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REVIEW ARTICLE

Click Chemistry-Based Injectable Hydrogels and Bioprinting Inks for Tissue Engineering Applications

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Abstract

BACKGROUND: The tissue engineering and regenerative medicine approach require biomaterials which are biocompatible, easily reproducible in less time, biodegradable and should be able to generate complex three-dimensional (3D) structures to mimic the native tissue structures. Click chemistry offers the much-needed multifunctional hydrogel materials which are interesting biomaterials for the tissue engineering and bioprinting inks applications owing to their excellent ability to form hydrogels with printability instantly and to retain the live cells in their 3D network without losing the mechanical integrity even under swollen state.

METHODS: In this review, we present the recent developments of *in situ* hydrogel in the field of click chemistry reported for the tissue engineering and 3D bioinks applications, by mainly covering the diverse types of click chemistry methods such as Diels–Alder reaction, strain-promoted azide-alkyne cycloaddition reactions, thiol-ene reactions, oxime reactions and other interrelated reactions, excluding enzyme-based reactions.

RESULTS: The click chemistry-based hydrogels are formed spontaneously on mixing of reactive compounds and can encapsulate live cells with high viability for a long time. The recent works reported by combining the advantages of click chemistry and 3D bioprinting technology have shown to produce 3D tissue constructs with high resolution using biocompatible hydrogels as bioinks and *in situ* injectable forms.

CONCLUSION: Interestingly, the emergence of click chemistry reactions in bioink synthesis for 3D bioprinting have shown the massive potential of these reaction methods in creating 3D tissue constructs. However, the limitations and challenges involved in the click chemistry reactions should be analyzed and bettered to be applied to tissue engineering and 3D bioinks. The future scope of these materials is promising, including their applications in *in situ* 3D bioprinting for tissue or organ regeneration.

Keywords Click chemistry · Hydrogels · 3D bioprinting · Tissue engineering · Regenerative medicine

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1 Introduction

"Click chemistry" is generally defined as the chemical reactions which occurs fast, spontaneous, versatile, extremely selective and which can give high yields of products when two molecular substances or components are mixed or reacted at mild reaction conditions [1, 2]. Among the hydrogels researched, the Cu(I)-catalyzed reactions for hydrogel synthesis were popular and commonly called as Click Chemistry. This specific term was first coined by

Sharpless' group in 2001 [3]. The terms such as bio-orthogonal or bio-click were used to describe the reactions which takes place in presence of macromolecules like proteins or live cells, etc. The main advantage of bio-orthogonal methods is they usually do not affect the other normal biochemical processes [4, 5]. Notable first click chemistry-based hydrogel was reported by Ossipov et al. [6] which was formed using poly(vinyl alcohol) (PVA) polymer. These kinds of hydrogel preparation methods have improved in the recent decade and provide the scientists a valuable tool to modify or synthesize novel polymers with multifunctional properties for numerous applications such as tissue engineering, drug delivery, regenerative medicine and other biomedical applications [7–10]. As explained by Sharpless' research group, click chemistry reactions needs to be modular, should have wide scope, very high yields, stable at physiological conditions, highly stereo-specific, non-toxic end products, under the simple reaction conditions being started with easily available materials and reagents. Furthermore, the reaction should provide product directly without using chromatographic techniques and toxic solvents [11].

Even though copper-based click chemistry was reported first for hydrogel synthesis using click chemistry, due to its copper ion toxicity and reactive oxygen species generation, this method was recently less preferred in tissue engineering and regenerative medicine [12]. Although scientists use ethylene diamine tetra-acetic acid (EDTA) for removing copper ions from the hydrogels, it may be difficult to use the same method in the cases of injectable hydrogels for regenerative medicine or localized drug delivery [13]. Hence, scientists started preferring copper-free click chemistry for synthesizing hydrogels for tissue engineering and regenerative medicine [14]. The copper-free click chemistry offers various functionalities without using any toxic catalysts or yield any toxic end products after gel formation [1]. The various copper-free click chemistry methods include strain-promoted azide-alkyne cycloaddition (SP-AAC) click hydrogels [14–16], Diels–Alder click chemistry hydrogels [17, 18], thiol-ene [19, 20], oxime [21–23], thiol-yne [24, 25], etc. The other class of click chemistry hydrogel synthesis method includes the pseudo click reactions which also gives high yield of products at moderate and extremely reactive reactions conditions [4]. These click chemistry-based hydrogel materials show immense potentials to be used in the latest technologies like 3D bioprinting for creating tissue and organ structures [26–29]. Even though scaffold-free approaches are investigated for tissue regeneration [30], injectable *in situ* hydrogels either with or without live cells, growth factors, biomolecules, nanoparticles and microspheres are promising for tissue engineering applications with improved mechanical properties and biocompatibility [31–40].

This review article focuses on the tissue engineering and potentially 3D bioprinting applications of click chemistry based on the main topics of copper-catalyzed click hydrogels, copper-free click hydrogels and pseudo-click hydrogels as described in Fig. 1. While copper-catalyzed click chemistry describes on alkyne-azide cycolo-addition reaction, copper-free click chemistry does strain-promoted azide-alkyne cycloaddition (SP-AAC), Diels–Alder, thiol-ene and oxime. Pseudo-click chemistry introduces thiol-Michael and aldehyde-hydrazide reactions. The chemical reaction strategies used in the copper-catalyzed reactions and copper-free click chemistry reactions are summarized in the Fig. 2 and Table 1.

2 Copper-catalyzed azide-alkyne cycloaddition (Cu-AAC) click chemistry-based hydrogels for tissue engineering

Cu-AAC is one of the commonly used metal-catalyzed methods for synthesizing cross-linked biohydrogels for live cell encapsulation and tissue engineering. The main advantages of the method are quick gelation and high yields of products [1, 2, 4, 41]. However, the limitations of Cu-AAC reactions are non-homogeneous viscosity of the hydrogels with defective regions, improper diffusion of precursors inside the hydrogel and oxidation of copper catalyst leading to lower efficiency [1, 2, 4, 42, 43]. *In situ* reduction of copper ions were tried using various methods; however, all methods failed to stop the accumulation of excess copper ions inside the hydrogels. Even photochemical method to reduce the copper ions was tried, but this also became less effective as the light exposure was not uniform throughout the hydrogel with less penetration [44, 45].

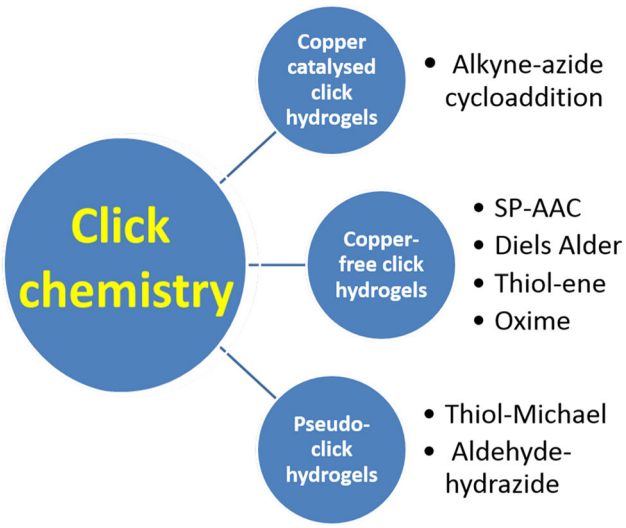

Fig. 1 Example methods of click chemistry-based hydrogels discussed in this article

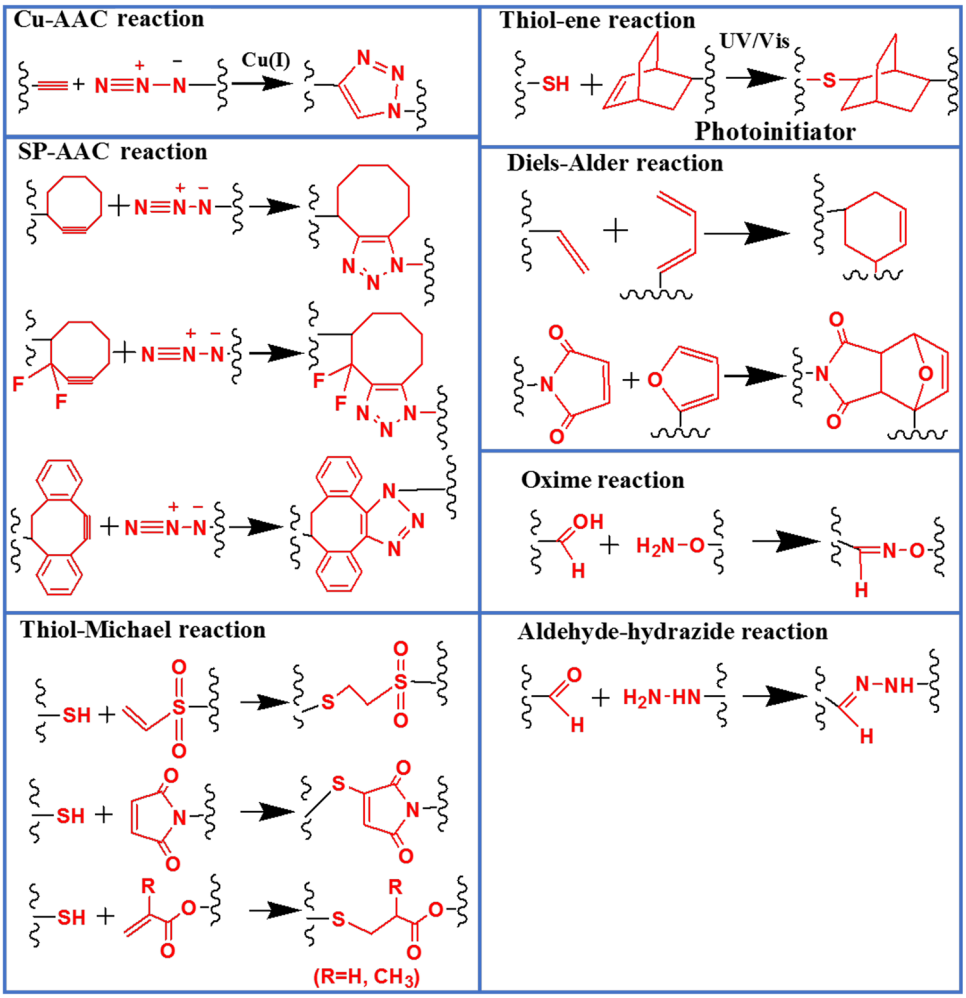

Fig. 2 General click chemistry-related chemical reactions for formation of hydrogels focused in this article. Adopted and reprinted from [4] Copyright (2018), with permission from Elsevier

One of the main problems is the release of copper ions from the catalyst after reaction, which is cytotoxic and curbs the use of this method directly in tissue engineering or regenerative medicine applications [2, 4, 46]. To overcome this challenge, many researchers started using chelating agents or ligands which are water-soluble in nature. Those chelating agents or ligands include bipyridine [47], bis(L-histidine) [48], bis[(*tert*-butyl triazoyl) methyl]-[(2-carboxy methyl triazoyl)-methyl]-amine [49], tris-(hydroxyl propyl triazolyl methyl) amine [50], etc. Even though these ligands or agents involve in protecting the encapsulated cells and biomolecules from oxidative stress by acting as sacrificial reducing agent, their research results reported that the reduced water solubility after chelation of copper ions also induced toxicity [47]. Recently, Guo et al. used 4-(2-hydroxy ethyl)-1-piperazine ethane sulfonic acid as a chelating agent for copper ions in Cu-AAC reactions to synthesize mussel-inspired citrate-based bio-adhesive hydrogels. Along with the high bio-adhesive properties (223.11 ± 15.94 kPa), these hydrogels possess excellent antimicrobial activity which can be positively considered for invasive applications. As the concentration of periodate was increased, the gelation time decreased slightly, and this may be attributed to the catechol group crosslinking than the click crosslinking [46]. In another interesting work, Lou et al. reported a hydrogel system consisting of interpenetrating hydrogel network resembling extracellular matrices. The high viscoelastic nature of hydrogels shows the immense potential of these hydrogels in stress relaxation, cell distribution, fiber redesigning and formation of focal adhesion points, which are not usually present in other 3D hydrogel systems. Gelation was faster in HA-aldehyde samples and they reached a stable modulus value within 15 min. Amplified modulus was achieved as the concentration of hyaluronic acid (HA) was increased [51]. Even though Cu-AAC-based click chemistry reactions yields high quantity of products with short gelation time, the toxicity associated with copper catalyst poses a big threat for using this method to prepare hydrogels for tissue engineering and regenerative medicine. However, efficient modification of catalysts, chelating agents, and further development in finding

Table 1 Representative click chemistry reaction methods, reactants, cell culture details, and their advantages and disadvantages. (In table format)

S.no	Hydrogel preparation methods	Reactants	Cells/*in vivo*/*in vitro*	Gelation time and degradation	Advantages	Disadvantages	References
1	Copper-catalyzed click chemistry reactions	HA-hydrazine, collagen, and HA-aldehyde or HA-benzaldehyde, Copper (II) sulfate pentahydrate	hMSCs	Gelation in 5 min	Enhanced focal adhesion, cell spreading, fiber remodeling	Copper ions toxicity, reactive oxygen species generation, regional variation in viscosity due to rapid gelation, non-homogeneous gels	[51]
2	Diels–Alder click reactions	Furan-linked gelatin against maleimide-linked PEG, chitosan-Pluronic F127)	Cardiomyocyte cells, *in vitro* and *in vivo*	Gelation in 2 h, slower degradation rate *in vitro*	Injectable gels, fully-interpenetrating network, thermosensitive, cell adhesive	Slow gelation, reduced solubility of the functional groups, inability to be injected *in vivo* in few cases	[59]
		N-maleoyl alanine terminated F127, furan grafted chondroitin sulfate, oxidized chondroitin sulfate, bone morphogenetic protein-4	Rat mesenchymal stem cells, bone cells (repair), *in vitro* and *in vivo*	Gelation in 3 days, 14 days degradation *in vitro*	Easily modifiable, improved viscoelastic properties and rheological properties. Swellable, injectable, self-healing ability		[61]
		Sodium alginate, bio glass and modified chondroitin sulphate	Cranial bone defect repair, *in vitro* and *in vivo*	Gelation in 3 days, 4 weeks at neutral pH, faster degradation at basic pH *in vitro*	Triple cross-linked injectable hydrogel, better physio-chemical properties		[60]
		Furfurylamine linked chondroitin sulfate, F127 linked maleimido and PEG-AMI, bone morphogenetic protein-4	Bone cells (repair)	Gelation in Less than one min	Non-covalent and covalent crosslinking, good biocompatibility		[17]
		Furan linked HA, dexamethasone and maleimide linked HA	Human adipose-derived stem cells, *in vitro*	Gelation in 60 min, more than 21 days for degradation *in vitro*	Thermo-responsible hydrogels, dexamethasone-controlled release in local environment, non-cytotoxic, can deliver adipogenic factors		[57]
		HA with furan adipic dihydrazide, HA with furan CHO and followed by addition of dimaleimide PEG	Chondrocyte cells (cartilage), *in vitro*	Gelation in 5 min	Mechanical properties, tissue adhesive, self-healing, pH responsiveness		[53]

Table 1 continued

S.no	Hydrogel preparation methods	Reactants	Cells/*in vivo*/*in vitro*	Gelation time and degradation	Advantages	Disadvantages	References
3	Strain-promoted azide-alkyne cycloaddition (SP-AAC) reactions	Azadibenzocyclooctyne-modified dextran and azide-modified dextran	Chondrocyte cells(cartilage), *in vitro*	Gelation in 1.1 to 10.2 min, slow degradation rate up to 21 days *in vitro*	Gelation time modifiable using concentration variation and substitution degree of dextran, encapsulation of cells and cell spheroids	Reaction rate is lower than copper catalyzed reactions, alkynes are larger, trigger high perturbation, less ligation rate, high side reactions	[72, 114]
		Dibenzocyclooctyl (DBCO)-modified HA, 4-arm PEG azide	Chondrocyte cells (cartilage), *in vitro* and *in vivo*	Gelation in 10–14 min, slow degradation up to 35 days *in vivo*	Nontoxic cross linker, good biocompatibility, *in situ* physical gelation, elastic modulus can be modified by varying concentration		[73]
		Hyperbranched poly(ε-caprolactone) (hyPCL)32-(1R,8S,9 s)-bicyclo[6.1.0]non-4-yn-9-ylmethanol (hyPCL32-BCN) and hyPCL32-azide (hyPCL32-N3)	Bone (MC3T3 preosteoblast) cells	Gelation in 30 min	Injectable hyperbranched PCL, biocompatible, excellent support for cell adhesion and proliferation		[71]
		4-dibenzocyclooctynol functionalized PEG, 4 arm PEG tetraazide, protein igands (laminin), neurogenic differentiation factor (interferon-γ)	Neural stem cell, *In vitro*	Gelation in less than 5 min	Additional differentiation factors not required in medium, high cell viability, no UV light required		[16]
4	Thiol-ene-based click chemistry reactions	PEGDA, dithiothreitol, borox	Endothelial cells and neural stem cells, *in vitro*	Gelation within few min of stirring	Injectable, 3D printable, sacrificial removal enables tubular structure formation	Polymer substrates lack alkenes with electron deficiency, acid condition required for the reaction	[77]
		4-arm PEG norbornene, PEG dithiol, Protein array printing of collagen I, collagen III, collagen IV, fibronectin, laminin, elastin and hyaluronan	Smooth muscle cells, *in vitro*		Cellular stiffness and increased functional contractility, cell attachment and 3D infiltration	UV source needed in radical mediated reactions which may damage cells, oxygen sensitivity may result in disulfide formation; the presence of cysteine and amine group residues may compete with thiol group while hydrogel formation occurs.	[82]
		Alkene-linked (allyl and norbornene residues), poly(oligoethylene glycol methacrylate)), cell adhesive peptides RGD and REDV	Human umbilical vein endothelial cells (HUVECs), *in vitro*		Photo-patterned peptides at the surface, development of cell array is achieved		[80]

Table 1 continued

S.no	Hydrogel preparation methods	Reactants	Cells/*in vivo*/*in vitro*	Gelation time and degradation	Advantages	Disadvantages	References
		Norbornene-linked pectin macromer, monocysteine RGD peptide, biscysteine peptide with matrix metalloproteinase cleavage site	Human neonatal dermal fibroblasts, *in vitro*	Degradation9 h in enzymatic treatment *in vitro*	Cell surface receptors in pectin may increase cell attachment and integration, simple, fast, robust, independent modification of the biochemical or biophysical cues in the system is possible		[79]
		8-arm-PEG thiol macromer, thioester di(vinyl ether), caged thioester catalyst, norbornene RGD	Primary hMSCs, *in vitro*		Dynamic, modifiable visco-elastic properties, using pH, stoichiometry and crosslinker structure the hydrogel network can be modified, thiol-ester resembles biological reactions		[78]
5	Oxime based click chemistry reactions	Aminooxy-terminated PEGs, aldehyde modified HA and collagen I	Schwann cells, HMSCs, *in vitro*		Less toxic to the cells, tunable properties, peptide, protein bonding is easier	Not bio-orthogonal	[22]

alternates for copper catalyst may yield better Cu-AAC based hydrogels with less toxicity for tissue engineering applications [2, 4, 9].

3 Copper-free click hydrogels

3.1 Diels–Alder (DA) click chemistry hydrogels for tissue engineering

Diels–Alder-based click chemistry reactions occur mainly between the diene and alkene (or dienophile) without the help of a catalyst or coupling agent. These reactions are highly accelerated in presence of water because of the hydrophobic effects [4]. DA reactions occurs in aqueous medium with high reaction rate, versatility, selectivity and efficiency which are characteristics for click chemistry reactions [52, 53]. They also do not yield any toxic end products. Hence, this robust method is highly used for developing cross-linked hydrogels for tissue engineering applications [2, 4]. Koehler et al. synthesized the DA-based hydrogels for drug release and osteogenic differentiation. DA reaction occurred between the maleimide poly(ethylene glycol) (PEG) macromer and furan dexamethasone peptide to form the hydrogels. The dexamethasone release study and osteogenic differentiation of human mesenchymal stem cells (hMSCs) were demonstrated in both 2D and 3D culture environments. The tested hydrogels also showed high alkaline phosphatase activity (6 times more) and increased mineralization [54]. Nimmo et al. reported HA-based furan-modified hydrogels crosslinked using DA reaction via dimaleimide-linked PEG. They suggested that the mechanical and other properties like degradation can be tuned by varying the proportion of furan and maleimide appropriately. Human epithelial cells were used to test the cyto-compatibility and degradation of the hydrogels [55]. Similarly, the Shoichet's group demonstrated the ability of the furan-modified HA and bis-maleimide PEG hydrogels formed by DA reaction to hold biomolecules (galactose) in spatially defined manner by photo-patterning through a processing of two-photon laser method. They also demonstrated the controlling of porosity and pore size distribution by cryo-gelation and thaw temperature, respectively. The mechanical property of the hydrogel was also controlled by modifying the furan substitution [56]. Yu et al. investigated a tissue-adhesive DA click chemistry-based hydrogels for cartilage tissue engineering. The double-crosslinked network hydrogel was formed between the HA with furan adipic dihydrazide, and HA with furan aldehyde and followed by addition of dimaleimide PEG. The hydrogels showed good mechanical, swelling and self-healing properties as well as cartilage-adhesive properties [53]. Similarly, Fan et al. reported furan-linked HA and maleimide-

linked HA as hydrogel components to form biodegradable DA-based click chemistry hydrogels for adipose tissue engineering. The human adipose-derived stem cells were incorporated into the hydrogels to test the cytocompatibility of the hydrogels. The hydrogels promoted stem cell proliferation, however, they did not promote differentiation of the stem cells [57]. Recently Bai et al. reported development of a dual crosslinked injectable self-reinforcing hydrogels for tissue engineering applications. The dual crosslinking was carried out with the help of non-covalent bonding (cyclodextrin, adamantane, poly(N-isopropyl acrylamide) (PNIPAM)) and DA-based click chemistry reaction (furfurylamine-linked chondroitin sulfate and maleimido-linked PEG). This dual crosslinking increased the mechanical strength of the hydrogels significantly and *in vivo* studies showed promising results for bone repair even without using any cells or growth factors in the hydrogel [58]. They also reported a dual-crosslinked injectable chondroitin sulphate hydrogel loaded with bone morphogenetic protein-4 via DA-based click chemistry for restoration of rat cranial defect. The components of the hydrogels were furfurylamine-linked chondroitin sulfate (ChS-Furan), F127-linked maleimido and PEG-AMI (Fig. 3), which offered both non-covalent and covalent crosslinking. The rat *in vivo* studies along with BMP-4 revealed the new bone formation in the injected area after 12 weeks of implantation [59].

Bai et al. introduced a triple cross-linked injectable hydrogel for repair of cranial bone defect. The hydrogel components were modified sodium alginate, bioglass and modified chondroitin sulphate. The triple cross-linking was achieved by cross-linking of non-covalent bond, acylhydrazone bond and DA-based click chemistry covalent. The hydrogel combination showed admirable physiochemical properties and bone regeneration abilities during *in vivo* studies [60]. Lu et al. reported a similar self-healing and injectable hydrogels for repair of *in vivo* cranial bone. [61]. Catechol-modified *N*-(furfural) chitosan (CFC) based dual cross-linked (co-ordination bond and DA click chemistry) hydrogels for tissue engineering application was reported. The co-ordination bonding between iron ions and catechol provide the ability to control the self-healing ability and degree of cross linking. The dual crosslinking also generated very good mechanical properties for the hydrogels [18]. Recently, Smith et al. introduced new click cross-linked hydrogels containing methyl-furan grafted hyaluronan which can form gels at physiological pH unlike hyaluronan-furan which can form gel only at low pH condition. This DA crosslinked hydrogels facilitate the encapsulation of live cells inside the hydrogels for tissue engineering and 3D cell culture applications. Furthermore, they observed hydrogen bonding interactions through computational analysis [62].

Another interesting hydrogel system reported by Abandansari et al. showed interpenetrating hydrogel (furan-linked gelatin against maleimide-linked PEG via DA click chemistry) with *in situ* gel forming ability and thermosensitive (chitosan-Pluronic F127) behavior along with high mechanical and biocompatibility properties for retention of encapsulated cardiac cells. The increased crosslinking degree, reduced gelation time and increased mechanical properties can be achieved by adding equivalent ratios of furan and maleimide. Hence, in this system by varying the PEG and gelatin ratio one can tune the

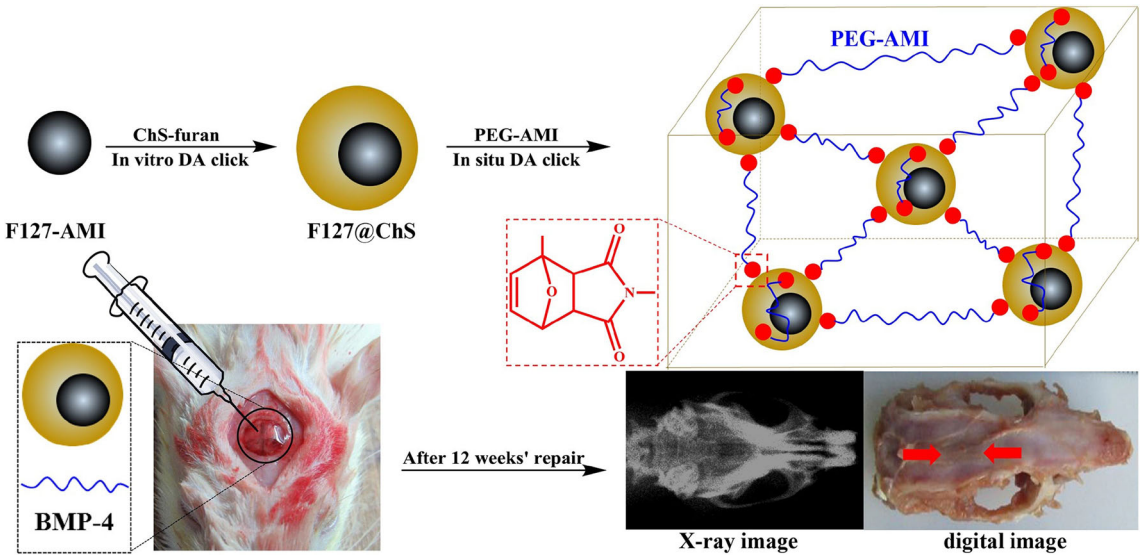

Fig. 3 Dual cross-linked injectable chondroitin sulphate-based hydrogel loaded with bone morphogenetic protein-4 via DA based click chemistry for restoration of rat cranial defect. Reprinted from [17] Copyright (2017), with permission from Elsevier

mechanical properties of the hydrogels [59]. As mentioned earlier, the main advantage of DA-based click chemistry is that they do not require any toxic initiators or coupling agents for the reaction. Drugs also can be incorporated for sustained release because of its exceptional site specificity and thermal reversible properties. However, there are some challenges to be addressed for using DA click chemistry hydrogels such as longer gelation time, reduced solubility of the functional groups and in few cases, less effectiveness for usage as injectable gels under physiological environment [2]. Yet, these DA-based click chemistry hydrogels show promising results towards regenerating cartilage, adipose tissue, cranial bone defects and it can be further applied to other tissue engineering applications.

3.2 Strain-promoted azide-alkyne cycloaddition (SP-AAC)-based click hydrogels for tissue engineering

To overcome the problems associated with copper-catalyzed click reactions, scientists started developing other methods which does not use copper as catalyst. This lead to the introduction of strain-promoted azide-alkyne cycloaddition click reactions. The method involves reaction of cyclooctyne molecules with azides by means of ring strain and electron withdrawal from fluorine substitutes [63]. "Strain-promoted" concept mainly came from the ring strain which accelerates the reaction between azide and cyclooctyne groups compared to other methods. This SP-AAC can decrease up to 18 kcal/mol of active energy during ring chain reaction because of bond angle distortion [14]. DeForest et al. introduced a robust synthetic hydrogel system consisting of macromolecular precursors (4 arm PEG modified with tetraazide and bis(di-fluorinated cyclooctyne moiety) di-functionalized polypeptide) as reactants for encapsulating cells directly without using copper. They also demonstrated high-resolution patterning of hydrogels with biological functionalities using photo-coupling of orthogonal thiol-ene. The advantages include tailoring the different properties of the hydrogels *in situ* for creating favorable environment for cells, direct functionalization and photo-patterning in presence of live cells [64]. In their subsequent work, they reported hydrogels containing patterned biomolecules with photo-reversibility. This hydrogel system was developed via SP-AAC reaction, which showed excellent spatiotemporal supremacy over the biomolecules in the 3D hydrogel system with the help of various bio-orthogonal photo-reactions [65]. Kloxin et al. demonstrated similar spatiotemporal control of biological molecules in the hydrogel systems using azide and cyclooctyne groups. This hydrogel with controlled micro-environment may help to understand the tissue morphogenesis and regeneration [66]. Another SP-AAC based hydrogel developed from azide-linked PEG-co-polymer and cyclooctyne-linked PEG was reported by Xu et al. This hydrogel system showed excellent cell viability than the photo-crosslinked PEG when bone marrow stromal cells were encapsulated in the hydrogel [67]. Zheng et al. investigated the ability of the hydrogels formed via SP-AAC reaction to encapsulate human mesenchymal stem cells with high cell viability. The hydrogel was prepared from 3 arm-glycerol exytholate triazide and dibenzocyclooctyne linked PEG. Gelation of the hydrogels with encapsulated cells were achieved by the cumulative effect which occurred because of the molecular interactions between the azide-terminated PEG and strained cyclocotyne units [68].

Takahashi et al. developed an *in situ* gel forming hyaluronan-based copper-free click chemistry hydrogels for tissue engineering and drug delivery applications. After modifying HA with azide group and cyclooctyne separately, SP-AAC crosslinked gels were obtained by mixing both solutions. *In vivo* studies revealed the biocompatibility and elimination of hydrogels without affecting the host. The gelation time of the hydrogel formation was less than 5 min. This gelation time was strongly dependent on the polymer concentration used for the preparation. However, when compared with other Schiff's base reactions, this reaction took more time for gelation. This was attributed to the low degree of HA modification compared to the reported reactions [69]. Jiang et al. reported a fast degrading, injectable hydrogel system made from alkyne-modified PEG and azide-modified PEG cross-linked via SP-AAC reaction. Their results showed excellent properties including *in situ* gel formation ability under *in vivo* conditions with mild immunogenic response [70]. Liu et al. revealed poly(ε-caprolactone)-based hyper-branched dendrimers which were cross-linked via SP-AAC reaction exhibiting high mechanical properties and biocompatibility. This hydrophobic dendrimer with multi-functional properties may be used in bone tissue repair and also in biomedical applications [71]. Similarly, Fu et al. described a SP-AAC based hydrogel formed from cyclooctyne-modified HA and azide-modified PEG. This hydrogel also displayed short gelation time, stability, bio-compatibility and slow degradation [15]. Another recent work includes the hydrogel formed from dextran modified with azide and cyclooctyne components using SP-AAC click chemistry. This injectable hydrogel was tested for cartilage tissue engineering by incorporating chondrocytes cells individually and as spheroids. However, spheroids induced higher production of chondrocyte extracellular matrices compared to the individual cells [72].

Han et al. reported a biocompatible, *in situ* cross-linking HA-based injectable hydrogel for cartilage regeneration. The high cross-linking was achieved by SP-AAC mechanism

where HA was modified with 4-arm PEG azide and dibenzyl cyclooctyne separately for gel formation. The copper-free click reaction formed gel with the help of reaction between azide and dibenzyl cyclooctyne groups. Different compositions were used to test the various properties required for the injectable hydrogels *in vitro*. They injected the hydrogels subcutaneously in mice and observed the change in volume of gel at different time periods (Fig. 4A, B). White solid tissue like hydrogels removed from the mice after *in vivo* experiments for cartilage regeneration were observed (Fig. 4C) [73]. This hydrogel system showed promising results for the regeneration of cartilage *in vivo*. Recently, a hydrogel system was fabricated using SP-AAC reaction composed of cyclooctyne and azide functionalized PEG-based polymer and protein (laminin) along with a neurogenic differentiation (interferon- γ) factor included in the hydrogel system. *In vitro* studies with protein-entrapped hydrogels containing neural stem cells showed differentiation without adding any growth factor in the cell culture medium [16]. However, the limitations of SP-AAC based click chemistry are the numerous steps that are involved in creating the cyclooctyne and their very low product yield compared to other methods [4]. Another notable challenge is the production of regio-isomeric combination of triazoles in the SP-AAC reactions. Even though these challenges remained, still this method holds promise for developing alternate hydrogel biomaterials with great degree of spatiotemporal functionalization and cell adhesive properties [14].

3.3 Thiol-ene-based click hydrogels for tissue engineering

Thiol-ene click chemistry reaction takes place between the thiol groups and the alkene groups of the reactants. This method provides many advantages such as high yield, high reaction rates, no initiator for initiation reaction, high selectivity, no sensitivity to oxygen or water and high biocompatibility, thus forming orthogonal networks [2]. The thiol-ene reaction mechanism comprises of a mixture of step growth reactions and polymer chain growth reactions. The initiator used in the reaction generates radicals first, which then takes up the thiol group protons from the reactants to form the thiyl radicals. Then, carbon-based radicals are formed by thiyl radicals which triggers the carbon–carbon double bond formation. Thus, formed carbon-based radical may either involve in chain transfer or react with new thiol group to form a new thiyl radical or multiply via carbon–carbon double bond [74]. The molecules bearing double bond (carbon–carbon) can be covalently cross-linked with thiol functionalized molecules. Thiol-based reactions are widely considered as safe and non-toxic in organic synthesis and currently, they are researched in the area of click chemistry for synthesizing novel polymeric materials [75].

Among the thiol-based reactions reported, thiol-ene and thiol-yne reactions are vastly used to develop functional hydrogel biomaterials for tissue engineering and regenerative medicine because of its non-toxic nature, where no metal catalysts are used [20]. Initially, Hawker et al. synthesized a PEG-based hydrogel system crosslinked via thiol-ene group with increased mechanical properties compared to that of the normal PEG hydrogel [76]. Poly(ethylene glycol) diacrylate (PEGDA) and dithiothreitol were used to prepare Thiol-ene-based glucose sensitive hydrogel with self-healing ability for neural tissue engineering application. This injectable hydrogel can act as sacrificial material when exposed to glucose to form branched tubular structures with in a 3D construct, where non-sacrificial materials act as the main matrix. Formation of vascularized neural tissue was demonstrated with the help of endothelial cells and neural stem cells after 14 days [77]. Brown et al. reported dynamic, viscoelastic, photo-

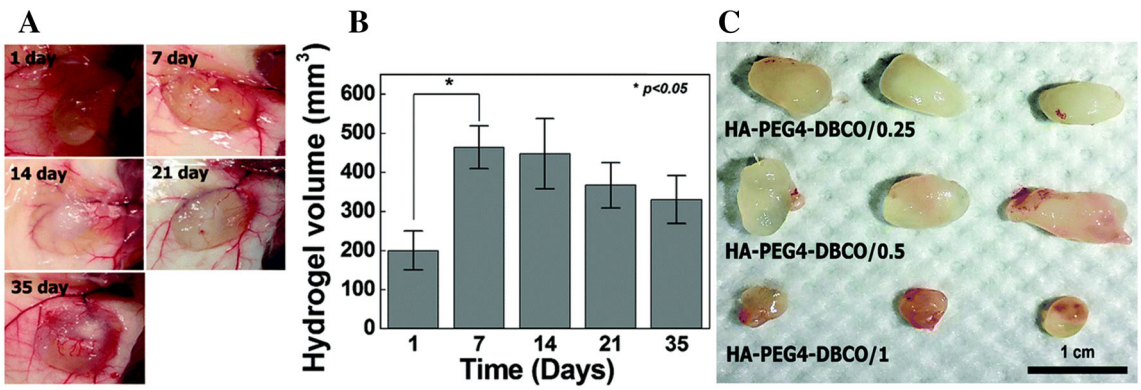

Fig. 4 **A** Hyaluronic acid-based copper-free click chemistry hydrogels implanted in Balb-c mice for up to 35 days for cartilage regeneration. **B** Volume of hydrogel *in vivo* at different time and **C** White solid tissue like hydrogels with chondrocytes removed from mice. Reproduced from [73] with permission from the Royal Society of Chemistry

polymerized hydrogel system from 8 arm PEG thiol macromer, thioester di(vinyl ether) as crosslinker, caged thioester as catalyst and norbornene-RGD for cell adhesion. *In vitro* experiments with primary hMSCs showed promising results for the hydrogel system. Because of the biological importance of the thiol-esters, it may be highly used in cell scaffold applications. These thiol-ene based photoclick reactions are known to form gels rapidly with homogeneous gelation and highly biocompatible hydrogels at normal physiological conditions [78].

Granja's group synthesized cellular degradable pectin-based hydrogel system via thiol-ene photo-crosslinking. The cell-instructive hydrogel system consisted of norbornene-functionalized pectin, monocysteine cell-adhesive ligands for integrin attachment and enzymatically cleavable biscysteine peptide crosslinker. Dermal fibroblast cells were encapsulated during the gel formation and *in vitro* skin tissue formation was evaluated. These thiol-norbornene based hydrogel network formation with high homogeneousness can help formation of tissues by providing enhanced environment. Also, thiol-norbornene based reactions did not involve any strained norbornene group chain growth or homo-polymerization. Further, these reactions yield hydrogels in a shorter time without affecting the cell viability under physiological conditions. Norbornene group modifications are mainly used for their speedy reactivity and high cytocompatibility [79].

Recently, another Thiol-ene-based reaction was used for developing cell arrays with alkene-linked (allyl and norbornene residues), poly(oligoethylene glycol methacrylate) and cell-adhesive peptides RGD and REDV. These systematically patterned biomolecules on the polymer enables us to control the cell adhesion in a controlled way. When human umbilical vein endothelial cells were tested on the patterned structure, RGD-patterned polymers showed higher cell adhesion [80]. Sharma et al. introduced a new microarray system based on thiol-ene photo-clickable peptides that can be used for understanding the cell behavior in 3D micro-environment which combines three technologies, mainly thiol-ene photo click chemistry, microcontact printing and electrospinning. They used thiol-ene method to crosslink the fibrous structures produced via electrospinning and to introduce peptides, a photo-click reaction mediated by norbornene was used in the fibrous matrix. This system with free norbornenes enables any reactant with thiol group to be linked into the 3D construct with high precision. Further, they used a normal contact printer to deposit peptides with cysteine moieties in a microarray model to test the concept with different cell lines [81]. In a similar work, Ding and coworkers demonstrated the ability of such combinatorial, clickable system by enhancing the contractile property of the human smooth muscle cells for vascular tissue engineering. The fibrous hydrogel system showed different biomimetic smooth muscle properties like modifiable structures, composition and mechanical stiffness. With the help of protein array technology and the hydrogels, they demonstrated the *in vitro* pharmacological responsiveness and contractility of the smooth muscles [82].

Zhou et al. reported a photo-polymerized Thiol-ene-based hydrogel for tissue engineering applications consisting of maleic chitosan, thiol-linked PVA and a biocompatible initiator. This hydrogel showed excellent mechanical properties and fibroblast cell compatibility [83]. Takemoto's group recently reported the crosslinking of hydrogels with live cells and tissues using azide-alkyne click chemistry reaction. They initially modified the alginate with alkyne group and live cells/tissues were modified synthetically with azide group targeting the sialic acid groups present on the cell surface and followed by incubating both cells with azide group and hydrogels with alkyne group for gel formation. The live cell containing hydrogels showed excellent cell viability [5]. These various works related to Thiol-ene-based click chemistry showed the ability of these reactions to provide hydrogels with optimum properties required for the various tissue engineering applications. However, one should choose the biocompatible reactants and method to develop the successful biomaterial for specific tissue engineering application.

3.4 Oxime-based click chemistry hydrogels for tissue engineering

The oxime click chemistry reaction occurs between an aldehyde or ketone group and an amino-oxy group. Reactions are rapid and contain similar orthogonal functionalities that are present in biomolecules and cells. The reaction does not require any toxic catalyst or UV light or no external temperature, and usually the byproduct of the reaction is water [3, 84]. The products obtained from the reactions consists of imine hydrazone and oxime chemical bonds, which are highly stable in physiological conditions [84]. With high stability than thiol groups, this oxime reaction is preferred for modifying different biomacromolecules like peptides, proteins and DNA [85]. Furthermore, they are used in polymer-protein ligation, cell surface modification and, also used to *in vivo* labeling of tissues [21, 86]. Grover et al. explored the oxime click chemistry-based hydrogels for live encapsulation of MSCs, using 8-arm-amino-oxy-PEG, RGD peptides and glutaraldehyde. They hypothesized that the formation of stable oxime bonds by the reaction of amino-oxy groups in aqueous condition lead to the increased stability of the hydrogels when compared to the normal imine bond formation by amine groups [21].

Amino-oxy modified PEG and aldehyde-modified HA-based oxime-crosslinked biodegradable, biocompatible hydrogels with tunable mechanical properties were evaluated by Hardy et al. for soft nerve tissue engineering. While cytotoxic assay was carried out with Schwann cells, cell adhesion study was performed using human MSCs for the hydrogels. Both showed promising results for the possible application in nerve tissue regeneration when collagen I was incorporated in the hydrogels [22]. DeForest's research group reported a biorthogonal oxime click chemistry-based hydrogel for 3D cell culture-related applications. In this study, they used mild UV light photo-polymerization for obtaining the cells-encapsulated hydrogel. This photo-mediated oxime reaction enabled them to immobilize proteins with alkoxyamines at specific regions where UV light is exposed. The photo-mediated oxime reactions allow us to polymerize selected areas in the samples based on our interest. The UV irradiated regions in the polymer solution formed gelation except in the photo-masked unexposed regions [23].

Hentzen et al. presented the crosslinking of model collagen peptides through oxime bond formation using 4-oxoacetamido-proline and 4-aminooxy-proline. These peptides were covalently linked, resulting in formation of stable collagen triple helices [87]. Tamura et al. reported a new affinity-guided oxime catalytic system involving pyridinium oxime and *N*-acyl-*N*-alkylsulfonamide for protein labelling. This system can be used to selectively label natural proteins present in test tubes and different cell lysates under normal physiological conditions. Cell membrane proteins can be fluorescently tagged and visualized in live cells using this catalytic system. They demonstrated the ability of this system in mouse brain slices [88]. Drawback of using oxime chemistry reaction in bioconjugation is normally oxime reactions require neutral or basic (pH) condition to reduce the possible oxime exchange reaction to occur [1]. Considering the various advantages over the limitations, these oxime-based click chemistry reactions may be used to development novel biomaterials for both *in vitro* and *in vivo* applications.

3.5 Thiol group-based (Thiol-Michael) pseudo-click hydrogels for tissue engineering

Thiol-Michael pseudo click chemistry involves an addition of thiol group into a double bond of vinyl sulfone, acrylate or maleimide, resulting in thioester bond formation with or without the use of a catalyst [89]. The advantages of this reaction include higher reaction rates, easier access to reagents functionalized with thiol and ene groups and high tolerance towards different functional groups [90–92]. Michael addition reaction-based cross-linked step growth hydrogels were first reported by Hubbell's group [93]. The reactants used were multi-acrylate linked PEG and dithiol linked PEG or thiol-linked peptides. Different Michael-type addition reaction-based hydrogels were developed in the past decade by various groups around the world. Various biomaterials were used for preparing hydrogels using this method such as chitosan-PEG, fibronectin, HA and gelatin-PEG [19, 94, 95]. Young and Engler reported cross-linked hydrogels, consisting of thiol-HA and PEGDA using Michael addition method for cardiac tissue engineering application [96]. Scientists used highly reactive vinyl sulfone instead of acrylate in the thiol-Michael reactions mainly because of its high electron withdrawing ability and formation of strong bonds [90]. Patterson and Hubbell reported thiol-Michael reaction-based PEG hydrogels for tissue regeneration using 4 arm PEG-linked with vinyl sulfone, cell-adhesive peptides and matrix metalloproteinase degradable peptides [97]. Jin et al. demonstrated the formation of collagen and chondroitin sulphate in the chondrocyte-cultured HA hydrogels prepared from thiol-linked HA and 4-armed PEG with vinyl sulfone, using thiol-Michael addition reaction for cartilage repair [98]. Fibronectin and RGD peptide were incorporated in PEG hydrogels consisting of thiol-maleimide and showed increase in the human fibroblasts cell adhesion and proliferation. The same group developed similar hydrogel with glutathione sensitivity using thiol-Michael reaction. The gel formation slowed down when the pH and temperature was reduced to 6.6 and 4 °C, respectively. This reduction in temperature and pH permitted the proper mixing using vortex machine and for loading samples [99]. Bang et al. reported chondroitin sulphate-based dual cross-linked hydrogels with gelatin grafting for improving the cell-adhesive properties of the chondroitin sulphate. They used the Michael type click chemistry reaction method and *N*-(3-diethylpropyl)-*N*-ethylcarbodiimide hydrochloride chemistry for the gel formation and the hydrogels were tested for its application related to tissue engineering and drug delivery [100]. These different works related to thiol-based click chemistry reactions show potentials of this method in developing novel hydrogel biomaterials for tissue engineering applications.

3.6 Aldehyde-hydrazide pseudo click hydrogels and amino-yne hydrogels for tissue engineering

Like other click chemistry reactions, aldehyde-hydrazide based pseudo reactions are simple, versatile, and do not generate toxic end products with high reversibility. In general, many polysaccharides were modified with aldehyde and adipic acid dihydrazide (ADH) derivatives to obtain various hydrogels. Bulpitt and Aeschlimann reported the first hydrogel using this method, by using HA-ADH and HA-aldehyde [101]. Tian et al. demonstrated the capability of the aldehyde-hydrazide cross-linked HA-based hydrogels in rat brain repair [102]. This work

showed an advanced potential of this method to develop such biomaterials for tissue regeneration. Alginate and HA-based *in situ* cross-linkable hydrogel was reported by Dahlmann et al. for cardiac tissue engineering. They demonstrated that the hydrogels were able to generate contractile cardiac tissue from rat heart cells [103]. Martinez-Sanz et al. developed an injectable bone morphogenic protein-2 (BMP-2) loaded HA hydrogel for *in vivo* bone tissue formation. They used synthetic procedure, including amidation and selective oxidation for the formation of stable gels. The gelation occurred within 30 s; however, BMP-incorporated hydrogel samples were placed for curing (3 h) at room temperature. The cured samples were used for *in vivo* experiments [104]. Similarly, many scientists recently reported different aldehyde-hydrazide hydrogels for tissue engineering applications that include PVA [105, 106], elastin-like protein-HA based hydrogels [107, 108], poly(*N*-isopropylacrylamide) (PNIPAM), [109, 110], HA-based hydrogels [111], and HA with growth factors for bone tissue engineering [112].

Recently, Huang and Jiang demonstrated the ability of the amino-yne click chemistry to form hydrogels with pH sensitivity for local drug delivery and tissue engineering applications. They reported the carboxymethyl chitosan-PEGDA based pH-responsive, degradable and injectable hydrogels for tissue engineering and biomedical applications without using a catalyst or initiator in normal physiological conditions. The mechanism of amino-yne click chemistry-based hydrogels' pH sensitivity is depicted in the Fig. 5. In presence of H^+ ions, enamine is converted to imine compound. Further, decomposition occurs to form aldehyde and amine group at low pH, ultimately resulting in complete release of drug at pH 2 [113]. Since, this injectable amino-yne based click chemistry hydrogel is simple, less expensive and spontaneously gel-forming, it may provide the much-needed biocompatible hydrogels for tissue engineering applications without using any toxic catalyst or photo initiator.

4 Application of click chemistry in development of bioinks for 3D bioprinting

Recently numerous novel multifunctional hydrogel biomaterials developed by various reaction methods are reported as bioinks for 3D bioprinting [26]. These

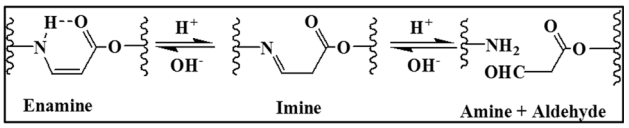

Fig. 5 Amino-yne click chemistry-based hydrogels: pH-sensitive mechanism. Reprinted (redrawn) with permission from [113]. Copyright 2018 American Chemical Society

hydrogels provide the much-needed microenvironment by biomimicking the extracellular matrices present in the native tissue structures [27]. Different natural and synthetic hydrogels with multifunctional properties like biocompatibility, high mechanical properties, pH sensitivity, temperature sensitivity, etc. and in combination with nanomaterials or composite materials or growth factors, biomolecules, etc. have been applied to create complex 3D structures and functional tissue structures using 3D bioprinting technology for tissue engineering applications [27]. Thus, a multidisciplinary approach involving of stimuli-responsive biomaterials, click chemistry reactions and cutting-edge 3D bioprinting technology may facilitate higher probability to create complex 3D structures which are functional and able to form tissue or organ structures mimicking native tissues [115]. In a review article by Kurzrock and Stewart, they discussed about the combination of click chemistry and 3D bioprinting related to chemical compound development and implications. They also envisioned that the natural proteins can be designed and tested in silico using supercomputers, whereas the click chemistry and 3D bioprinting technology can help us to obtain the desired chemical compound with different structures and high precision [116]. Similar proof-of-concept studies was already reported related to these methods [117, 118].

Likewise, click chemistry reactions have been used to develop hydrogels for 3D bioprinting bioinks [27]. Bertlein et al. reported thiol-ene photo-clickable gelatin-based hydrogels as bioink for fabrication of 3D structures using 3D bioprinting technology [119]. Li et al. demonstrated Cu-AAC based click chemistry reactions for developing peptide functionalized poly(ester urea) scaffolds for bone tissue engineering applications. In this work, they used L-phenylalanine functionalized with propargyl groups-based poly(ester urea) scaffolds with BMP-2 and osteogenic growth peptide. These scaffolds showed increased osteogenic activity and hMSC differentiation than the non-functionalized scaffolds [120]. Stichler et al. reported a similar combination of thiol-ene click chemistry and 3D bioprinting for developing 3D structures recently. They used polyglycidol-based hydrogels which were cross-linked by UV light, and cytotoxicity tests were performed using hMSCs obtained from bone marrow. They added high molecular weight HA to adjust the rheological properties and printed 20 layered structures with high reproducibility [121]. This combinational approach using click chemistry and 3D bioprinting were reported by many scientists recently for various tissue engineering applications [122–126].

5 Conclusion

In this review, we explained the diverse click chemistry-based hydrogel syntheses, mechanisms, typical hydrogels reported for tissue engineering applications as well as their advantages and disadvantages in details. High reaction rates, spontaneous reactions, selectivity, versatility, high product yields, biocompatibility, *in situ* gel formation and injectability are some examples of the advantages of click chemistry reactions. Overall, click chemistry-based hydrogels for tissue engineering applications are promising and immensely explored all over the world in the recent years for various biomedical applications like 3D bioprinting, drug delivery, diagnosis, tissue engineering and regenerative medicine, etc. Considering the various advantages and disadvantages of diverse synthesis routes, one should design the experiments for creating novel biomaterials for specific applications. For example, thiol-based hydrogels with residual initiators are reported to induce mild immunogenic response at cellular level. Hence, high care should be taken to remove toxic catalyst or initiators completely during hydrogel synthesis, or cost-effective purification techniques should be employed to obtain better-quality products from the click chemistry reactions. One important criteria for developing such hydrogels with superior properties is the combination of advantages from diverse click chemistry methods or its key biomaterials to obtain multifunctional and biocompatible biomaterials. At the same time, the reactions should not affect or interfere with other processes or it should not have any side effects or cross reactions with unexpected reaction groups. New approaches like the surface modification of live cells with ligands for crosslinking with biocompatible hydrogels are introduced recently, which shows the rapid growth in this area. The use of click chemistry in the synthesis of bioinks for 3D printing are envisioned to provide highly precise 3D tissue structures with live cells embedded in the hydrogels. Future of click chemistry for developing novel multifunctional biomaterials for tissue engineering and regenerative medicine applications are promising and encouraging.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical statement There are no animal or human experiments carried out for this article.

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