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Review

Click hydrogels, microgels and nanogels: Emerging platforms for drug delivery and tissue engineering

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ABSTRACT

Hydrogels, microgels and nanogels have emerged as versatile and viable platforms for sustained protein release, targeted drug delivery, and tissue engineering due to excellent biocompatibility, a microporous structure with tunable porosity and pore size, and dimensions spanning from human organs, cells to viruses. In the past decade, remarkable advances in hydrogels, microgels and nanogels have been achieved with click chemistry. It is a most promising strategy to prepare gels with varying dimensions owing to its high reactivity, superb selectivity, and mild reaction conditions. In particular, the recent development of copper-free click chemistry such as strain-promoted azide-alkyne cycloaddition, radical mediated thiol-ene chemistry, Diels–Alder reaction, tetrazole-alkene photo-click chemistry, and oxime reaction renders it possible to form hydrogels, microgels and nanogels without the use of potentially toxic catalysts or immunogenic enzymes that are commonly required. Notably, unlike other chemical approaches, click chemistry owing to its unique bioorthogonal feature does not interfere with encapsulated bioactives such as living cells, proteins and drugs and furthermore allows versatile preparation of micropatterned biomimetic hydrogels, functional microgels and nanogels. In this review, recent exciting developments in click hydrogels, microgels and nanogels, as well as their biomedical applications such as controlled protein and drug release, tissue engineering, and regenerative medicine are presented and discussed.

1. Introduction

Hydrogels, microgels and nanogels with excellent biocompatibility, a microporous structure with tunable porosities and pore sizes, and dimensions spanning from human organs, cells to viruses have emerged as a most versatile and viable platform for sustained protein release, targeted drug delivery, and tissue engineering [1–7]. In the past decade, various physical and chemical cross-linking strategies have been developed to fabricate hydrogels, microgels and nanogels [8–10]. The physical hydrogels (e.g. thermosensitive hydrogels, stereocomplexed hydrogels, and ionically crosslinked hydrogels), though formed under particularly mild conditions, are typically weak and exhibit poor long-term stability in tissues [9,11–13]. In contrast, chemical hydrogels, formed from photo-polymerization and enzymatic cross-linking for example, are generally characterized by better stability, durability, and mechanical properties [14,15]. It should be noted, however, that chemical hydrogels often require use of an initiator or enzyme that might introduce potential toxicity concerns. In addition, they may suffer from low specificity, leading to unwanted cross-reactions with drugs, proteins and cells.

In recent years, click chemistry due to its high reactivity, superb selectivity, and mild reaction conditions has appeared as a most promising strategy to prepare hydrogels with varying dimensions and patterns (Scheme 1). The unique bioorthogonality of click reaction renders thus formed hydrogels highly compatible with encapsulated bioactives including living cells, proteins and drugs. For example, hyaluronic acid (HA) hydrogels developed via copper(I)-catalyzed azide-alkene cycloaddition (CuAAC) have been used as drug reservoirs and cell scaffolds [16]. Bisphosphonate-functionalized dextran nanogels crosslinked via CuAAC achieved significant localization in both femur and spine, and provided a possible anti-osteoporotic effect towards bone disease [17]. In

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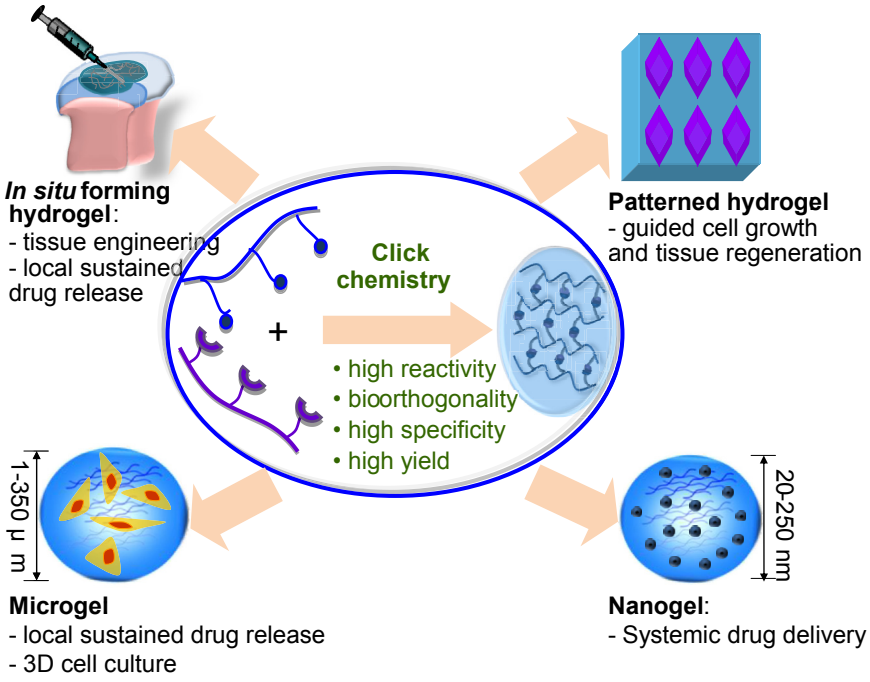


Scheme 1. Preparation and potential biomedical applications of click hydrogels, microgels and nanogels.

particular, recent development of copper-free click chemistry such as strain-promoted azide-alkyne cycloaddition, radical mediated thiol-ene chemistry, Diels–Alder reaction, tetrazole-alkene photo-click chemistry, and oxime reaction renders it possible to form hydrogels, microgels and nanogels without use of potentially toxic catalysts. Anseth et al. reported that cell-laden hydrogels could be formed from azide functionalized PEG and difluorocyclooctyne (DIFO) functionalized peptide without a copper catalyst [18]. Notably, the biochemical properties of hydrogels could be manipulated with precise spatiotemporal control using photo-initiated thiol-ene reactions to introduce new biomolecules into the hydrogel network.

In this review, exciting developments in click hydrogels, microgels and nanogels, as well as their applications for controlled protein and drug release, tissue engineering, and regenerative medicine are presented and discussed. It is remarkable to note that in less than ten years since the first report, click hydrogels have become a unique and versatile family of biomaterials that provide new opportunities to guide cell phenotypes and function and to control drug and protein release. In particular, recent development of click microgels and nanogels has further offered versatile platforms with varying dimensions for controlled drug release, and (stem) cell culture and management in 3D scaffolds. There are several excellent reviews on the synthesis of biomaterials including polymers, dendrimers and hydrogels via click chemistry [19–24], but none have focused on click hydrogels, microgels and nanogels. Here, we first give an overview on the development of click and pseudo-click strategies for the fabrication of different types of hydrogels (Table 1). Then, preparation and biomedical applications of click microgels and nanogels are presented. Finally, spatiotemporal incorporation of biological cues in hydrogels via click chemistry to construct 3D biomimetic micropatterned scaffolds is discussed.

2. Copper(I)-catalyzed azide-alkyne (CuAAC) click hydrogels

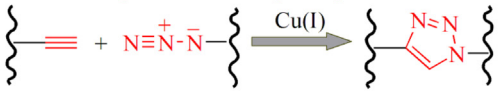
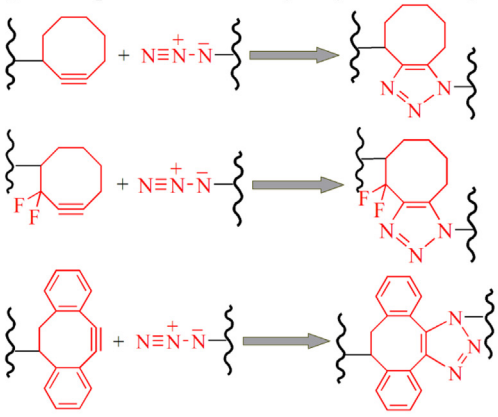
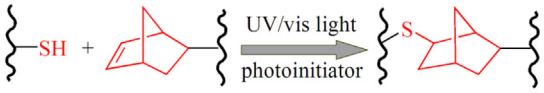
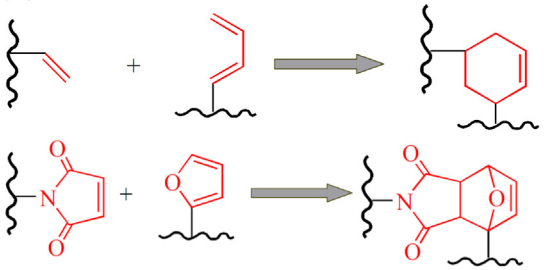
Since the first reports in 2002 by Sharpless and Meldal [25,26], the copper(I)-catalyzed azide-alkyne (CuAAC) click reaction has been viewed as ideal for chemical synthesis, drug discovery, bioconjugation, and biochemistry due to its fast reaction rate, high efficiency, excellent regiospecificity and bioorthogonality [20,27–30]. In particular, alkynes and azides are not present in nature, which renders CuAAC reaction compatible with different drugs, proteins and cells. The reaction kinetics of CuAAC is fast under physiological conditions and typically yields gels within a few seconds to tens of minutes. The first click hydrogels were reported in 2006 by Hilborn et al. from azide and alkyne functionalized poly(vinyl alcohol) (PVA) [31]. The hydrogels were formed with high gel fractions and elastic moduli ranging from 2 to 18.8 kPa using copper sulfate and sodium ascorbate as catalysts. Hawker et al. prepared PEG hydrogels from diacetylene- and tetraazide-functionalized PEG via a CuAAC click reaction [32]. Interestingly, these click hydrogels showed significantly higher tensile strength (up to 2.39 MPa) and strain compared to photochemically cross-linked hydrogels, likely due to the more efficient click reaction. In addition, additives such as carbon black, 4-phosphonooxy-2,2,6,6-tetramethylpiperidyloxy nitroxide and titanium dioxide nanoparticles had little influence on hydrogel formation, supporting that CuAAC click reaction is specific and tolerant of other matters in the reaction mixture. Yang et al. designed and fabricated RGD functionalized PEG hydrogels based on tetraacetylene PEG and diazide-functionalized RGD peptides using $CuSO_4$ and sodium ascorbate as catalysts [33]. The gelation time ranged from 2 to 30 min depending on polymer concentration, temperature and catalyst concentration. The results showed that an increase of RGD peptide concentration from 0.54 to 2.7 mM in hydrogels led to significantly enhanced attachment and proliferation of primary human dermal fibroblasts. Liskamp et al. obtained enzymatically degradable PEG hydrogels from an alkyne-functionalized star-shaped PEG and protease-sensitive diazide-functionalized peptide [34]. These hydrogels were degradable in trypsin and plasmin, and their swelling ratio and storage modulus could be tailored by polymer concentration, molecular weight, and architecture (4- *vs.* 8-arm PEG). Biodegradable hydrogels were also developed from azide-functionalized PEG and alkyne-functionalized poly(trimethylene carbonate) (PTMC) or

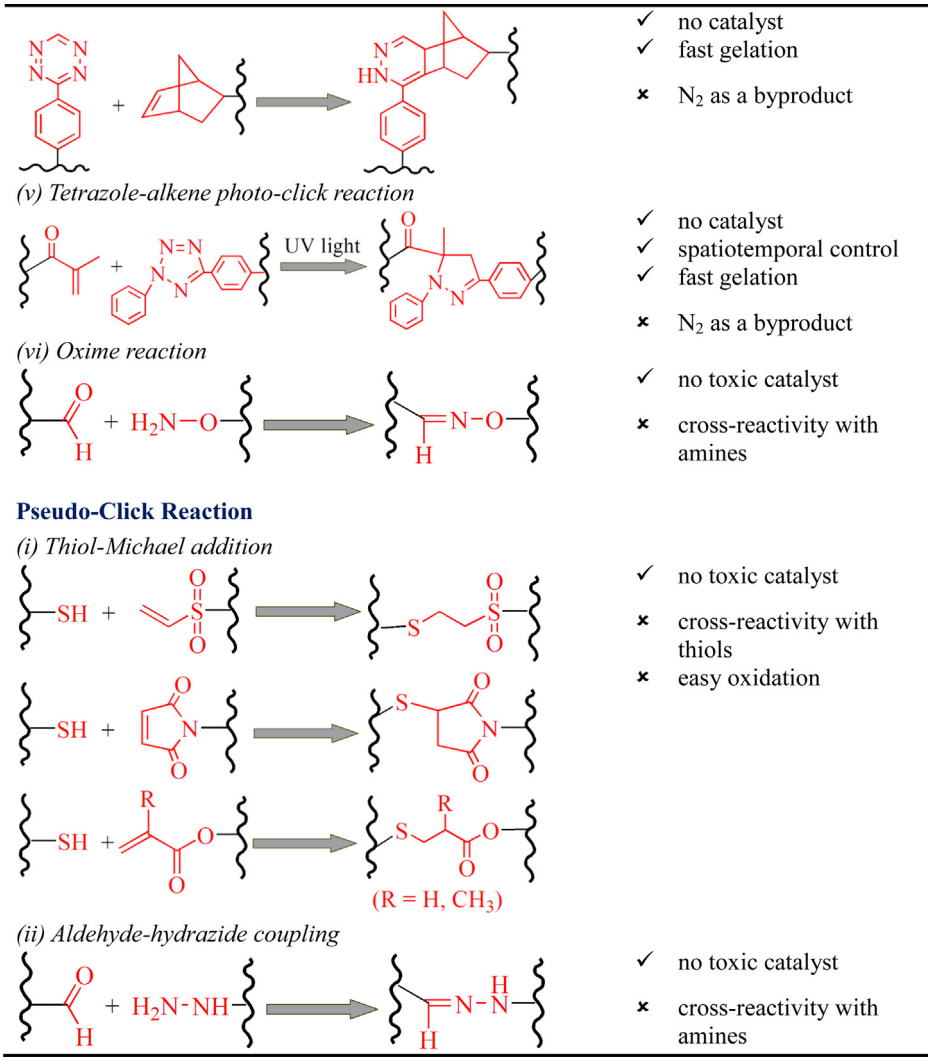
polyaspartamide derivatives [35,36]. Interestingly, Adzima et al. reported that photo-initiated Cu(II) reduction lends excellent spatial and temporal control of the alkyne-azide cycloaddition, with which patterned PEG hydrogels could readily be fabricated from PEG-dialkyne and PEG-tetraazide in the presence of $CuSO_4$ and Irgacure 2959 photoinitiator using standard photolithographic techniques [37].

Thermoresponsive hydrogels were formed from alkyne- and azide-substituted poly(N-isopropylacrylamide-co-hydroxyethylmethacrylate) (P(NIPAAm-co-HEMA)) by CuAAC click reaction in the presence of $CuSO_4$/sodium ascorbate and *N,N,N′,N′,N″*-pentamethyldiethylenetriamine (PMDETA) for 24 h at room temperature [38]. These click hydrogels exhibited higher swelling ratio and faster shrinking/swelling kinetics compared with PNIPAAm hydrogels crosslinked via free radical polymerization. In follow-up studies, thermo-responsive hydrogels were prepared from azide-functionalized PNIPAAm and alkyne-substituted β–cyclodextrin (β-CD), or alkyne-functionalized PNIPAAm and azide-substituted cellulose [39,40]. The release of fish DNA from these thermo-sensitive hydrogels was accelerated by an increase of temperature. Fleury et al. obtained thermo-sensitive hydrogels via click reaction of alkyne-functionalized guar and α,ω-diazido-poly(-ethylene glycol-co-propylene glycol) [41]. The resulting hydrogels demonstrated a fast and reversible thermo-sensitive swelling behavior. *In vitro* release studies showed that DOX·HCl release from hydrogels was temperature-dependant.

Hydrogels based on natural polymers (also called natural hydrogels) due to their excellent biocompatibility and biodegradability have attracted great interest for drug delivery and tissue engineering. Crescenzi et al. prepared click hydrogels based

Table 1
Summary of click chemistry strategies employed to form hydrogels.

Click Reactions	Features
Cu(I) Catalyzed Azide-Alkyne Cycloaddition (CuAAC) 	✓ bioorthogonality × toxic copper catalyst
Copper-Free Click Reaction	
(i) Strain-promoted azide-alkyne cycloaddition (SPAAC) 	✓ no catalyst ✓ bioorthogonality × difficult synthesis of cyclooctynes
(ii) Thiol-ene photocoupling 	✓ spatiotemporal control ✓ fast gelation × potential toxicity from photoinitiators and radicals × cross-reactivity with thiols
(iii) Diels-Alder reaction 	✓ no catalyst ✓ accelerated by H_2O ✓ thermoreversible × slow gelation
(iv) Inverse electron demand Diels-Alder reaction	



on azide- and alkyne-functionalized hyaluronic acid (HA) [16]. *In vitro* release studies showed that benzidamine and doxorubicin (DOX) were released from HA hydrogels over a period of hours to several weeks depending on cross-linking densities. Cell culture experiments revealed that yeast cells entrapped in HA hydrogels maintained about 80% proliferating activity after 24 h. Dentini et al. prepared HA hydrogels from azide-functionalized HA and a series of dialkyne reagents (1,4-diethynylbenzene, 1,6-heptadiyne, 1,8-nonadiyne) with different lengths [42]. The hydrogels formed from longer dialkynes demonstrated higher cross-linking densities, stronger storage moduli, and lower swelling ratios. To retain CD44 recognition sites, Huerta-Angeles et al. prepared click HA hydrogels from HA azide and alkyne derivatives conjugated through the hydroxyl groups of HA [43]. These hydrogels were found to support the proliferation of chondrocytes for up to 15 days. Koschella et al. obtained cellulose hydrogels from water-soluble azide- and alkyne-functionalized cellulose [44]. In order to mimic the natural cartilage extracellular matrix, Gao et al. developed triple biopolymer hydrogels from alkyne-functionalized gelatin, azide-functionalized HA and chondroitin sulfate (CS) using Cu(I) as a catalyst [45]. The resulting hydrogels exhibited a fast gelation rate, a highly swollen state and an elastomer characteristic. *In vitro* cell culture studies showed that a confluent layer of cells were formed after 3 days, indicating that these triple biopolymer hydrogels could well support the adhesion and proliferation of chondrocytes.

A major limitation of the CuAAC reaction in biomedical applications is the potential cytotoxicity of copper ions and reactive oxygen species (ROS) generated by copper ions, which might lead to severe structural damage of biomolecules such as proteins, nucleic acids, polysaccharides, and lipids [46]. Despite the fact that residual Cu(I) catalyst in preformed hydrogels may be removed by EDTA, this is not ideal, nor is it a suitable option for injectable material use; therefore the formation of click hydrogels without use of a copper catalyst would be more desirable.

3. Copper-free click hydrogels

3.1. Strain-promoted azide-alkyne cycloaddition (SPAAC) click hydrogels

In the past years, several cyclooctyne molecules were found to react quickly with azides without a copper catalyst as a result of ring strain and electron-withdrawing fluorine substituents [47,48]. Anseth et al. fabricated enzymatically degradable hydrogels through a strain-promoted azide-alkyne cycloaddition

(SPAAC) reaction between 4-arm PEG tetraazide and a difluorinated cyclooctyne (DIFO)/alkene-functionalized peptide containing (GPQG↓ILGQ) sequence at physiological conditions, allowing for the direct encapsulation of 3T3 cells (Fig. 1) [18]. Subsequently, orthogonal thiol-ene photocoupling chemistry was employed to enable patterning of biological functionalities within the gel in real time and with micrometer-scale resolution in the presence of cells. The modulus (1000–6000 Pa) and chemical functionality of the hydrogels could be precisely and independently tuned by the azide/DIFO ratio, the PEG molecular weight or the dosage of light, providing an avenue to direct cell function throughout specific regions within a 3D material [49]. Interestingly, hydrogels with photoreversible patterning of biomolecules were developed by SPAAC reaction between PEG tetracyclooctyne, and bis(azido)-functionalized allyloxycarbonyl-protected polypeptide in an aqueous medium [50]. The alkene groups in the hydrogels were utilized to introduce biochemical cues by the thiol-ene photo-conjugation reaction. The biochemical cues could also be removed by exposure to UV light, giving excellent spatiotemporal control over the presentation of

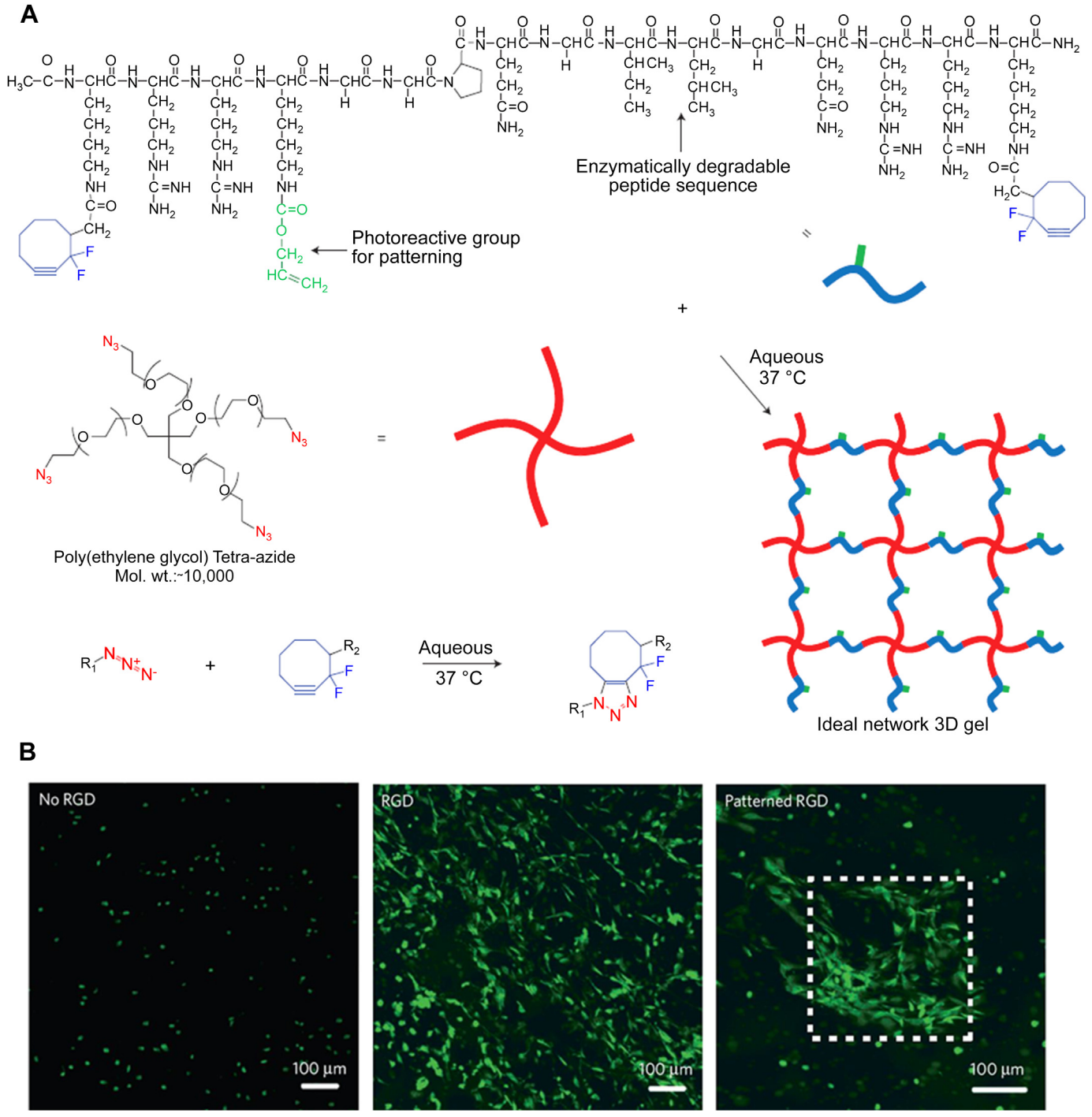


Fig. 1. Preparation and post-modification of cytocompatible SPAAC click hydrogels. (A) Preparation of 3D network hydrogels via SPAAC reaction from 4-arm PEG tetra-azide and difunctionalized peptide sequence; (B) effect of patterned RGD on 3T3 population within 3D click hydrogels that formed using full mask, no mask or full mask with a 250-μm-square opening (illustrated by the dashed lines); cells adopt a spread morphology only in user-defined regions of RGD [18].

biologically relevant chemical cues within the hydrogels. In another study, photo- and enzyme-labile hydrogels were developed from PEG-tetracyclooctyne and diazide peptide, which facilitated spatiotemporal control over the geometry and connectivity of cellular microenvironments [51]. These functional hydrogels were exploited for dynamic culture of lung epithelial cells. In particular, alveolar-inspired shapes were created to investigate their influences on epithelial cell differentiation.

Song et al. reported that biodegradable hydrogels with varying mechanical properties and degradability were formed within minutes through SPAAC click chemistry under physiological conditions from azide-containing PEG-co-poly(5,5-bis(azidomethyl)-1,3-dioxan-2-one) and dibenzocyclooctyne (DBCO)-functionalized PEG [52]. Bone marrow stromal cells encapsulated in these click hydrogels exhibited higher viability than those in photo-crosslinked PEG dimethacrylate hydrogels. Becker et al. reported that human mesenchymal stem cells (hMSCs) encapsulated in *in situ* forming hydrogels from a SPAAC reaction between DBCO functionalized PEG and three-arm glycerol exytholate triazide remained high viability (89% ± 2%) after 24 h [53]. Santi et al. developed SPAAC hydrogels with tunable drug release and degradation rates from 4-arm azido-PEG and 8-arm DBCO-derivatized PEG, in which one β–eliminative linker was used to tether the drug to the hydrogel and the other, with a longer half-life, was used to control hydrogel degradation [54]. The results showed that exenatide-conjugated hydrogels enabled once-a-month administration, instead of twice-daily injections for free exenatide. Ito et al. reported that *in situ* forming hydrogels based on azide- and cyclooctyne-modified HA without any catalyst under physiological conditions showed good biocompatibility following intraperitoneal and subcutaneous administration [55]. The main drawback of SPAAC is that the synthesis of cyclooctyne is comprised of over ten steps with a low overall yield, which makes this strategy unsuitable for large-scale synthesis.

3.2. Thiol-ene click hydrogels

The thiol-ene click reaction involves a radical-mediated addition of a thiol to a double bond under light irradiation, which is efficient, high yielding, and tolerant of different functional groups [56,57]. Lin and Anseth reported that hydrogels were formed in less than 3 min from norbornene-functionalized 4-arm PEG (PEG-4-Norb) with dithiol peptide, in which both the mechanical and biochemical properties of hydrogels could be controlled by thiol/ene ratio, PEG molecular weight and architecture, polymer concentration, or peptide sequences [58,59]. In addition, hydrogel degradation could be tuned from bulk degradation to surface erosion by introducing enzyme-sensitive peptides [58]. The surface eroding thiol-norbornene hydrogels were exploited to release various proteins including bovine serum albumin and carbonic anhydrase on the same time scale in response to human neutrophil elastase (HNE) at sites of inflammation [60]. The protein release rate was controlled by the peptide reaction constant, the concentration of HNE, and the content of peptide within the gel. Notably, proteins released from the hydrogels retained nearly 100% of their bioactivity [61]. Alternatively, drugs like glucocorticoid dexamethasone (Dex) were conjugated to the *N*-terminus of a MMP-degradable peptide, which was easily copolymerized into PEG gel scaffolds via thiol-norbornene reaction to achieve cell-mediated delivery of bioactive small molecules [62]. The conjugated Dex was locally sequestered, and only available for uptake by local co-encapsulated hMSCs that secreted MMPs to cleave the peptide tether, subsequently stimulating cellular responses (elevated alkaline phosphatase activities and calcium deposition levels) for over 21 days.

Anseth et al. developed peptide-functionalized PEG hydrogels through thiol-ene photo-polymerization of PEG-4-Norb with bis-cysteine peptides for the encapsulation, culture, migration and differentiation of hMSCs in 3D environments [63–65]. The high viability (>95%) of hMSCs encapsulated in the hydrogels after up to 24 h and the well-formed actin fibers and β1 integrin staining at the ends of stress fibers observed by immunohistochemistry illustrated strong cell adhesion to the network. The CRGDS peptide could be easily incorporated via spatiotemporal photopatterning techniques within predetermined 3D regions of the PEG hydrogels, providing a functional cylinder microenvironment with which to control cell spreading of hMSCs (Fig. 2) [63]. CRGDS-functionalized hydrogels were also utilized to investigate the migration of hMSCs in a 3D microenvironment; it was found that hydrogels with low crosslinking density (0.18 ± 0.02 mM) and high adhesivity (1 mM CRGDS) permitted higher cell migrating speeds (17.6 ± 0.9 μm h^{-1}), greater cell spreading (elliptical form factor = 3.9 ± 0.2) and more sustained polarization [64]. In addition, hMSCs encapsulated in these hydrogels were observed to express osteogenic, chondrogenic and adipogenic markers under the appropriate culture conditions, and the expression of these markers tended to increase with gel degradability [65]. In a follow-up study, the thiol-norbornene

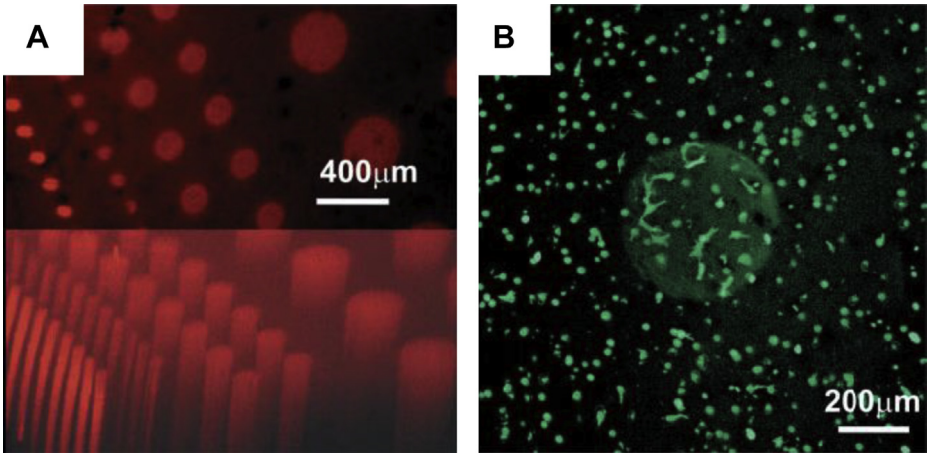


Fig. 2. PEG-peptide hydrogels with 1 mM excess of norbornene functional groups formed from PEG-4-Norb and KKCGGPQGIWGQGCKK peptide via thiol-norbornene reaction. (A) 3D projection of an 800-μm-thick section of gel, in which CRGDS-rhodamine cylinders were photopatterned and angled view of the same (bottom); and (B) 3D projection of a 400-μm gel section. hMSCs are encapsulated in MMP-degradable hydrogel with CRGDS displayed only where photopatterned (day 6) [63].

hydrogels were utilized to repair a calvarial defect and exhibited significant improved bone regrowth, although the addition of MSCs did not further enhance bone regeneration [66].

Lin et al. reported that PEG hydrogels based on PEG-4-Norb and chymotrypsin-sensitive bis-cysteine-peptides could be used as bioactive and immuno-isolating barriers for the encapsulation of insulin secreting pancreatic β-cells [67]. In this system, cells formed spherical clusters naturally and were retrieved via rapid chymotrypsin-mediated gel erosion. The recovered cell spheroids were observed to release insulin in response to glucose treatment, indicating that thiol-ene hydrogels are cyto-compatible. In another study, human pancreatic ductal epithelial cells (PANC-1) encapsulated and cultured in 3D thiol-ene PEG hydrogels were observed to form clusters within 4 days, in contrast to a monolayer morphology on 2D culture [68]. After culturing for 10 days, PANC-1 cells formed a large ductal cyst-like structure in MMP-sensitive or RGDS-immobilized gels, whereas they were more compact and adopted an epithelial cell phenotype in MMP-insensitive or YIGSR-immobilized gels.

MMP-sensitive PEG hydrogels based on PEG-4-Norb and bis-cysteine-peptides were also employed to characterize the spreading, proliferation, and migration of valvular interstitial cells (VICs) in a 3D environment [69]. VIC morphology and rate of process extension were observed to increase through decreasing the hydrogel matrix density presented to the cells. Notably, the expression of alpha smooth muscle actin (αSMA) and ECM (collagen) of VICs were found to be dependent on biochemical functionality (i.e. peptide sequence, growth factor) and mechanical properties (i.e. gel modulus) of hydrogels, indicating that gel platforms could offer a physiologically relevant microenvironment for VIC activation and characterization [59,69]. Recently, Anseth et al. demonstrated that PEG hydrogels crosslinked by MMP-degradable peptide via thiol-ene reaction could serve as a 3D platform for the culture of both aggregated and single mammalian motor nerve cells that not only permitted cell survival over more than a week of culture, but also allowed for the robust extension of motor axons [70]. These 3D hydrogels appeared to be an improved cellular delivery system for the treatment of neuro-degenerative diseases.

Crosby et al. reported that highly resilient hydrogels were fabricated from hydrophilic PEG and hydrophobic polydimethylsiloxane (PDMS) polymers by using thiol-ene chemistry [71]. Cyclic tensile testing showed that the hydrogels maintained a resilience greater than 97% across all measured strains (up to 300%), which was similar to resilin (one of the most resilient materials known) probably due to uniform network structure, low crosslink density and lack of secondary structure in the polymer chains. In addition, the swelling capacity, mechanical properties, and fracture toughness of the hydrogels could be easily tuned by manipulating the volume fraction of PDMS [72].

Inspired by conjugating thiol-containing peptides to alkene moieties in hydrogels using visible light [50,73], Lin et al. developed visible light-mediated thiol-ene hydrogels from PEG-4-Norb and dithiothreitol [74]. The gels were formed within 4 min using a visible light source (400–700 nm) with eosin-Y (0.1×10^{-3} M) as the only photo-initiator. This photo-click technique preserved the rapid and efficient step-growth cross-linking without use of any cytotoxic initiator, ensuring high cyto-compatibility for hMSCs and pancreatic β-cells encapsulated in hydrogels. The visible light-mediated thiol-ene reaction was also employed to create hydrogels with multilayer structures and a wide range of thicknesses ranging from tens of micrometers to a few millimetres [75].

3.3. Diels–Alder (DA) click hydrogels

The Diels–Alder (DA) reaction is a highly selective [4 + 2] cycloaddition between a diene and a dienophile without any catalyst or byproduct, and it is greatly accelerated in water due to increased hydrophobic effects. Wei et al. reported that thermosensitive hydrogels were prepared by an aqueous DA reaction of poly(N,N-dimethylacrylamide-co-furfuryl methacrylate) with dimaleimide-PEG without the use of an initiator, catalyst, or coupling agent [76]. The gelation time (10–160 min) and hydrogel swelling ratio decreased with increasing temperature. Marra et al. prepared HA hydrogels with a modulus of 18 Pa from maleimide-modified HA and furan-modified HA via DA click reaction [77]. The hydrogels were formed in 40 min and allowed direct encapsulation of positively and negatively charged proteins. *In vitro* release studies showed that insulin (negatively charged) and lysozyme (positively charged) encapsulated into hydrogels achieved sustained release over 21 days.

The DA click reaction could be reversed at high temperature through the retro-DA reaction, which opens a way to controlled drug release [78]. Bowman et al. reported DA-mediated controlled release of peptide/dexamethasone from PEG hydrogels by conjugating furan-RGDS/dexamethasone onto PEG hydrogel with an excess of maleimide functionalities [79]. By changing the temperature, varying extents of release were attained over the time course of several days, ranging from 40% release at 37 °C to complete release at 80 °C. In addition, increasing the amount of free maleimide, and therefore the number of potential DA reaction sites, effectively slowed the release of peptide from the hydrogel. The dynamic nature of the DA reaction was also exploited for controlled release of covalently tethered dexamethasone from PEG hydrogels [80]. DA-mediated dexamethasone release stimulated hMSCs to undergo robust osteogenic differentiation within an hMSC-laden hydrogel, as indicated by significantly increased alkaline phosphatase (ALP) activity and substantial mineral deposition. Recently, Goepferich et al. developed DA click PEG hydrogels based on 4-arm PEG-maleimide and 8-arm PEG-furan that were readily degraded by retro-DA reactions at body temperature within days to several weeks, providing a potential platform for controlled protein release [81]. The gelation time ranged from 14 to 171 min and modulus ranged from 2.8 to 37.1 kPa, depending on concentration, branching factor, and molecular weight of the macromonomers.

Shoichet et al. prepared HA hydrogels with independent control over mechanical properties, architecture topography, and biomolecule distribution through DA click reaction of furan-modified HA with dimaleimide-PEG [82,83] (Fig. 3A). The shear modulus ranging from 275 to 680 Pa, similar to that of central nervous system tissue, could be controlled by either changing the polymer concentration or the furan substitution on the HA backbone. Porosity and pore size were controlled by cryogelation and thaw temperature, respectively. Biomolecules were photopatterned into the hydrogels by two-photon laser processing, resulting in spatially defined growth factor gradients to direct cell function (Fig. 3B and C) [83]. Furthermore, endothelial cells on hydrogels exhibited high viability (>98%), and appeared to interact with the HA matrix after 14 days of culture *in vitro*, demonstrating excellent cytocompatibility of hydrogels [82].

3.4. Click hydrogels formed by inverse electron demand Diels–Alder reaction, oxime reaction, and tetrazole-alkene photo-click reaction

Recently, Anseth et al. introduced the tetrazine-norbornene inverse electron demand Diels–Alder reaction as a cross-linking chemistry to prepare cell-laden hydrogels from a tetrazine-functionalized 4-arm PEG and a dinorbornene peptide [84]. The fast reaction rate and irreversible nature of this click reaction allowed for hydrogel formation within minutes. The cytocompatibility of the polymerization led to high viability (92%) of hMSCs at 24 h postencapsulation, and the specificity of the tetrazine-

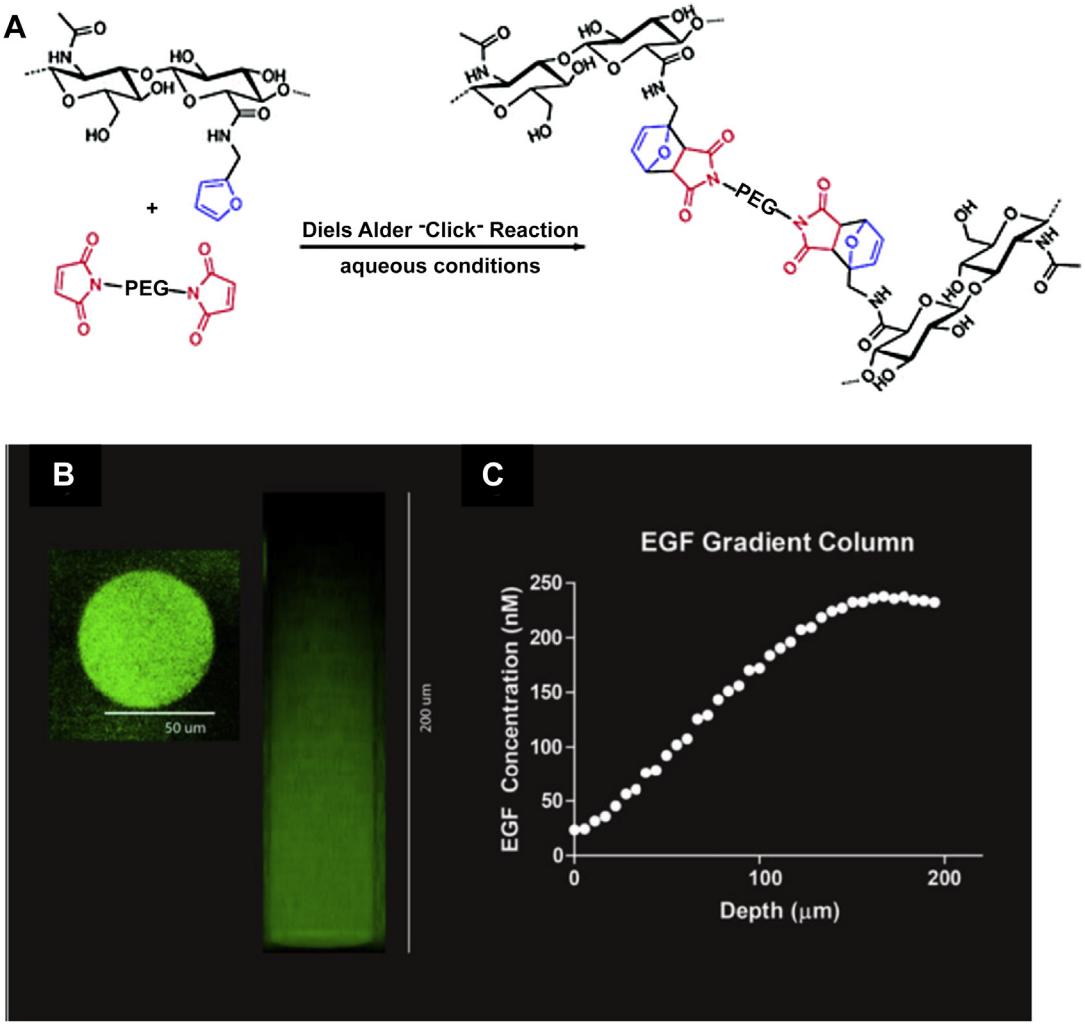


Fig. 3. Three-dimensional patterning of epidermal growth factor (EGF) within a HA hydrogels. (A) Preparation of HA hydrogels from HA-furan and dimaleimide-PEG via Diels–Alder (DA) click reaction; (B) creation of a linearly immobilized gradient of EGF labeled with AlexaFluor 488. From the top of the hydrogel, the number of scans by the multiphoton laser is increased as it penetrates the sample, corresponding to an increase in fluorescence intensity and hence and increase in protein immobilization; and (C) the concentration of immobilized protein in the gradient was quantified by the fluorescence intensity, showing a change in concentration from 25 nM at the top of the hydrogel to 250 nM at a depth of 150 μm in the hydrogel [83].

norbornene reaction was exploited for sequential modification of the network with proteins like BSA via thiol-ene photochemistry. Maynard et al. developed hydrogels from 8-arm aminooxy PEG and glutaraldehyde via oxime click chemistry [85]. The oxime bond formation is acid catalyzed and thus the gelation time could be tuned from 5 min to over 30 min by changing the pH of the hydrogel precursor solution from 6.0 to 8.0. MSCs encapsulated within the oxime cross-linked PEG hydrogel were viable and metabolically active for at lease 7 days. We recently reported on the preparation of *in situ* forming PEG hydrogels from PEG-4-MA and PEG-4-tetrazole via tetrazole-alkene photo-click reaction [86]. The gelation times ranged from 50 s to 5 min, and storage moduli varied from 0.65 to 25.2 kPa depending on polymer concentrations and degrees of tetrazole substitution in PEG-4-tetrazole conjugates. *In vitro* release studies showed that cytochrome C (CC), γ-globulins (Ig), and recombinant human interlukin-2 (rhIL-2) all were released from tetrazole-alkene hydrogels in a sustained and quantitative manner over a period of 14–20 days. Importantly, released CC and rhIL-2 exhibited comparable biological activities to native CC and rhIL-2, respectively, supporting that the tetrazole-alkene photo-click reaction is highly specific and compatible with proteins.

4. Pseudo-click hydrogels

Pseudo-click chemistry including thiol-Michael reaction and aldehyde-hydrazide coupling reaction has also been broadly employed for hydrogel preparation due to its high reactivity, simple reaction conditions, and high yield. Pseudo-click reaction is usually characterized by moderate orthogonality.

4.1. Thiol-Michael pseudo-click hydrogels

A thiol-Michael addition reaction consists of adding of a thiol across a double bond in acrylate, vinyl sulfone, or maleimide, giving thioethers with or without the help of a basic catalyst [87]. The reaction has been employed for quite some time as a powerful and versatile technique to fabricate hydrogels due to its fast reaction rate, high coupling efficiency, tolerance to a wide variety of functional groups, and easy access to thiol and ene functionalized reagents [88–90].

Hubbell et al. were the first to construct step-growth hydrogels using the Michael addition reaction as a cross-linking chemistry [91–93]. The hydrogels were formed from PEG-multiacrylate and

PEG-dithiol, dithiothreitol, or thiol-containing peptides. The gelation rate could be controlled by the basic catalyst, the precursor concentration, and the local electrostatic environment of the thiol. When the thiol group is close to a positively charged amino acid (e.g. arginine), it's pK_a would be decreased and the Michael addition reaction would be accelerated [92,94]. The negatively charged amino acid (e.g. aspartic acid) showed the opposite effect. Release studies revealed that albumin encapsulated in PEG hydrogels was released in a zero-order kinetics over a period of about 4 days [91]. Recently, Pritchard et al. developed injectable PEG hydrogels with de-swelling properties from PEG diacrylate (M_n = 400 g/mol) and ethoxylated trimethylolpropane tri-3-mercaptopropionate (ETTMP) that avoided undue swelling associated with clinically available injectable hydrogels [95]. The resulting hydrogel was reduced to 59.7% of its initial volume under simulated physiological conditions, achieved a sustained release of methylprednisolone sodium succinate for more than 500 h, and promoted the adhesion of murine MSCs through the incorporation an oligolysine peptide.

Natural polymers due to their excellent biocompatibility, biodegradability and bioactivity have been introduced into PEG hydrogels for functional cell delivery. Hoffman et al. reported that the proliferation of fibroblasts inside hybrid hydrogels based on thiolated heparin and PEG diacrylate was significantly increased (more than 5-fold) by the addition of heparin-binding molecules like fibrinogen [96]. Similarly, chitosan-PEG hybrid hydrogels were developed to encapsulate cells like smooth muscle cells [97,98]. Fibronectin-HA-PEG hydrogels were exploited to stimulate wound repair since fibronectin facilitates dermal fibroblast migration during normal wound healing [99]. Kao et al. developed gelatin-PEG hydrogels via thiol-Michael reaction for 3D MSC administration to accelerate wound closure, neovascularization and re-epithelialization in full thickness skin wounds in Sprague–Dawley rats [100]. Thiol-Michael hydrogels based on thiolated HA, thiolated gelatin and PEG diacrylate have been investigated for the repair of ischemic myocardial infarcts and osteochondral defects [101–104]. The cells in the hybrid hydrogels displayed improved cell spreading, viability and activity. Interestingly, Engler et al. recently developed HA hydrogels with time-dependent mechanical properties from thiol-functionalized HA and PEG diacrylate to mimic temporal tissue stiffness changes during the development of cardiac muscle [105]. Results showed that when hydrogels stiffened from 1.9 kPa to 8.2 kPa over 456 h post-gelation, similar to heart muscle development, pre-cardiac cells grown on the dynamic hydrogels exhibited a 3-fold increase in mature cardiac specific markers and formed up to 60% more mature muscle fibers over 2 weeks as compared to static polyacrylamide hydrogel controls.

Vinyl sulfone (VS) was reported to be far more reactive than acrylate in the thiol-Michael reaction due to its higher electron withdrawing capability [88]. The addition of thiol to VS gives a very stable thioether sulfone bond. Stein et al. fabricated injectable hydrogels from PEG copolymers containing multiple thiol groups and di-VS terminated PEG for controlled protein release [106]. *In vivo* release studies showed that erythropoietin, RANTES and three PEG-conjugated RANTES derivatives were released in a sustained manner for 2–4 weeks. These protein-releasing hydrogel systems demonstrated prolonged biological activity in rats. Leach et al. developed hydrolytically degradable PEG hydrogels with mesh size of 13–35 nm from 4-arm PEG-VS (PEG-4-VS) and PEG-diester-dithiol and investigated for BSA delivery [107,108]. The degradation rate of hydrogels could be tuned by the molecular weight and number of methylene groups between the thiol and the ester of PEG-diester-dithiol. The protein released from these PEG hydrogels maintained its secondary structure. Feijen and Zhong reported that degradable dextran hydrogels were rapidly formed *in situ* by thiol-Michael reaction of dextran VS conjugates and thiol-functionalized PEG or dextran [109–111]. *In vitro* release studies demonstrated that varying proteins such as immunoglobulin G (IgG, d_h = 10.7 nm), BSA (d_h = 7.2 nm), lysozyme (d_h = 4.1 nm), and fibroblast growth factor (bFGF)) were released in a controlled manner over 14 days. The released proteins had fully preserved biological activity.

Hubbell et al. developed cell-responsive degradable PEG hydrogels from PEG-4-VS, monocysteine cell-adhesion peptides and MMP-degradable bicysteine-peptides via a thiol-Michael reaction and investigated their applications for tissue regeneration [112–115]. Cell culture showed that fibroblasts could spread and migrate into the networks and that the migration rate depended on the adhesion ligand density and the proteolytic activity of the incorporated peptide sequences. The hydrogels used to deliver recombinant human bone morphogenetic protein-2 (rhBMP-2) to the site of critical defects in rat crania were completely infiltrated by cells and were remodeled into bony tissue within five weeks, resulting in efficient and highly localized bone regeneration. Similarly, recombinant proteins combining enzymatic degradability with cell adhesion were cross-linked with divinyl sulfone-terminated PEG to form cell-adhesive and proteolytically degradable hydrogel matrices that achieved success in healing critical-sized defects of rat calvaria [116,117]. Segura et al. encapsulated DNA/PEI polyplexes uniformly into MMP-degradable PEG hydrogels that were formed through the thiol-Michael click reaction of cysteine-containing MMP sensitive peptides with 4-arm PEG-VS pre-modified with cell adhesion RGD peptides [118]. The results of gene transfer to MSCs in 3D hydrogel scaffolds showed that seeded cells afforded cumulative transgene expression over 7 days. Feijen et al. reported that HA hydrogels based on thiol-functionalized HA and PEG-4-VS have a high potential for cartilage regeneration, as evinced by accumulated collagen and chondroitin sulfate after culturing chondrocytes in these hydrogels for 21 days [119].

The Michael reaction between maleimide and thiol has been shown to be quantitative and highly selective [120]. Kiick et al. designed and prepared degradable hybrid hydrogels from maleimide-functionalized heparin and thiol-functionalized PEG via thiol-maleimide reaction for the binding and controlled release of growth factors [121]. *In vitro* protein release showed little burst release and 15–30% of bFGF release over 7 days. The incorporation of RGD peptide and fibronectin into the thiol-maleimide PEG hydrogels enhanced the adhesion and spreading of human aortic adventitial fibroblasts, which were critical for blood vessel remodeling [122]. In a follow-up study, glutathione-sensitive hybrid hydrogels formed through the thiol-Michael reaction of aromatic thiols of PEG and maleimides of heparin were developed and exhibited a 4–5 fold faster degradation rate than cysteine-maleimide crosslinked hydrogels, and a 10-fold slower degradation rate than disulfide-crosslinked hydrogels [123].

García et al. prepared a set of functional hydrogels from different Michael acceptors such as PEG-4-acrylate, PEG-4-VS, and PEG-4-MAL that were pre-modified with RGD peptide using dithiol protease-cleavable peptide crosslinker [124]. The results revealed that the maleimide derivative required two orders of magnitude less triethanolamine to form hydrogels, with faster cross-linking times of 1–5 min and tighter network structure, than its counterparts. Importantly, gels formed at a low PEG-4-MAL polymer weight percentage (4 w/v%) could promote the spreading of encapsulated C2C12 murine myoblasts, which was comparable to naturally-derived extracellular matrices such as collagen matrix (Fig. 4A). These PEG-4-MAL hydrogels integrated well into the tissue with a penetration depth of 50 μm after being injected onto the pericardium of an excised rat heart (Fig. 4B–C), indicating that these hydrogels have a great potential for application as a myocardial surface patch.

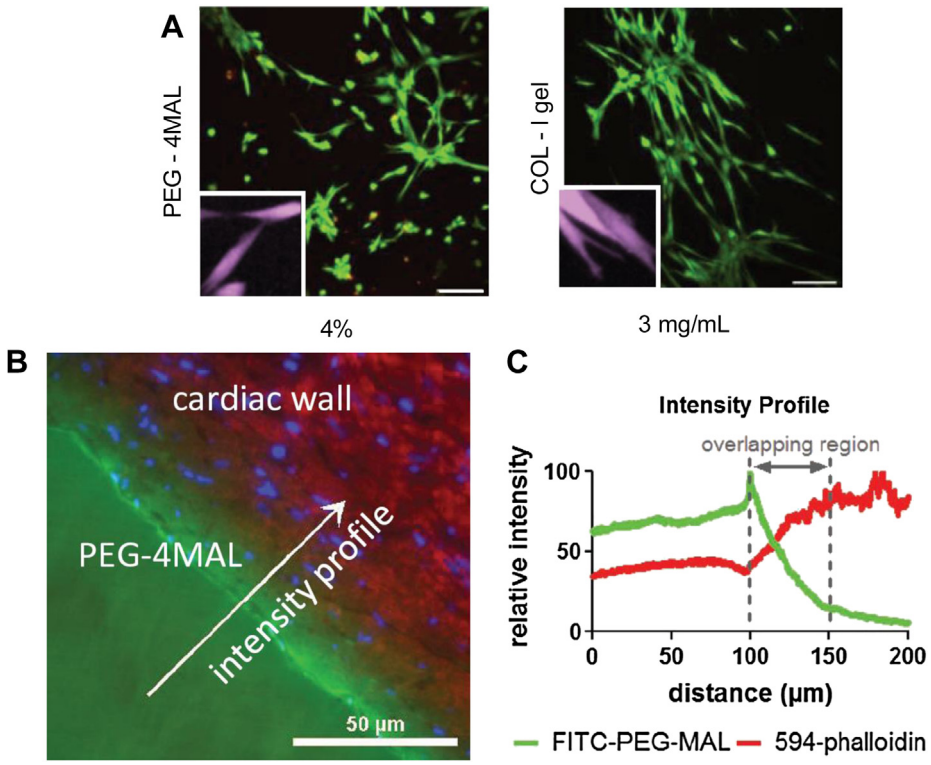


Fig. 4. Cell encapsulation in PEG-4-MAL hydrogels. (A) Live/Dead staining of C2C12 murine myoblasts after 3 days encapsulation in 4 w/v% PEG-4MAL hydrogels (scale bar = 100 μm), inset higher magnification showing individual cell spreading; (B) PEG-4-MAL hydrogels incorporating 1 wt/v% FITC-PEG-MAL in rat myocardial wall counterstained with Alexa-Fluor 594 phalloidin and DAPI; and (C) fluorescence intensity profiles for FITC-PEG-MAL and 594-phalloidin illustrated that hydrogels were incorporated into the tissue to a depth of approximately 50 μm [124].

4.2. Aldehyde-hydrazide pseudo-click hydrogels

The aldehyde-hydrazide pseudo-click reaction is attractive for hydrogel formation due to its simplicity, versatility, absence from toxic reagents and side products, and reversibility. Various hydrogels have been fabricated from the polysaccharide adipic acid dihydrazide (ADH) and aldehyde derivatives via the aldehyde-hydrazide reaction. HA-ADH is usually synthesized by reacting the carboxylic groups in HA with an excess (30-fold molar) of ADH in the presence of EDC and HOBt, and HA-aldehyde (HA-CHO) is obtained by reacting HA with equimolar sodium periodate [125]. Kohane et al. demonstrated that HA hydrogels synthesized from HA-ADH and HA-CHO could be employed to deliver bupivacaine, providing prolonged local anesthesia [126]. It was found that 2 w/v % HA hydrogels doubled the duration of sciatic nerve block of bupivacaine alone without a statistically significant increase in myotoxicity. Similarly, tissue-type plasminogen activator (tPA)-loaded HA hydrogels showed high efficiency in preventing recurrent adhesions using a rabbit model, although over 80% tPA was released within 2 days [127]. The incorporation of gelatin into the hydrogels could markedly extend the duration of FITC-albumin and interleukin-2 (IL-2) release to over 3 weeks with retention of protein activity (over 70% for IL-2) [128]. Drugs like dexamethasone could also be covalently conjugated to HA hydrogels via an acyl-hydrazine bond to achieve sustained drug release [129]. With degradation of the hydrogel, dexamethasone was released over 5 days, and its biological activity was preserved. Cui et al. developed a pH-sensitive releasing system by covalently attaching IgG to HA hydrogels via the hydrolytically unstable hydrazone linkage for reparation of the injured brain in rat [130].

Hydrazone crosslinking hydrogels have also exhibited a great potential in tissue regeneration. Prestwich et al. prepared HA hydrogels from HA-ADH and PEG propiondialdehyde via hydrazone crosslinking at neutral pH and investigated their use as bio-interactive dressings for wound healing [131]. Full thickness cutaneous wounds treated with HA hydrogels had more dermal collagen regeneration and organization by day 10 compared to those treated with Tegaderm™. Gruh et al. developed *in situ* forming hydrogels from HA and alginate for myocardial tissue engineering [132]. These natural hydrogels allowed for the generation of contractile bioartificial cardiac tissue from cardiomyocyte-enriched neonatal rat heart cells, which resembled the native myocardium. Interestingly, Ossipov et al. developed a disulfide-based protection strategy for the *in situ* formation of mechanically stable HA hydrogels, which avoided intra/intermolecular cross-linking of HA chains, excess of dihydrazide reagents, and intermediate purification [133]. This strategy allowed for the incorporation of reactive hydrazide and thiol groups in HA orthogonally, permitting *in situ* formation of hydrogels with HA-CHO derivatives and conjugation of small molecular drugs such as bisphosphonate (BP) and pyrene for localized delivery at the site of implantation [134,135]. The incorporation of Ca^{2+} into the HA-BP hydrogel promoted an efficient and fast mineralization of the matrix through the strong interaction between BP residues and Ca^{2+} ions that served as nanometer-sized nucleation points for further calcium phosphate deposition within the HA hydrogel [136]. Varghese et al. developed a mild synthetic procedure for HA-CHO by incorporating an amino-glycerol side chain via amidation reaction and selective oxidation of the pendent group, which upon mixing with HA-ADH formed stable hydrogels within 30 s [137]. These HA hydrogels loaded with BMP-2 achieved bone formation subperiosteally in 8 weeks after implanting in the rat calvarium. In a similar way, the hydrazone crosslinking technique has been employed to prepare hydrogels based on dextran [138] and synthetic polymers such as

PVA [139], polyaspartylhydrazide [140], poly(N-isopropylacrylamide) (PNIPAM) [141], and recombinant elastin-like protein [142]. Notably, Varghese et al. obtained highly stable HA hydrogels from HA-CHO and a HA-carbodihydrazide derivative (HA-CDH) via a resilient hydrazone bond that was about 15 fold more stable than the other hydrazones at pH 5.0 [143]. These HA hydrogels exhibited exceptional hydrolytic stability, mechanical properties, low swelling, and controlled enzymatic degradation. The *in vivo* evaluation of these HA hydrogels revealed that neo-bone with highly ordered collagen matrix mimicking natural bone regeneration was formed.

5. Click nanogels and microgels

Nanogels and microgels are crosslinked spherical hydrogel particles that have nano- (typically 20–250 nm) and micro- (typically 1–350 μm) scale dimensions, respectively. They have excellent biocompatibility, high water content, tunable sizes, large surface area for multivalent bioconjugation, and abundant space to accommodate bioactives such as drugs and live cells. This makes them particularly appealing for various biomedical applications such as tissue engineering, biomedical implants, bio-nanotechnology, and drug delivery [10,144]. In recent years, click chemistry has emerged as a unique and versatile approach to fabricate nanogels and microgels with controlled sizes and functionalities.

5.1. Click nanogels for controlled drug delivery

Haag et al. designed and developed biodegradable dendritic polyglycerol (dPG) nanogels based on azide- and alkyne-substituted dPGs with acid-labile acetal linkers by inverse nano-precipitation and *in situ* CuAAC crosslinking for the encapsulation and release of proteins [145]. This mild synthetic approach allowed for the encapsulation of asparaginase with no adverse effect on protein structural integrity and activity. *In vitro* release studies showed that asparaginase release was significantly inhibited at pH 7.4 in 35 h, while almost 100% of the protein was released in 5 h at pH 4 or in 35 h at pH 5. Taking advantage of orthogonal chemoselective reactions of aldehyde and thiol groups, Ossipov et al. prepared multifunctional HA nanogels (~200 nm) in one pot from aldehyde/thiol dual-functionalized HA, acid-labile PVA-carbazate-DOX prodrug, and pyrene-pyridyl disulfide [146]. *In vitro* drug release studies showed that 50% and 19% DOX was released from the nanogels in 100 h at pH 5.0 and pH 7.2, respectively. Fluorescence microscopy studies revealed that DOX-loaded HA nanogels were more efficiently uptaken by CD44 receptor-overexpressing MDA-MB-31 cells than MCF-7 cells (low expression of CD44), indicating HA-presenting nanogels could selectively recognize CD44 positive cells. Anderson et al. developed dextran nanogels (~70 nm) with controlled functionalization and targeting using an inverse emulsion method via an astoichiometric CuAAC reaction (dextran-alkyne:dextran-azide = 3:1 or 1:3) (Fig. 5A) [17].

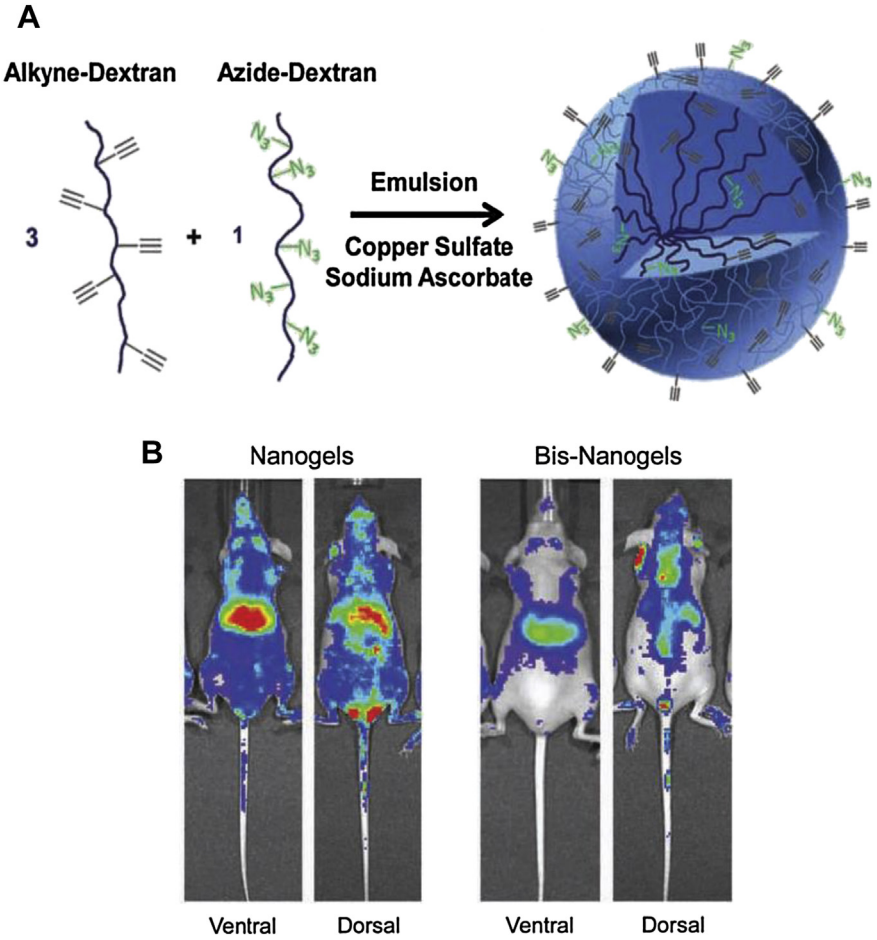


Fig. 5. Preparation and biodistribution of alkyne-rich nanogels. (A) Synthesis scheme for alkyne-rich nanogels: alkyne- and azide–functionalized dextrans react via CuAAC within an inverse emulsion; (B) *in vivo* fluorescence of alkyne-rich nanogels and biphosphonate-functionalized nanogels (bis-Nanogels) at 24 h in SKH-1 hairless mice [17].

Functionalization of the nanogels with a bisphosphonate ligand reduced kidney and liver uptake by 43%, and achieved significant nanogel binding to the inner walls of the marrow cavity in both the femur and spine (Fig. 5B). The targeted nanogels depleted F4/80-positive cells within bone marrow, suggesting that these functional nanogels might contribute to a depletion of osteoclasts and provide an anti-osteoporotic effect.

Fu et al. prepared well-defined fluorescent PEG nanogels with a size of 30–120 nm from alkyne-functionalized Ga-porphyrin complex and azide-functionalized PEG by combining the CuAAC click reaction and reverse-emulsion methods [147]. The resulting nanogels exhibited an emission maximum at a wavelength of 700–800 nm, indicating that they might have potential application in near infrared imaging. Elbert et al. reported that clickable nanogels with a diameter of 100 nm, synthesized from azide and alkyne functionalized PEG monomers using CuAAC reaction, were conjugated on mercaptosilanated glass via a UV thiol-yne reaction, yielding a single monolayer of nanogel coatings [148]. These nanogel coatings exhibited significantly less fibrinogen absorption than PEG coatings.

Matyjaszewski et al. developed nanostructured HA hydrogels through a thiol-Michael reaction of thiolated HA with acrylate-functionalized nanogels that were prepared via combination of atom transfer radical polymerization (ATRP) and inverse mini-emulsion followed by converting pendent hydroxyl groups into acrylate groups [149]. These nanogels showed controlled release of biomolecules like rhodamine B and dextran. Gama et al. reported dextrin nanogels imbedded in dextrin hydrogels for protein delivery [150]. The dextrin nanogels self-assembled from alkyl chain modified dextrin could be incorporated in click dextrin hydrogels based on dextrin-aldehyde derivatives (Dex-CHO) and adipic acid dihydrazide. This nanogel-hydrogel system could efficiently load interleukin-10 and insulin.

5.2. Click-based microgels for controlled drug delivery and 3D cell culture

Paradossi et al. reported that core–shell PVA-HA microgels with a diameter of 2 μm were prepared from azide- and alkyne-substituted PVA by combining the inverse emulsion technique with a CuAAC reaction at an azide/alkyne molar ratio of 1:2, followed by reacting the unreacted alkyne groups at the surface with azide-substituted HA [151]. The loading and release of DOX was controlled by HA shells. The microgels exhibited a remarkable targetability to CD44 receptor-overexpressing adenocarcinoma colon HT-29 cells and DOX was effectively released locally. Anseth et al. developed photodegradable PEG microgels with a mean diameter of 22 μm from PEG di-photodegradable-acrylate (PEG-diPDA) and PEG tetrathiol (PEG-4-SH) through thiol-Michael reaction using an inverse suspension polymerization technique [152]. Multiple proteins were loaded into batches of microgels and focused cytocompatible irradiation was used to degrade individual particles selectively to release specific proteins of interest. The authors showed that transforming growth factor beta β1 (TGF-β1) loaded into the microgels was released with visible light to a reporter cell line (PEG25), demonstrating that the released protein maintains its bioactivity. Sivakumaran et al. developed a hybrid hydrogel consisting of thermoresponsive microgels physically entrapped or covalently cross-linked to dextran hydrogels via a hydrazide-aldehyde reaction to facilitate long-term release of a small molecule drug [153]. The overall rate of bupivacaine release and the magnitude of the burst release were significantly decreased when microgels had higher internal crosslinking densities and/or were covalently cross-linked with the bulk hydrogel. Seiffert et al. reported that monodisperse cell-laden microgels with a diameter of 250–350 μm could be fabricated from dithiolated PEG and acrylated hyperbranched polyglycerol (hPG) by combining droplet-based microfluidics with the thiol-Michael reaction [154]. The size, elasticity, and swelling of the microgels could be precisely controlled by the molecular weight of the PEG crosslinker and the polymer concentration. Yeast cell experiments revealed that microgels prepared from higher molecular weight PEG induced higher cell viability due to increased diffusion of nutrients and metabolites. The viability of fibroblasts in the microgels could increase up to 89% with increasing cell density, probably due to intensified paracrine signaling.

Very recently, Haag et al. designed and developed pH-sensitive microgels (170 μm) from PEG-dicyclooctyne and dendritic poly(-glycerol azide) (dPG-azide) by combining the SPAAC click reaction and droplet-based microfluidics (Fig. 6) [155]. dPG-azide was prepared with different acid-labile benzacetal linkers that allowed for precise control of the microgel degradation kinetics in a pH range between 4.5 and 7.4. NIH 3T3 cells could be readily encapsulated into these bioorthogonal microgels and more importantly cells could be released on demand by lowering the pH. The released cells exhibited a high viability (over 94%), good proliferation and spreading. Lutolf et al. reported that reactive PEG microgels with precisely controlled dimensions (90–700 μm) and physicochemical properties were obtained through a thiol-Michael reaction of VS-terminated 8-arm-PEG (PEG-8-VS) with an astoichiometric PEG-4-SH using droplet microfluidic technology [156]. Microgels with VS or SH groups could further be tethered with bioactive molecules (e.g. RGD, gelatin, avidin, and IgG), providing a versatile platform for the culture and manipulation of various types of (stem) cells.

6. Biomimetic micropatterned hydrogels prepared via click chemistry

In order to mimic the cellular microenvironment and ultimately control cell fate and guide tissue regeneration, the spatiotemporal incorporation of biological cues such as peptides, growth factors, and proteins into hydrogel scaffolds may be needed. The click chemistry has recently appeared as a most interesting and practical approach to create 3D biomimetic micropatterned hydrogels. Bowman et al. reported that 3D patterned PEG hydrogels were prepared from PEG-4-SH and PEG-dialkyne via thiol-yne reaction to form an alkyne-rich hydrogels followed by conjugating an azide-labeled fluorophore to the hydrogels through a photoinducible CuAAC reaction using standard photolithographic techniques [37]. The results showed that 25 μm wide patterned fluorescent features within spatially defined regions of the gel could be readily formed with only 50 s of irradiation (400–500 nm, 10 mW/cm^2). Muir et al. demonstrated that surface patterning of PEG hydrogels could be accomplished via combined photoinducible CuAAC and radical crosslinking reactions of alkyne-functionalized PEG on an azide-functionalized plasma polymer surface [157]. The thickness of PEG hydrogel patterns ranged from tens to several hundreds of nanometers depending on UV exposure time and PEG molecular weights. These PEG hydrogel patterns resulted in highly functional surfaces that spatially controlled cell attachment.

Shoichet et al. reported that exposure of *S*-2-nitrobenzyl-cysteine (S-NBC)-functionalized photolabile agarose hydrogels to a focused UV laser beam generated thiol-containing cylindrically patterned channels (approximately 150 μm in diameter and 2–3 mm in length), which could modified with maleimide-containing biomolecules like RGD peptide via thiol-maleimide click reactions for guided 3D neurite extension and cell migration within hydrogel matrices [158]. Similarly, platelet derived growth factor-AA (PDGF-AA) could be immobilized on thiol-containing channels in agarose hydrogels to support the adhesion and preferential differentiation

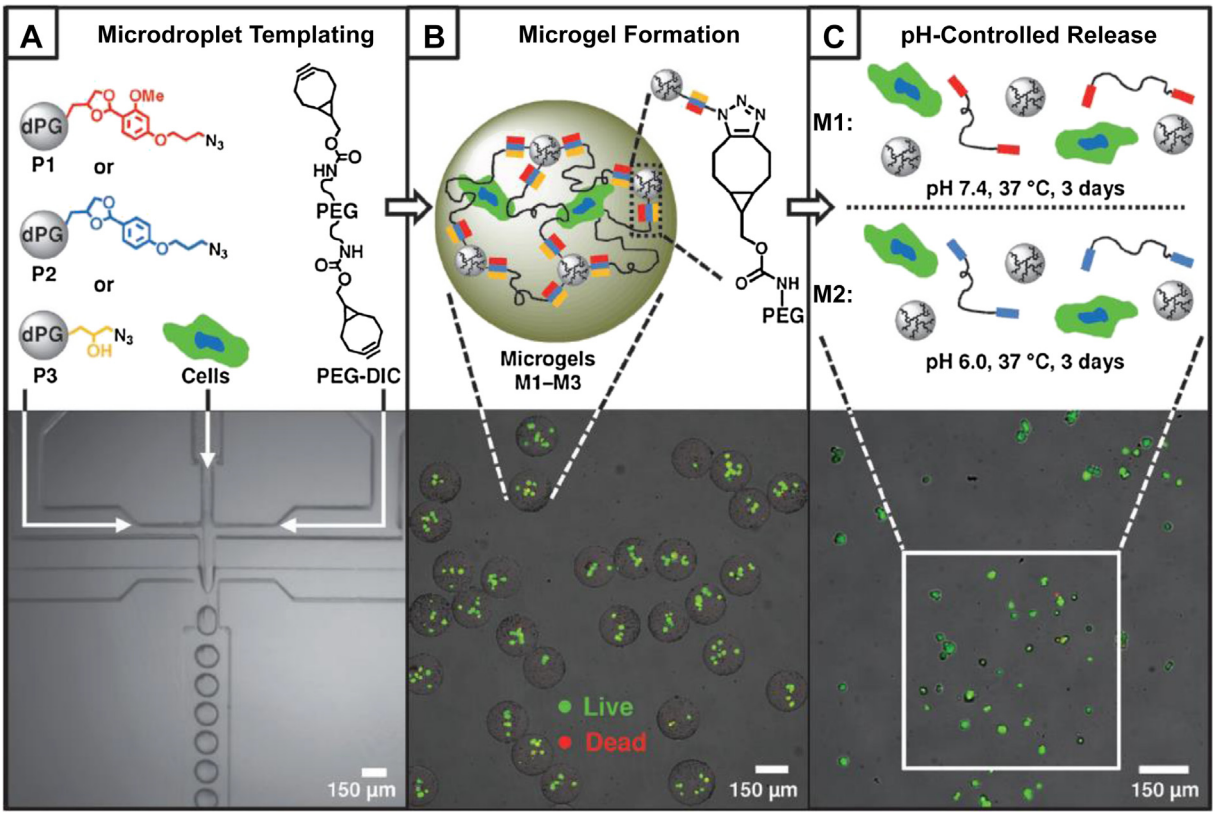


Fig. 6. Encapsulation and release of live cells from click microgels. (A) A set of dPG-azide precursors P1–P3 with acid-cleavable linkers that had different hydrolysis kinetics and PEG-dicyclooctyne were prepared. One of the dPG macromonomers, the PEG-dicyclooctyne crosslinker, and NIH 3T3 cells were injected into a microfluidic device followed by droplet gelation via SPAAC; (B) cell-laden microgels M1-M3 were formed, the viability of cells encapsulated into microgels was at least 94%; and (C) incubation of the microgels M1-M3 at 37 °C and 5% CO_2 for three days. Microgel M1 showed complete degradation at pH 7.4 and the released cells had unchanged residual viability of 96%, microgel M2 showed complete degradation at pH 6.0 and the released cells had unchanged residual viability of 94%, while microgel M3 showed no degradation between pH 6.0–7.4 [155].

of neural stem/progenitor cells (NSPCs) to oligodendrocytes [159]. Burdick et al. developed a patterned hydrogel network structure through the thiol-Michael reaction of 50% of the total available acrylate groups in HA with cell adhesive cysteine-containing peptide followed by UV light-induced radical polymerization of the remaining acrylate groups using photomasks, in order to spatially control cellular remodeling and stem cell fate within the 3D hydrogels [160,161]. With use of this patterning, the outgrowth from chick aortic arches and the spreading of encapsulated MSCs were only observed in the regions without UV irradiation. Moreover, network structures were shown to dictate adipogenic/osteogenic MSC fate decisions by controlling cell spreading.

Two orthogonal click reactions can also be employed in a sequential fashion to fabricate patterned hydrogels, in which one reaction is used to form hydrogels while the other reaction is used to introduce specific biochemical functionalities. Anseth et al. reported that combining CuAAC chemistry for gelation and a thiol-ene reaction for complex patterning allowed independent tuning of the physical and biochemical properties of materials [162]. A standard photolithographic technique was employed to generate two- and three-dimensional patterns as well as controlled biochemical gradients within the preformed hydrogels using fluorescently labeled RGDSC peptide. In a follow-up study, copper-free SPAAC and thiol-ene click reactions were utilized sequentially to synthesize and pattern a cell-laden PEG hydrogel based on PEG-4-azide and difluorinated cyclooctyne (DIFO)/alkene-functionalized peptide [18]. The copper-free SPAAC reaction allowed for the direct encapsulation of cells within PEG hydrogels, while the thiol-ene photocoupling chemistry enabled patterning of biological functionalities within the gel in real time and with micrometer-scale resolution, providing an avenue to observe cell processes including migration, proliferation and morphological changes in a 3D environment. The degree of patterning could be controlled by the dosage of light (intensity and exposure time) imposed on the system, and the initiator concentration [49]. Notably, cytocompatible hydrogels with dynamically tunable properties were developed from PEG-4-cyclooctyne and bis(azide)/alkene-functionalized photodegradable peptide via SPAAC reaction [73]. The biomechanical and biochemical properties of hydrogels were regulated independently by photocleavage of crosslinks under UV irradiation (365 nm) and photoconjugation of pendant functionalities using visible light (490–650 nm), respectively, which allowed manipulation of fibroblast outgrowth and spreading within the RGD photopatterned region. The photoreversible patterning of biomolecules within the cytocompatible SPAAC PEG hydrogels was accomplished by incorporating thiol-functionalized photodegradable peptide via thiol-ene reaction [50]. The results showed that seeded NIH 3T3 cells only adhered to and spread on the peptide-functionalized regions. Removal of the adhesive ligand 24 h after cell seeding through masked UV light exposure resulted in detachment of the cells from the user-defined regions within minutes of exposure.

Multiphoton excitation is an important depth-sectioning tool in microscopy and relies on the large power density present at the focal point of a pulsed laser to excite chromophores using two or more low-energy photons. The 3D control of excitation, deep penetration and reduced phototoxicity has rendered multiphoton excitation a powerful technique to create patterns with submicrometer feature sizes in hydrogels. Shoichet et al. developed intricate cell-sized volumes of free thiol below the surface of agarose hydrogels with a 6-bromo-7-hydroxycoumarin sulfide

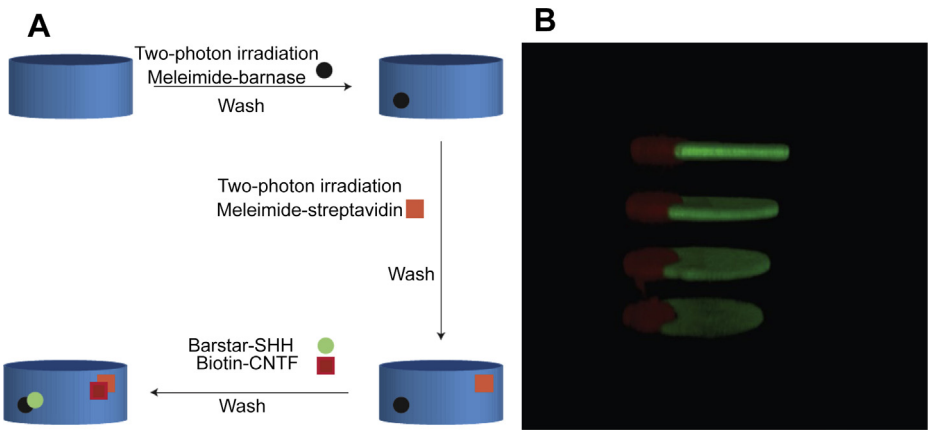


Fig. 7. Spatially controlled simultaneous patterning of multiple growth factors in three-dimensional hydrogels. (A) Schematic illustration for the simultaneous immobilization of barstar-SHH and biotin-CNTF; and (B) representative figure for the simultaneous 3D patterning of biotin-CNTF-633 and barstar-SHH-488. Maleimide-barnase was patterned in layers in the shape of a truncated (green) circle 400, 500, 600 and 700 μm below the surface of the hydrogel. Maleimide-streptavidin was then patterned in a smaller (red) oval shape inserted into the truncated circle of the maleimide-barnase pattern [165]. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

derivative (hundreds of micrometers depth) using a multiphoton confocal microscope [163]. The free thiol allowed for site-specific covalent immobilization of bioactive molecules in hydrogels without causing hydrogel cross-linking or changes in its physical properties. Endothelial cells on agarose hydrogels with RGD peptide and an immobilized gradient of vascular endothelial growth factor (VEGF165) showed stereotypical tip and stalk cell morphology and tubule-like structures, indicating that patterned hydrogels have a great potential in vascular tissue engineering [164]. With this technique, spatially controlled simultaneous patterning of multiple stem-cell differentiation growth factors, sonic hedgehog (SHH) and ciliary neurotrophic factor (CNTF), was accomplished in 3D hydrogels (Fig. 7A) [165]. Coumarin-caged thiols in agarose hydrogels could be selectively deprotected and subsequently employed for the sequential immobilization of maleimide functionalized barnase and streptavidin via a thiol-Michael reaction. Barstar-SHH and biotin-CNTF were then simultaneously immobilized in the hydrogel via their physical binding interactions with barnase and streptavidin, respectively (Fig. 7B). The agarose hydrogels with a SHH gradient of 100–500 ng/mL^{-1} over the first 100 μm were shown to guide the migration of neural precursor cells (NPCs) to a depth of 85 μm, which is a depth appropriate for thin tissues such as retina.

7. Conclusions and perspectives

In the past several years, click reactions have emerged as an innovative and versatile strategy to construct novel functional hydrogels, microgels and nanogels for biomedical applications. The high coupling efficiency and specificity, bioorthogonality, and mild reaction conditions of click chemistry lend click hydrogels, microgels and nanogels unique and highly compatible with bioactives such as live cells, proteins, peptides, and therapeutics. The click hydrogels, and in particular the *in situ* forming hydrogels, have demonstrated a great potential as functional extracellular matrices for tissue engineering and as injectable systems for long-term sustained drug and protein release. The click microgels have been shown to provide a biomimetic 3D environment for facile encapsulation and growth of live cells as well as a platform for local protein release. The click nanogels are ideally suited for targeted intracellular delivery of drugs, especially therapeutic proteins. In addition, click chemistry has allowed for the design and development of increasingly instructive micropatterned and biomimetic hydrogel scaffolds for dictating stem cell differentiation and/or guiding cell attachment and growth. Click hydrogels, microgels and nanogels are emerging as indispensable platforms for controlled drug delivery, tissue engineering and regenerative medicine.

It has to be realized that the development of click hydrogels, microgels and nanogels is a newly burgeoning field. The wide variety of work reported has demonstrated the beauty of click chemistry in creating novel hydrogel materials with dimensions spanning from human organs, cells to viruses. Studies on the biomedical applications of these materials to date are, however, mostly in proof-of-concept. There is limited information on the *in vivo* biocompatibility and fate of hydrogels, microgels and nanogels prepared by different click reactions. In the future, injectable, bioactive and degradable click hydrogels and microgels should be developed based on well-established non-cytotoxic materials and investigated for the engineering of functional soft tissues such as cartilage and myocardium *in vivo*. The release and therapeutic efficacy of protein drugs from injectable hydrogels and microgels requires systemic *in vitro* and *in vivo* studies. For targeted drug delivery, click nanogels should be designed and prepared with a high drug loading level, a prolonged circulation time, a specific ligand for recognition by target cells, and stimuli-sensitive degradation properties to trigger and control drug release *in vivo*. In particular, click nanogels with little adverse effect on fragile biomacromolecules are appealing for intracellular delivery of therapeutic proteins like apoptotic proteins for cancer therapy. We are convinced that with rational materials design, click hydrogels, microgels and nanogels may in one day be translated effectively to the clinic.

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