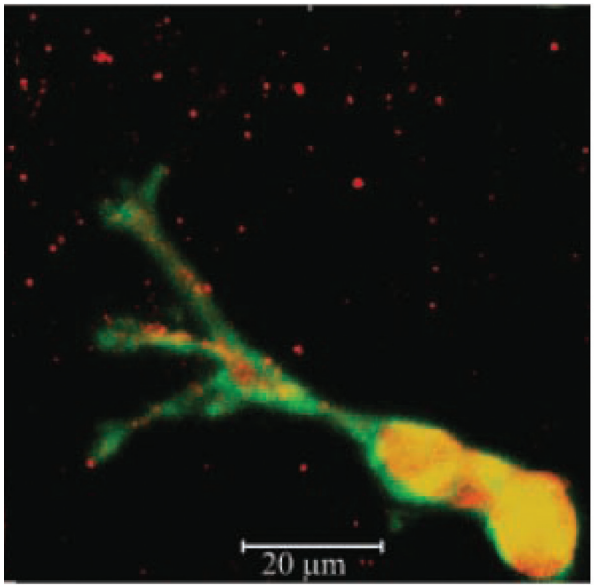


Figure 5. Primary endothelial cells guided in 3D patterned agarose hydrogel.[76] VEGF165 was immobilized in a concentration gradient within the scaffold using the thiol-maleimide click reaction. (Copyright Wiley-VCH Verlag GmbH & Co. KGaA; reproduced with permission.[76])

The "click" nature of thiol addition to a maleimide unit was also exercised by Kosif et al.[77] with the immobilization of proteins in PEG-methacrylate based hydrogels. Hydrogel synthesis was achieved using AIBN initiated thermal polymerization, by which they directly incorporated a furan-protected maleimide containing monomer. This furan-protected maleimide represents a DA adduct which is susceptible to thermal cycloreversion. Postgelation, a thermal cycloreversion step activated the maleimide group to its thiol-reactive form. To evaluate this system as a potential template for bioconjugation, thiolated-biotin was covalently attached to the hydrogels, and its affinity for streptavidin investigated. This system is particularly intriguing as it incorporates the retro-DA click reaction, which is not commonly applied in regenerative biomaterials.

■ CONCLUDING REMARKS

Click chemistry has long been recognized as a powerful tool within biotechnology applications, and has since begun to gain popularity for the preparation of functionalized materials applicable in regenerative medicine strategies. These elegant, versatile reactions represent attractive building blocks for engineered hydrogel networks, decorated 2D cell culture

surfaces, and patterned 3D biomimetic scaffolds. Click chemistry is particularly compelling for hydrogel formation and modification because it is water-based chemistry. Moreover, the click reaction provides impeccable control over the conformation of the protein or peptide immobilized, thereby maximizing its bioactivity. With the formation of stable covalent bonds, one can be confident of a well-defined system with which to study cellular response.

Each click reaction, however, has limitations that warrant consideration. The obvious shortcoming of CuAAC is the copper requirement. Copper has been shown to be cytotoxic, and increased copper intake has been linked to hepatitis, Alzheimer's disease, and other neurological disorders.[78] Another disadvantage of CuAAC is alkyne homocoupling. Although rare, alkynes may react with another alkyne instead of an azide.[9] The evolution of SPAAC has played a substantial role in catalyzing the development of click reactions beyond CuAAC; however, the difluorinated cyclooctynes can be difficult to synthesize[14] thereby complicating what would normally be a simple reaction. Collaboration between chemists and bioengineers, as well as increased commercial access to starting materials, will overcome some of these synthetic limitations.

The DA click cycloaddition is a "reagent-free" click reaction that does not require catalyst, photoinitiator, or radical initiation. Moreover, the DA reaction is accelerated in aqueous solutions, an ideal characteristic for biomaterial design. Despite these strengths, the DA click reaction has been much less explored relative to the CuAAC and thiol-click reactions, perhaps due to longer reaction times. Although it has not been specifically reported, the reaction time for the formation of HA hydrogels via Diels–Alder click chemistry is likely greater than 8 h since hydrogels are allowed to form overnight.[42] The rate of reaction can be accelerated with heat or photoinitiation if required. DA adducts can also undergo cycloreversion, but this is only relevant at higher temperatures, and thus should not pose a problem to biomaterials used *in vitro* or *in vivo*.

Recently, the thiol–click reaction has emerged as a prominent reaction within the field of regenerative biomaterials, particularly as a tool for protein conjugation, given the simplicity of immobilization via functional groups common to proteins. However, it is difficult to control protein immobilization via thiol-Michael addition due to cross-reactivity. In terms of 3D patterning, this can be avoided by spatially controlling where free thiols are exposed within a photolabile matrix using two-photon laser technology. The anionic chain process of thiol-Michael click reactions is thought to reduce its efficiency and rate. On the other hand, the photoinitiated thiol–ene click reaction occurs by free-radical addition, offering enhanced spatial and temporal control. However, this method often requires a photoinitiator, which can be cytotoxic and hence reduce the biocompatibility of the material. Finally, free thiols are susceptible to disulfide bond formation via oxidation, which further limits the efficiency of thiol–ene click reactions.

What other bioorthogonal reactions are likely to emerge within the field of regenerative medicine? Popik and co-workers have recently developed a photoinducible azide–alkyne cycloaddition reaction circumventing the copper catalyst requirement.[82] Generation of a dibenzocyclooctyne from a photoprotected cyclopropenone provides spatial and temporal control over the reaction under ambient conditions. While this chemistry has not been applied to biomaterials to date, use of this reaction would harness spatial and temporal control to the already bioorthongal reactants without the use of a copper catalyst. A highly effective click reaction for protein immobilization is the Staudinger ligation.[83] The simple nature of this chemistry makes it an excellent candidate for the synthesis of 2D substrates used to study stem cell proliferation/differentiation. While we have reviewed several examples of thiol–ene and thiol–Michael click reactions applied to regenerative biomaterials, the thiol–yne reaction not yet been tested in regenerative medicine. This reaction is capable of consecutive reaction with two thiol functional groups.[84] Application of this chemistry to hydrogel synthesis would provide scaffolds with high cross-link densities. Finally, while not traditionally defined as "click", the orthogonal chemistry of peptide binding pairs has recently been used to design hydrogels with 3D immobilized proteins.[85] Physical binding pairs, barnase–barstar and streptavidin–biotin, were exploited to simultaneously immobilize differentiation factors within an agarose hydrogel. Application of this technology in the future will avoid multistep protocols, while preserving protein bioactivity.

In a short period of time, the use of click chemistry within the field of regenerative medicine has exploded. The ability to synthesize biomaterials with pristine definition and architecture to ultimately control cell fate is unprecedented. To this day, click reactions continue to evolve with the identification of new orthogonal chemistries. Bioengineers will continue to translate these improved chemoselective methodologies to their regenerative materials. Already, we have seen a considerable increase in the use of click reactions for biochemically patterning 3D hydrogels, which we anticipate will increase further. Sequential click reactions will likely emerge as a key approach to design sophisticated biomaterials in a simplistic manner.

AUTHOR INFORMATION

Corresponding Author

*E-mail: molly.shoichet@utoronto.ca, Phone: 416.978.1460, Fax: 416.978.4317.

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