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"Click" reactions to construct bioactive peptide conjugates

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ARTICLE

"Click" Reactions: a Versatile Toolbox for the Synthesis of Peptide-Conjugates

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Peptides that comprise the functional subunits of proteins have been conjugated to versatile materials (biomolecules, polymers, surfaces and nanoparticles) in an effort to modulate cell responses, specific binding affinity and/or self-assembly behavior. However, the efficient and convenient synthesis of peptide-conjugates, especially the constructs with multiple types of peptide functionality remains challenging. In this *critical review*, we focus on "click" reactions that have been used to synthesis peptide-functionalized conjugates, introducing their reaction conditions, specifically elucidating parameters that influence reaction kinetics and total conversion, and highlighting examples that have been completed recently. Moreover, orthogonal "click" reactions that synthesize multi-functional biomaterials in a one-pot or sequential manner are noted. Through this review, a comprehensive understanding of "click" reactions aims to provide insight on how one might choose suitable "click" reactions to constitute peptide-functionalized molecules/surfaces/matrices for the development of advanced biomaterials.

1. Introduction

Peptides, the varied sequence of amino acids that compose the functional modules of proteins, have become increasingly important in the design and fabrication of bioactive materials for medical applications.[1-4] There are several advantages to use peptides as functional motifs in biomaterials. Peptides have a highly refined structure, and yet gram-scale synthesis of peptides containing up to 50 amino acids is facile by solid phase peptide synthesis (SPPS) methods. They are degradable in physiological conditions, and the degradation products are amino acids metabolites that are non-toxic and readily resorbed or excreted. The most intriguing feature of peptides is their diversity in functional properties as a result of a small subset of 20 amino acid building blocks found in nature. Numerous peptide subunits with specific bio-functions have been identified from the active portions of proteins that influence cell behavior and minimize undesired immune responses. For example, the RGD peptide found in fibronectin, a protein conserved in the extracellular matrix (ECM) of a number of species, is well-known to bind with integrins on the surface of cells and has been widely used to enhance cell adhesion to biomaterial surfaces.[5] Meanwhile, thanks to the development of viral phage display technologies, peptides that bind specifically with certain cell receptors or surfaces, ranging from proteins[6] to synthetic polymers,[7,8] and to inorganic surfaces,[9] have been identified, providing a vast library of receptor-binding peptides to serve as surface-targeting or protein-homing motifs for use in bio-imaging and biomedicine.[10] Moreover, peptides readily self-assemble to form secondary structures, such as α-helix, β-sheet, trihelix, etc., making them useful in the fabrication of hierarchical structures with thermal stability or pH sensitivity.[11] In summary, each peptide comes with a particular property, regardless of its bioactivity, receptor-binding ability or self-assembly behavior, which originates from the sequence. These sequences when combined with polymers or biologically relevant molecules possessing unique and novel properties can be applied to construct bioactive materials with a wide range of applications.

Many peptide-conjugates have been found to improve the bioactivity of materials by enhancing cell/tissue target specificity and decreasing side effects to the host. Peptide-conjugates fall roughly into three categories depending on the components to which the peptides are linked with (Fig. 1): 1) peptide-conjugates with other biological molecules, such as carbohydrates, peptides, nucleic acids, drugs or diagnostic probes; 2) peptide-conjugates with polymers; 3) peptide-conjugates with non-polymeric surfaces, either as 2-D substrates or 3-D nanoparticles (NPs). Each type of peptide-conjugate was designed and synthesized for a particular application. For instance, glycopeptides have been designed and synthesized for the development of cancer vaccines.[12-14] Peptide-dendron conjugates have been designed to utilize multivalency in order to dramatically strengthen the binding affinity and specificity of peptide clusters to targeted cells or biomaterial surfaces, which is useful in drug delivery.[15-18] Modular peptides link peptide subunits containing different bio-functions together, and have proven to be an effective method to construct conjugates that mimic proteins for regulating cell behavior.[19] Peptide-polymer conjugates have variable applications, from the fabrication of antifouling implant surfaces,[20] to peptide-functionalized hydrogels[21-23] and scaffolds[24-26] which provide better material integration with surrounding host tissues. Peptide-functionalized NPs can be used to increase target cell uptake.[27,28] Many peptide-functionalized 2-D surfaces have been fabricated for *in vitro* studies of cell behavior.[29,30] It is clear that peptide-conjugates have been reported throughout the literature for

many diverse applications in therapeutics, diagnostics, drug delivery, tissue engineering and the fundamental study of cell behavior.

Because peptide-conjugates have vast applications in biomedicine, synthetic technologies with high chemical specificity and reaction efficiency are required to obtain high yield with cost efficiency. "Click" reactions have emerged as ideal candidates to serve in this mission. "Click" reactions are conjugation reactions that fulfill the following prerequisites: i) high yield, nearly quantitative conversion; ii) biologically benign conditions (aqueous solution, ambient temperature, and near physiologic pH); iii) limited or no residual byproduct. Ever since Cu(I)-catalyzed azide-alkyne cycloaddition (CuAAC), the first widely accepted "click" reaction, was reported, researchers from diverse disciplines have eagerly sought to use the highly efficient coupling reaction to synthesize different conjugates. Many reviews have been written to summarize the utilization of CuAAC in synthesis of molecules with different architectures and functional groups.[31-34] Over time, several other "click" –type reactions have emerged, including strain-promoted azide-alkyne cycloaddition (SPAAC), thiol-ene reaction, Diels-Alder reaction, oxime ligation and others for development of complicated structures. These "click" reactions constitute a toolbox for efficient coupling methodologies for the synthesis of bioconjugates. Moreover, within this toolbox, several "click" reactions are orthogonal to each other, meaning different types of "click" reactions can happen in a one-pot approach or in a sequential manner. These characteristics and advantages meet the increasing demand for multi-functional biomaterials that can mimic complicated synergistic signaling cascades inherent within biological environments. To better harness this "click" reaction toolbox for the synthesis of peptide-conjugates, it is important to understand each type of reaction, including the reaction conditions, additives (initiator, catalyst or reducing agent), byproducts and its advantages and disadvantages when used for synthesis of bioconjugates.

In this review, we focus on recent progress that researchers have made by using "click" reactions to synthesize versatile peptide-conjugates. First, methods that introduce functional groups for "click" reactions to peptides will be summarized. Different types of "click" reactions that have been applied to synthesize peptide-conjugates will be introduced (see Table 1). CuAAC, SPAAC, thiol-ene reaction, thiol-Michael addition, oxime ligation, Diels-Alder reaction, Staudinger ligation and native chemical ligation will be the focus in this paper due to their popularity and versatility in the synthesis of peptide-conjugates. The reaction conditions, additives that initiate the reaction or catalysts to accelerate the reaction, as well as parameters that influence reaction rate and yield will be discussed. Examples that successfully utilized "click" reactions to make peptide-functionalized biomaterials, including peptide-conjugates with small biomolecules, polymers and surfaces will be highlighted. Lastly, orthogonal "click" reactions that have been used to make multi-functional biomaterials will be elucidated. Through this review, we aim to provide a summary on how one might choose suitable "click" reactions to constitute peptide functionalized molecules/surfaces/matrices for the development of advanced biomaterials.

2 Methods to tag peptides with "clickable" functional groups

One simple way to tag peptides with functional groups that undergo "click" reactions is to directly couple a non-canonical amino acid containing a pre-selected functional group within the peptide during synthesis. If the functional position is at the N-terminus, acids with "clickable" groups are readily available for use following cleavage. Thanks to the development of microwave-assisted SPPS, it is straight forward to prepare peptides containing up to 50 amino acid residues. The obvious advantage of this method is the precise control of functional position along the peptide chain by simply altering the coupling sequence. The only limitation is that the desired functional groups must be compatible with the reaction conditions for peptide coupling, deprotection and cleavage; otherwise, certain protection of the functional groups is required. Azide,[29] alkyne,[36] alkene,[37] thiol,[38] maleimide,[39] and amineoxy[40] have successfully been introduced into peptides through this strategy.

In SPPS, residues of amino acids that are reactive or unstable during the coupling and deprotection steps are often protected with orthogonal protecting groups. By carefully choosing the protecting groups on the side chain residues of amino acids, one may selectively functionalize the peptide at a specific site. A review that summarizes protection groups for amino acids and their corresponding deprotection conditions was recently written by Dr. Albericio *et al.*[41] This is a complementary method with direct coupling of a non-canonical amino acid with peptide, due to the difficulty of synthesizing non-canonical amino acids. For example, the protection group (4,4 - dimethyl - 2,6 - dioxocyclohex - 1 - ylidine)ethyl (Dde) in Fmoc-Lys(Dde)-OH can be deprotected using mild conditions while other protected amino acid residues are stable, generating an amine group that can be functionalized with an alkene or a di-fluorocyclooctyne group.[37,42]

Among different types of functional groups, peptides with an azide tag are used most frequently, mainly due to their high bioorthogonal selectivity and the versatile reactivity in different "click" reactions. The "click" reactions using an azide group are summarized in Table 1, including CuAAC, SPAAC, Staudinger ligation and tandem [3+2] cycloaddition-retro-Diels-Alder reaction (tandem crD-A).[35] Peptides with an azide group were synthesized either by directly coupling the peptide with azido acid,[43-45] or by post transfer of bromine to azide.[46] For the direct coupling of an azido amino acid or an azido acid with a peptide, there are many candidate molecules that have been reported in the literature.[45,47] Considering the safety issues with organic azides, the coupling step of a peptide with an azido amino acid is using manual (not microwave) SPPS methods. The cleavage cocktail needs to avoid the usage of thiol-scavengers, because the azide would be reduced to an amine by 1,2-ethanedithiol during the cleavage reaction.[48] The procedure of post transfer of a brominated-peptide to an azido-peptide was also reported,[46] although it requires two cycles of dialysis and freeze drying to purify the peptide, the coupling step can be assisted using microwave conditions which may benefit the synthesis of long chain azido-peptides.

The other functional group that is notable due to its versatile reactivity in several types of "click" reactions is the thiol group. Peptides with a thiol group were readily prepared by coupling with

cysteine, a natural amino acid containing a thiol in the side group. It is also possible to reduce the disulfide bonds in proteins with a reductive agent to obtain free thiol groups *in situ* for further functionalization.[49,50] As shown in Table 1, a thiol group is capable of reacting with an alkene under irradiation through a thiol-ene reaction,[51] with an alkyne under irradiation through a thiol-yne reaction,[52,53] with an electron-deficient alkene through Michael addition, and with dipyridyl disulfide.[50] In addition to the noted reactions, the cysteine at the N-terminus of a peptide is able to couple with a thiol ester by native chemical ligation (NCL), and with an aldehyde through thiazolidine ligation. Because of the diverse reactivity with different functional groups, peptides with thiol groups have been widely used to synthesize peptide-conjugates with carbohydrates,[54-57] polymers,[49,58-61] and fluorescein.[62]

3 "Click" reactions to synthesize peptide-conjugates

3.1 Cu(I)-catalyzed azide-alkyne cycloaddition (CuAAC)

The concept of "click" reaction was first introduced to refer broadly to CuAAC by Sharpless in 2001,[63] although the Huisgen 1,3-dipolar cycloaddition was reported in the organic literature much earlier.[64] The reaction has nearly quantitative yields and a wide range of solvent tolerance and the resulting triazole product is highly stable under biological conditions. All of these attributes make it a good candidate to synthesize peptide-conjugates. The only drawback is the usage of Cu(I) as the catalyst if one desires stereoselectivity. The Cu(I) catalyst in CuAAC leads to several problems, including cytotoxicity,[65] disrupting DNA double helix strands,[66] denaturation of proteins[67] and reduction of the quantum yield of quantum dots (QDs).[68] To avoid the oxidation of Cu(I) to Cu(II), degassing and inert gas protection may be needed, as well as the addition of reducing agent like Na ascorbate and suitable ligand to prevent Cu(I) oxidation and accelerate the reaction rate. Meanwhile the removal of Cu(I) salts requires tedious purification, such as dialysis, microfiltration, or high performance liquid chromatography (HPLC) to eliminate trace copper for subsequent biological tests of the peptide-conjugates.

Parameters that influence CuAAC reaction efficiency

To obtain peptide-conjugates with high purity and alleviate tedious purification work, it is very important to achieve high conversion in reactions. Although CuAAC usually results in almost quantitative conversion in organic synthesis, as the peptide chain becomes longer, the secondary structure of the peptide may hinder the efficiency of the reaction. Depending on the peptide sequence and conjugated moieties, the yield of CuAAC coupling peptide with other bioactive molecules can range from 5% to 90%. Microwave irradiation and increased temperature have been applied to increase the reaction rate and the yield of the final products in the synthesis of RGD peptide dendrons.[45,69,70] However, when the dendron valency increases to four, the total yield dramatically drops to about 20%.[45,70] This reduction may arise from hindered reactive sites of the partially formed peptide-dendron that slow the reaction kinetics, while elongated reaction time leads to decomposition of the peptide chain. To overcome these problems, a more effective method is to add Cu(I) binding ligands, which protect the Cu(I) from oxidation and increase the reaction kinetics and subsequently the final conversion after a given time.[71-75] Many works on developing Cu(I) ligand to boost reaction kinetics and conversion have been carried out. Finn *et al.* used THPTA (structure shown in Fig. 2) as the CuAAC-accelerating ligand, and successfully obtained protein-decorated cowpea mosaic virus conjugates within 1 h.[73] The Wu group developed the ligands BTTES and BTTAA (structure shown in Fig. 2), which dramatically accelerated the CuAAC kinetics in aqueous solution in comparison with THPTA, and were proven to be safe for use in labeling target glucose on the cell surface in living system.[72,74] Researchers have also studied the effects of different alkynes on reaction kinetics, and found that propargyl compounds showed the most reactivity to azide molecules.[76] However, the conjugation of propargyl acid to peptides was found to be incompatible with microwave-assisted SPPS in this study. Ultrasound irradiation[77] and electrochemical activation of Cu(I)[78] have also been used in studies to make peptide conjugations with higher conversion.

Peptide-conjugates synthesized by CuAAC

Many peptide-conjugates have been coupled to biologically relevant small molecules through CuAAC. Typical examples are shown in Fig. 3. The Brimble group utilized CuAAC to synthesize molecule **1**, an azido-phosphate that was "clicked" to an alkyne-peptide to make a phosphopeptide which plays an important role in the biomineralization process.[79] Analogous to this, two azido-saccharides have been conjugated to an alkyl-peptide, generating a glycopeptide, molecule **2**.[80] The high efficiency of CuAAC is well demonstrated in their later work in the construction of more complicated macromolecules, molecule **3**, a multivalent neoglycopeptide conjugate.[81] To synthesize molecule **3**, a tetrasaccharide dendron is initially synthesized through CuAAC with 89% yield, and is then reacted with a peptide equipped with an alkyl residue to obtain the final product with 32% yield. The synthesis of peptide-functionalized dendrons can also be successfully carried out with CuAAC reaction. In the Becker group, a series of dendrons with tunable numbers of azide functional groups on the dendron surface were reacted with alkyne-bearing hydroxyapatite (HA)-binding peptides to obtain peptide-functionalized dendrons. For instance molecule **5**, that interacts with HA surface through multivalent binding, dramatically strengthened the binding affinity of near 1000-fold compared with HA-binding peptide. This is the most strong binding motif to HA surface that has been reported, which is significantly useful in drugs for bone healing.[36] Not only have dendrons with a single type of peptide functionality been constructed, but heterofunctionalized dendrons, such as molecule **4,** have also been successfully synthesized. As a modular peptide, molecule **4** was developed as a mimic of osteocalcin, containing a hydroxyapatite-binding domain, the peptide-functionalized dendron, and a cell interacting domain by way of bone morphogenetic protein-2 (BMP-2) derivative peptide. Peptide-grafted polymers with diverse architecture have also been easily synthesized through CuAAC reaction. A comb-shaped peptide decorated polymer, such as molecule **6**,[46] and chain end conjugate, like molecule **7**,[82] have both been implemented successfully.

When peptides bearing more than one reactive site are reacted with polymers equipped with multiple corresponding functional groups, peptide-functionalized hydrogels are achieved (Fig. 4). In

peptide-functionalized hydrogels, the peptide plays two roles. One role is a result of its intrinsic bioactivity, such as using the RGD peptide to enhance cell attachment (Fig. 4A)[43] or using a protease-sensitive peptide for enzyme-controlled biodegradation (Fig. 4B and 4C).[37,83] The other role is for the diazido- or dialkyl-peptide to act as a crosslinker for hydrogel formation. The gel formation time is tunable depending on the concentration of reagents and temperature, from 2 min to 30 min.[43] The mechanical properties of the hydrogel depend on polymer architecture and molecular mass.[83] However, hydrogels formed by CuAAC contain Cu(I) which is known to be cytotoxic, so extensive dialysis is required to eliminate the trace amount of Cu(I) for further cell behavior evaluation.[43]

A peptide-functionalized substrate can serve as an important model system to study the effects of certain peptides on cell behavior. CuAAC provides an efficient method to covalently link the peptide onto an azide- or alkyne-bearing surface. Fig. 5 presents some typical examples of peptide-functionalized surfaces with tunable concentration, gradient profile or patterned topology. The Murphy group has prepared substrates bearing a controlled RGD peptide density by a CuAAC mechanism through use of a mixed self-assembled monolayer (SAM) of azide-terminated hexa(ethylene glycol) alkanethiolates and tri(ethylene glycol) alkanethiolates, as shown in Fig. 5A. The RGD concentration was altered by changing the composition of alkanethiolate compounds, and the influence of varied RGD surface density on cell adhesion, spreading and focal adhesion complex formation was studied in detail.[84] To study the concentration effect of peptides on cell behavior more effectively, a surface possessing a concentration gradient was desired. In Fig. 5B, the Becker group utilized gradient ozone treatment to prepare a surface with a gradient concentration of alkyne functional groups, and further immobilized the RGD peptide onto this substrate. In this method, it is most impressive that the concentration effect of the RGD peptide on cell adhesion could be quantitatively evaluated using a single slide, potentially reducing the costs for materials and experimental time.[29] An electrochemical method has also been applied to make both gradient and patterned substrates. Yeo *et al.* recently reported the preparation of a patterned RGD-modified substrate by on-demand activation of a cobalt complex-masked alkyne on SAMs using electrochemical treatment following a CuAAC reaction with an azide-terminated peptide. In this strategy, the region of the presenting alkyne for "click" reaction can be controlled by the shape and position of the electrode. Therefore, it is very convenient for sequentially revealing the masked alkyne and for modification of different functional moieties on a surface, as shown in Fig. 5C.[85] Larsen and co-workers also reported using the electrochemical method to prepare gradient peptide-functionalized surfaces. In their approach, the gradient formation stemmed from the local generation of Cu(I), the critical catalyst for CuAAC, from Cu(II) and Cu.[30] Moreover, they combined micropatterned stamp technology with electrochemical treatment, and obtained a striped-patterned substrate with two different peptide sequences immobilized on alternating stripes respectively, as shown in Fig. 5D.[86]

Through the CuAAC mechanism, bioactive peptides have been coupled onto the surface of NPs for development of hybrid devices to be used in the fields of drug delivery, diagnostics and biosensors. An excellent example that demonstrates the use of the "click" reaction to construct functional NPs in drug delivery was carried out by the Bhatia group. In Fig. 6A, cyclic LyP-1 targeting peptides were linked to iron oxide NPs *via* CuAAC. Due to multivalent binding, the peptide-functionalized NPs were able to penetrate the tumor interstitium to specifically bind with p32-expressing cells in tumors *in vivo*, while NPs lacking such peptide functionality remained in the blood vessels or within their immediate periphery. To achieve targeted drug delivery, nanoengineered polymer capsules that encapsulate drugs inside and are equipped with a targeting peptide outside can serve as an ideal drug vehicle.[27] In Fig. 6B, Caruso *et al.* reported a general approach to functionalize polymer NPs with antibodies using CuAAC, and the obtained NPs showed greater specificity toward colorectal cancer cells even when the target cells only constituted 0.1% of the total cell population. While most researchers have directly grafted recognition peptides onto the surface of NPs, peptide-functionalized NPs have also been prepared through exploitation of the self-assembly of peptide-bearing block copolymers.[87] Hawker and Anderson demonstrated this approach by combining the use of multiple functionalized macromonomer and living free radical polymerization. As shown in Fig. 6C, within the block copolymer that spontaneously forms NPs in aqueous conditions, there is a targeting unit, the RGD peptide, as well as a diagnostic unit, that is the tetraazacyclododecane. The advantage of this system is that RGD loading of NPs is easily tunable, ranging from 0 - 50 %. Accordingly, the effect of RGD loading on NP biodistribution *in vivo* was studied, providing insight on the optimal design of multivalent NPs for applications in therapeutic and imaging applications.[28]

3.2 Strain-promoted azide-alkyne cycloaddition (SPAAC)

To overcome problems associated with the use of a metal catalyst in CuAAC reactions, Bertozzi and Boons have developed several strained cyclooctynes to replace the alkyne in the Huisgen 1,3-dipolar cycloaddition. Because of intramolecular strain, a cyclooctyne reacts readily with an azide in the absence of metal catalyst with high yields at ambient temperature.[88-91] SPAAC has been used to synthesize many different kinds of conjugates, including dendrimers,[92] proteins,[93] polymers,[94] metal-organic frameworks[95] and functional surfaces.[96] More importantly, SPAAC has been used to image targeted biomolecules in living systems.[89,97-99] In short, SPAAC is a very important "click" reaction for the construction of hybrid biomaterials and for labeling biomolecules in a living system. The disadvantages of SPAAC are correlated with the high reactivity of the strained cyclooctyne. Unlike CuAAC, which is highly regioselective with a product of 1,3-triazole, the regioselectivity of SPAAC is poor. Moreover, thiols in the cysteine residue react with cyclooctyne through a thiol-yne reaction which leads to non-specific labeling.[100] In addition, cyclooctynes are expensive and difficult to synthesize greatly limiting use of this technique.

Parameters that influence SPAAC reaction efficiency

The reaction kinetics and conversion of SPAAC largely depend on the chemical structure of cyclooctyne. The commonly used cyclooctyne structures are shown in Fig. 7. Bertozzi's group reported

the first cyclooctyne (molecule **8**) to undergo SPAAC with azide under physiological conditions.[88] The reaction kinetics were quite slow compared with CuAAC, and it was not until much later that they found molecules **9** and **10**, with electron-withdrawing groups (fluoro substitution) in the α position of the triple bond that dramatically increased the reaction kinetics. In fact, molecule **10** (DIFO) shows similar reactivity with azide as CuAAC under the same conditions.[89] Boons's group synthesized dibenzocyclooctyne (DIBO), molecule **11**, that possesses almost the same reaction rate as DIFO.[91] Later Delft and Popik reported that by replacing the carbon atom in the cyclooctyne ring with nitrogen, as in molecule **12**, the reaction kinetics were improved even more than with DIBO.[101,102] Molecule **13**, which is a cyclopropenone-masked dibenzocyclooctyne, generates DIBO under ultra-violet (UV) irradiation and is suitable for fabrication of patterned surfaces.[103] In some cases, steric hindrance also plays a role in the total reaction conversion. When reacted with azide-pendant molecules to obtain comb-shaped functionalized polymers, alkyne through CuAAC afforded faster reaction kinetics and almost total conversion of azide in the polymer chain, while DIBO through SPAAC showed slower reaction kinetics and only partial conversion of azide.[104,105]

Peptide-conjugates synthesized by SPAAC

Since its discovery, SPAAC has been quickly adopted for synthesis of diverse peptide-conjugates, including peptide-functionalized detective probes, polymers, particles and substrates, due to its fast, robust and efficient chemistry. The Kim group synthesized various ^{18}F-labeled peptide for positron emission tomography (PET) molecular imaging and diagnosis.[106] With high efficiency and no need for a metal catalyst in SPAAC, the procedure only took 30 min with a yield above 90%, and the ^{18}F-labeled peptides were obtained in a directly injectable form with no need for HPLC purification. They successfully demonstrated PET molecular imaging *in vivo* with the ^{18}F-labeled cRGD peptide, as shown in Fig. 8A. Analogous to this work, a ^{64}Cu-labeled peptide as a PET imaging probe has been synthesized by Conti and coworkers with a yield of 98%. In this work, SPAAC was critical for successful synthesis because Cu(I) in CuAAC interferes with the ^{64}Cu radiolabeling agent. Some experiments *in vivo* showed specific uptake of the ^{64}Cu-labeled cRGD peptide in target tissues.[107] The first work to utilize SPAAC to fabricate three-dimensional hydrogel networks for cell culture was carried out by the Anseth group as shown in Fig. 8B. Therein, an enzymatically degradable peptide was equipped with two DIFO groups at the chain ends, and was then mixed with a four-arm poly(ethylene glycol) (PEG) tetra-azide. Using SPAAC, the hydrogel formed within 1h in aqueous conditions at 37 ^{0}C. Moreover, a photoreactive group and enzymatically degradable component in the peptide sequence provided a way to tailor the biophysical and biochemical properties of the hydrogel independently, which made it an ideal platform to study the cell behavior and for further application in tissue engineering.[42]

In the Becker group, the stability of DIBO during electrospinning process was studied. As shown in Fig. 8C, poly(γ-benzyl-L-glutamate) bearing one DIBO at the chain terminus was electrospun to fabricate fibers approximately 1 μm in diameter. From the UV spectra, it was evident that DIBO functional groups survived the electrospinning process, and that the resulting nanofibers were capable of reacting with moieties bearing azido groups for post-fabrication surface modification. This capability provides researchers an easy and efficient way to fabricate peptide-functionalized nanofiber scaffolds for tissue engineering applications.[94] Substrate modification through SPAAC has been reported by the Chaikof group when they used a DIBO-derivatized IKVAV peptide to react with an azide-bearing polymer-functionalized surface, in order to create an efficient and fast approach to decorate the cell surface as well as to fabricate peptide microarrays (Fig. 8D).[108] The RGD peptide-functionalized block copolymers, which form micelles that can be loaded with drugs, were made by Boons's group and the mechanism of drug release was studied (Fig. 8E).[109] As mentioned, SPAAC is a more suitable approach to decorate QDs compared with CuAAC, because Cu(I) interferes with inorganic matrix and induces luminescence inhibition. Through SPAAC, glucose-functionalized QDs with improved luminescent properties have been synthesized,[68] demonstrating that SPAAC is a more favorable approach to make peptide-functionalized QDs without sacrificing the luminescence quantum yield.

3.3 Thiol-ene reaction

The addition of a thiol to an alkene through radical intermediate is referred as the term thiol-ene reaction. In the synthesis of bioconjugates, the photoinitiated thiol-ene reaction is preferred because the reaction can be triggered in aqueous solution under physiologic conditions by ultra-violate (UV) irradiation. Without the addition of a toxic metal catalysis as in CuAAC, and with the low cost of thiol- or alkene-containing molecules, the thiol-ene reaction has many advantages over both CuAAC and SPAAC in bioconjugation chemistry. Moreover, the photoinitiation feature endows the thiol-ene reaction with the capability of spatial and temporal control of functionality on materials, which is useful in the fabrication of patterned substrates or hydrogels. However, there are several side reactions involved, such as the polymerization of alkene which leads to the formation of complex byproduct and oxidation of thiol that generates disulfide.[110] As such, carefully tuning the reaction parameters is essential to obtain product with high yield and conversion.

Parameters that influence thiol-ene reaction efficiency

The yield of thiol-ene reactions is dependent on many aspects, including the reactivity of reagents, reaction media (solvent and pH), amount of additive photoinitiator, wavelength of UV light, and the ratio of alkene to thiol. The adjacent chemical environment of the double bond plays a dominant role in the reaction kinetics. Generally, electron-rich alkenes have higher reactivity and faster kinetics compared with electron-deficient alkenes because the step of the thiyl radical adding to the alkene is an electrophile attack. Specifically, norbornene shows very high reactivity among alkenes due to its bond angle distortion and ring strain. According to the work by Coffey *et al.*, the reactivity of alkenes with methyl mercaptan is norbornene $\geq$ vinyl silane > allyl ether $\geq$ vinyl ether > fumarate > propene > maleimide > methacrylate > crotonate > styrene > acrylonitrile > butadiene.[111] In fact, electron-deficient

alkenes (e.g. maleimide, acrylate) react with thiols through thiol-Michael addition which is another type of widely used "click" reaction described later in this review. Despite the effect of different reagents, solvent also influences the reaction conversion. For example, synthesis of peptide-glucose conjugates in dimethyl formamide leads to a much higher yield than in dichloromethane or dichloroethane.[54] In acidic conditions, pH ~ 4, the disulfide formation is effectively hindered, which affords a higher conversion than in basic conditions, pH ~ 10.[55] The reaction kinetics are also wavelength-dependent; at 254 nm, the rate is faster than that at 365 nm.[112] While a longer wavelength is friendlier to cells and organs, in many cases UV light at 365 nm has been applied to minimize potential photodamage.[113] The reaction time necessitates careful monitoring to achieve complete conversion since long reaction time has led to more oxidized disulfide byproduct.[114]

Peptide-conjugates synthesized by thiol-ene reaction

The thiol-ene reaction has been widely applied in the synthesis of peptide-functionalized conjugates; some representational examples are shown in Fig. 9. The glycopeptide, structure shown in Fig. 9A,[115] was synthesized by reacting an alkene-bearing glucose with a thiol-containing peptide with 80 mol% photoinitiator under 365 nm for 1h. Liquid chromatography–mass spectrometry (LC-MS) confirmed the existence of final product; however, it was difficult to separate from byproducts at higher molecular weight. The Brimble group implemented direct peptide lipidation through thiol-ene coupling, and the generated lipopeptide is an important motif for the design and construction of self-adjuvanting vaccines, as shown in Fig. 9B. In this study, the thiol-ene reaction conditions were carefully tuned and the authors found that by the addition of 1,4-dithiothreitol (DTT) as the chain transfer agent, the final product was obtained with 90% conversion and 95% purity. This is impressive, since use of thiol-ene chemistry to synthesize peptide-conjugates entails multiple potential built-in side reactions.[116] Due to its high efficiency, thiol-ene chemistry has been applied to synthesize multivalent glycopeptide-decorated bovine serum albumin with extremely high molecular weight that can serve as a vaccine by way of a tumor-associated glycopeptide antigen, as shown in Fig. 9C. However, although MALDI-ToF MS analysis indicates the success of obtaining the target molecules, it remains a challenge to separate them from the byproducts.[117] The synthesis of peptide-polymer conjugates through thiol-ene reactions has also been exploited. Post-polymerization modification of poly(allylmethacrylamide) with a thiol-terminated peptide (CVPGVG) under heat was done by Klok and coworkers.[118] Later they used the same strategy to synthesize a series of polyvalent peptide−synthetic polymer conjugates as inhibitors against human immunodeficiency virus-1 (HIV-1) entering into a host cell.[119] The thiol-ene reaction was also utilized as the method to construct and functionalize three-dimensional hydrogel networks as synthetic ECM mimics. Through a thiol-norbornene reaction, a four-arm polymer with norbornene at the chain termini was reacted with a di-thiol peptide and formed a hydrogel within minutes as a result of the fast reaction kinetics.[120] A hydrogel conjugated with a bioactive peptide *via* thiol-ene reaction post-functionalization was implemented by DeForest and Anseth. Precise spatial and temporal control as a result of photoinitiation in thiol-ene chemistry enabled feasible fabrication of bioactive peptide-functionalized patterned hydrogels and gradient hydrogels which are useful to control the biochemical microenvironment of the cell matrix, as shown in Fig. 9D. Moreover, the introduction of a photocleavable group in the peptide chain facilitated the photorelease of bioactive peptides into the surrounding medium, which is potentially useful in controlled drug release.[121] A sophisticated peptide-functionalized surface was fabricated through thiol-ene photochemistry by Niemeyer and Waldmann. A protein microarray with micrometer-sized features (5 – 100 μm) was prepared by applying a photomask during the reaction process of an alkene-presenting surface with a thiol-bearing protein, as shown in Fig. 9E left.[122,123] To further minimalize the structural features of the pattern, they combined a laser source with confocal microscope and directly patterned the peptide with 650 nm line width as shown in Fig. 9E right.[123] These site-specific immobilization techniques provide promising routes to fabricate peptide-functionalized substrates with controlled hierarchical structures.

3.4 Thiol–Michael addition

The thiol–Michael addition is a popular "click" reaction in material chemistry and organic synthesis.[125] In this reaction, a thiol, acting as a nucleophile attacks an alkene linked with electron-withdrawing groups and forms a thiol ester bond in the final product. The reaction happens in a weakly basic aqueous solution at room temperature with fast reaction kinetics and almost quantitative conversion. Compared with CuAAC, the lack of a metal catalyst makes this reaction preferable in situations where metal ions interact with the materials being coupled; compared with thiol-ene reactions, without requiring irradiation this reaction is useful in circumstances where UV light causes damage to materials and cells; compared with SPAAC, it shows higher reaction kinetics, making it more suitable to use for labeling molecular targets *in vivo*.

Parameters that influence thiol-Michael addition reaction efficiency

The main parameters that influence the reactivity of thiol-Michael addition reactions include the structures of the vinyl groups, thiols and any catalyst used.[126] This review focuses on peptide-conjugate synthesis, and in most circumstances the thiol group comes from the cysteine residue. Therefore, the reactivity of different thiols will not be discussed here. However, it is notable that the adjacent amino acid in the peptide sequence does influence the reaction kinetics and conversion. Generally speaking, positive charged amino acids, like arginine, decrease the p*K*a of the neighboring thiol and accelerate the reaction, while negatively charged amino acids, like aspartic acids, show the opposite effect.[127] The reaction kinetics are highly correlated with the structure of electron deficient vinyl groups. The order of reactivity among types of C=C bond in thiol–Michael addition is as follows: maleimide > vinyl sulfone > acrylates / acrylamides > acrylonitrile > methacrylates/methacrylamides.[125] To accelerate the reaction kinetics, amine and phosphine compounds have been applied as basic catalyst or nucleophilic catalyst. Nuleophilic catalysts, such as tri-n-propylphosphine, offer high conversion in faster kinetics compared to basic catalysts, for instance triethylamine.[126] However, in bioconjugation, the use of a catalyst is

not ideal due to solubility and cytotoxicity of the catalyst in the living system. The pH of aqueous solution does influence the reaction kinetics in that basic conditions prompt higher reaction rates, and the common pH used for bioconjugate synthesis is 7-8.5.[128]

Peptide-conjugates synthesized by thiol–Michael addition

Thiol–maleimide reactions are the most effective thiol–Michael addition reactions, proceding with rapid kinetics and quantitative conversion in physiological conditions. It is the most efficient "click" reaction to synthesize large peptide-conjugates with high yield. In Fig. 10, molecule **A**, which is a 36-mer peptide conjugated with a glucose dendrimer, was obtained in 1h with a yield of 82%;[129] Molecule **B**, that is a ^{68}Ga-labeled hexadecameric cRGD-decorated dendron was obtained in 10 min with 32% yield, which is very impressive for making multivalent peptide-functionalized high generation dendrons.[130] Thiol-maleimide addition has become the critical coupling step to synthesize the 23 kD protein shown in Fig. 10C. In this work, the cyclic peptides (22-mer in blue, 22-mer in red and 25-mer in yellow) were linked to the linear template 21-mer peptide (in black) sequentially through thiol-maleimide reaction with quantitative conversion.[131] Peptide-conjugates with natural and synthetic polymers have also been efficiently synthesized using this reaction. For example, a hyaluronic acid–peptide (CWRYMVm) conjugate for formyl peptide receptor with a potential application as a peptide drug has been reported.[132] Another example is using thiol-Michael addition to graft β-sheet peptides onto poly[*N*-(2-hydroxypropyl)methacrylamide] to form a hydrogel in aqueous solution through self-assembly. The bioconjugation efficiency of peptide grafted onto comb-shaped polymers is related to the grafting density in the polymer chain. High grafting density requires a large feed ratio and leads to low conjugation efficiency.[132] This is similar in other studies that synthesize comb-shaped peptide-polymer conjugates and the reason is due to steric hindrance. Polymers with peptides at the chain termini have also been synthesized efficiently through thiol–Michael addition.[133,134] The reaction yield for a 4-armed PEO reacted with Cys-peptide has been reported to be as high as 75% with 99.9% purity.[134] Cell-responsive synthetic hydrogels have been developed *via* thiol-Michael addition. Representative works include multi-armed vinyl sulfone- and diacrylamide-functionlized PEO reacted with cysteine-bearing peptides made by the Hubbell group,[128,135,136] and amphiphilic block copolymers capped with methacrylate or maleimide reacted with a cysteine-presenting RGD peptide by the Gazit group.[137] Among different vinyl groups, maleimide again shows its advantages in the fabrication of bioactive hydrogels because of its high efficiency and rapid kinetics. A bioactive peptide-functionalized poly(ethylene oxide) (PEO) and a heparin hydrogel were prepared through thiol-maleimide addition, as shown in Fig. 10E. The hydrogel formed within a minute and the composition was tunable to contain different types of peptides. Experiments *in vitro* demonstrated its ability to induce morphogenesis of human vascular endothelial cells and dorsal root ganglia.[134] The thiol-maleimide reaction has also been utilized in the fabrication of some peptide-functionalized surfaces.[138-141] In Fig. 10D, maleimide was first immobilized onto the substrate by chemical vapor deposition polymerization. By applying micropattern stamps, a patterned peptide-functionalized surface was achieved, which provided restricted regions for cell attachment. Maleimide-bearing polymer grafted from a Ti surface was used to make a peptide-functionalized surface by Kizhakkedathu *el al.* The peptide density was altered by tuning the grafting density of polymer brushes on the surface, and proved to greatly influence the antimicrobial activity of the peptide-functionalized surface.[141]

3.5 Oxime ligation

Oxime ligation is the condensation reaction between a carbonyl group (aldehyde/ketone) with an aminooxy group, generating a conjugated molecule linked with oxime bond and one molecule of water as byproduct. The advantages of oxime ligation are: i) no metal catalyst is involved; ii) highly selectivity and is generally compatible with other functional groups in biomolecules; iii) almost quantitative conversion; iv) the oxime bond is reversible and pH sensitive. The pH-sensitive stability of oxime ligation makes this method unique among "click" reactions, which is useful to make pH responsive biomaterials. In the pH range of 4 to 8, the oxime bond is quite stable, and apparent decomposition is not observed until the pH goes below 3 or above 9.[142,143]

Parameters that influence oxime ligation reaction efficiency

Generally, when synthesizing peptide-conjugates using oxime condensations, the reaction happens in aqueous solution at pH 4.5-5.5. Under these conditions, the amines along the peptide chain, which may also react with aldehyde/ketone, are protonated, while the aminooxy group serves as a nucleophile to attack the electron-deficient carbon in aldehyde/ketone with high reactivity. The equilibrium constant of oxime ligation is related to pH. Under physiological conditions, it falls in the range of $>10^8$ M^{-1}.[144] The reaction kinetics are also highly related to pH. Faster reaction rates occur in acidic conditions while much slower reaction rates are observed in neutral conditions.[145] In a study by Tam's group, they optimized the pH for oxime ligation to be around 5.[142] However, in some circumstances, like when the peptide or protein is not stable under acidic conditions, the pH of the reaction medium must be close to physiological pH making the oxime ligation reaction very slow. To solve this problem, Dawson's group reported that at neutral conditions, in the presence of 100mM aniline, a peptide in μM concentration could efficiently be labeled with fluorescein in slight excess, while almost no reaction happened without the aniline catalyst under the same conditions.[144] Besides pH sensitivity, the appearance of organic cosolvent, like DMSO was also reported to enhance the reaction rate by 20 fold.[142]

Peptide-conjugates synthesized by oxime ligation

Oxime ligation has been used in the synthesis of peptide-functionalized biomolecules, polymers, hydrogels, NPs and substrates. Peptide-conjugates with small molecules, such as daunorubicin for targeted cancer therapy,[146] glucose as tumor-related antigens (yield 60-70%),[147] ureidopyrimidinone which self-assembled into fibrous structures *via* four-fold hydrogen bonding (yield 33-73%)[148] were obtained with high purity after RP-HPLC purification. More impressing, oxime ligation has been successfully applied to make peptide-conjugates with high molecular mass. Four-

armed peptide-functionalized conjugates have been synthesized using glucose and dendron as frameworks respectively, with purification yields between 15-64%.[130,142,149] Peptide-oligonucleotide conjugates, often used as important therapeutic agents, have been successfully synthesized using oxime ligation with a yield of 45-65%.[143,150,151] Moreover, bis-conjugation of oligonucleotides *via* CuAAC and oxime ligation was realized to make peptide-oligonucleotide conjugates with other bioactive molecules (yield ~ 50%).[152] Because SPPS is generally limited to peptides with 50 amino acid residues or less, the construction of peptides containing large numbers of amino acid residues requires highly efficient conjugation reactions.[153] Oxime ligation has been demonstrated to be able to fulfill this requirement. Sequential oxime ligation has been used to conjugate a 20-mer peptide with another 20-mer peptide, and finally with a 9-mer peptide to finally obtain a protein mimic that showed a distinct immune response against tumor cells (yield ~ 15-25%).[154] More impressively, oxime ligation was adopted in the total synthesis of insulin which is an important therapeutic molecule. In their work, the proinsulin was achieved in 30 min with HPLC purification yield of 21% by incorporating a 25-mer aminooxy-bearing peptide with a 34-mer ketone-functionalized peptide through oxime ligation.[155] From all of the successful synthesis of well-defined large peptide-conjugates with high purity and yield, oxime ligation revealed its abnormal efficiency in making bioconjugates. Oxime ligation has been exploited to synthesize comb-shaped polymers[156] and protein-polymer conjugates.[157] Bioactive hydrogels that are formed by mixing 8-armed aminooxy PEG with glutaraldehyde and ketone-functionalized RGD peptide was reported by Maynard and coworkers recently. Encapsulated cells in the formed hydrogel showed high cell viability and proliferation, demonstrating the non-toxic intrinsic of the oxime hydrogel.[158] Recently, in the Becker group, a systematical study was carried out on the influence of pH and catalyst on the gelation time and mechanical properties of PEG-based hydrogels generated *via* oxime ligation. The gelation time was adjustable from seconds to hours depending on pH and catalyst. As shown in Fig. 11A, hydrogels formed at pH 4.5 are significantly stiffer than those formed at pH 7.4 at similar time intervals. Moreover, azide functional groups and alkene functional groups were incorporated with the aminooxy-bearing crosslinker. Three dimensional patterning of peptides within the hydrogel matrix was achieved by photoinitiated thiol-ene reaction.[40] Oxime ligation has also been employed to functionalize the surface of superparamagnetic iron oxide nanoparticles (SPIONs) with a γ-amino-proline-derived cell penetrating peptide in order to increase the cell uptake of SPIONs as molecular imaging agents and drug carriers, as shown in Fig. 11B.[159] Engineered peptide-functionalized substrates with patterned topography or gradient concentration profiles are an essential type of platform for the study of cell behavior in biomaterials and tissue engineering. Patterned peptide-presenting surfaces were realized by the photo-activation of capped-aminooxy group in the Yousaf group [160] and capped-aldehyde group in the Barner-Kowollik group.[161] After revealing of aldehyde or aminooxy groups on surface with site-specific UV-irradiation, aminooxy or ketone-bearing peptides were immobilized onto the substrate through oxime ligation effectively. The Maynard group reported another approach to preparing patterned peptide-functionalized substrate by electron-beam lithography technique as shown in Fig. 11C. In their study, patterned micro-sized hydrogels with aminooxy groups was fabricated by direct electron-beam etching of the spin coated polymer film on substrate. And then ketone-bearing RGD peptide was immobilized onto the substrate through oxime ligation. Cell culture on the patterned surface demonstrated that the peptide retained its bioactive function, such as increasing cell attachment, and influencing the cell morphology, including cell shape and occupied area.[162]

3.6 Diels-Alder reaction

In organic chemistry, the Diels-Alder reaction refers to a [4+2] cycloaddition that happens between a conjugated diene and a substituted alkene, usually termed as dienophile, and forms a cyclohexene derivative as the product. The reaction is reversible at elevated temperatures. Depending on the structure of the diene and dienophile, the reaction may require heat. In this review, only reactions that occur at room temperature will be discussed because peptides are unstable at high temperatures. Some commonly used reagents are shown in Table 1. The generated product consists of both retro- and stereo- isomers, however this issue is not discussed here since the Diels-Alder reaction is simply serving as an efficient conjugation reaction and the formation of isomers doesn't influence the bioactivity of peptide-conjugates. The advantages of this reaction are: i) no toxic catalyst is needed; ii) high selectivity among functional groups exists in the bio-system, iii) the reaction kinetics can be tuned by applying different reagents. By using the inverse-electron-demand Diels-Alder reaction, the reaction rate constant can be as high as 2000 $M^{-1}s^{-1}$, which is much higher than in all other "click" reactions.[163]

Parameters that influence Diels-Alder reaction efficiency

The chemical structure of dienes and dienophiles dramatically influences the reaction kinetics and conversion. For the traditional Diels-Alder reaction, when the diene is electron-rich and dienophile is more electron-deficient, the reaction rate is faster. Ring-strain also promotes the reaction. However, the Diels-Alder reaction at room temperature usually is not fast. For example, the reaction between diene and maleimide takes 3 days to obtain 80-85% conversion when the reagent is in the mM concentration regime.[164] To accelerate the reaction kinetics, the hetero Diels-Alder (HDA) reaction and the inverse electron demand Diels-Alder (IEDA) reaction, also shown in Table 1, have been developed. In hetero Diels-Alder reaction, an electron-deficient phosphoryl or pyridinyl dithioester is reacted with a suitable diene. When the diene chosen is cyclopentadiene, the reaction can achieve quantitative conversion in 10 min in a μM concentration without additive catalyst.[165] In the IEDA reaction, an electron deficient conjugated double bond in tetrazine is reacted with a strained dienophile, such as norbornene and *trans*-cyclooctene, followed by a retro Diels-Alder reaction, and one molecule of nitrogen is released. As such, this reaction is irreversible upon heating.[163] The kinetics of IEDA reaction is the highest among all "click" reactions. As a result, this kind of reaction has been applied in live cell imaging.[166-168] It is notable that tetrazine also reacts with cyclooctyne,[169] so when combining inverse electron demand Diels-

Alder reaction with SPAAC, the addition sequence of reagents into the system should be carefully considered.

Peptide-conjugates synthesized by Diels-Alder reaction

The Diels-Alder reaction has been applied to synthesize fusion peptides, peptide-imaging agent conjugates, peptide-polymer conjugates, peptide-functionalized hydrogels and patterned surfaces. The ligation reaction condition of diene-peptide with maleimide-peptide was studied by Waldmann *et al.* in order to develop a novel synthetic technology for the site-specific functionalize of peptides and proteins.[170] Different peptide sequences, solvent, and reaction time were tuned carefully, and generally after 1-2 days, the yield after HPLC purification was around 70%.[170,171] This is much higher than commonly used CuAAC and thiol-ene reactions, which is attributed to the fact that metal catalyst is not needed and there are no side reactions in Diels-Alder reactions. One drawback about the diene-maleimide reaction system is that the maleimide functional group also reacts with a thiol group if present in the protein or peptide, and a step of blocking or protecting the thiol groups is required. Peptide-conjugates with imaging agents have also been realized through diene-maleimide Diels-Alder reaction and been served in the strategy of two-step labeling of endogenous enzymatic activities.[164] IEDA reactions have been exploited to synthesize peptide-cancer drug conjugates, when the concentration of two reagents was several μM, the yield was reported to be 98% after 24 hours.[172] Peptide-polymer conjugates synthesized through HDA reaction was carried out by the Barner-Kowollik group.[173] In their work, natural polymer (cellulose) or synthetic polymer bearing a cyclopentadiene reacted with peptide equipped with a thiolamide, respectively, in homogeneous solution or in a heterogeneous system, and generated peptide-decorated polymers. With a very small amount of acid or Lewis acid as catalyst, the conjugation reaction achieved nearly 90% conversion in 2h. Recently, tetrazine-norbornene IEDA reactions have been applied to make peptide-functionalized hydrogels.[174] Thanks to the fast reaction kinetics, the gelation time was around 2 minutes, and norbornene-thiol thiol-ene reaction was followed in order to fabricate patterned hydrogels, which provide several options to moderate the microenvironment that cells live in. Patterned peptide-functionalized surface has also been fabricated based on Diels-Alder reaction combined with electric[175] or light[176] treatment.

3.7 Staudinger ligation

Staudinger ligation is the reaction between an azide and a phosphine compound that forms a native amide bond and a phosphine oxide as the byproduct with release of a molecule of nitrogen. This reaction, developed by Bertozzi and coworkers, originated from the Staudinger reaction,[177-179] and can be conducted in aqueous solution with no metal catalyst, while the azide and phosphine groups rarely appear in natural biological environments. All of these features make the Staudinger ligation a good candidate for synthesizing bioconjugates[180] and for labeling biomolecules in living organisms.[181-183] However, there are some side-reactions that may occur, including oxidation of the phosphine and Staudinger reduction, which may hinder quantitative conversion.[90,184]

Parameters that influence Staudinger ligation reaction efficiency

Staudinger ligation reactions can be divided into two categories, non-traceless and traceless Staudinger ligation, as shown in Table 1. In non-traceless Staudinger ligation, the phosphine oxide is contained in the conjugated product, while in the traceless Staudinger ligation, a simple amide bond is formed between two reagents, avoiding the existence of phosphine oxide in the conjugation molecule. Traceless Staudinger ligation links two biomolecules with an amide bond, making it more favorable to use for synthesis of peptide-conjugates, especially for construction of proteins from several peptide fragments. The reaction kinetics and final yield are determined by the structure of phosphine in traceless Staudinger ligation[185,186] Interestingly, the reaction yield can be above 90% when a glycine residue is present at the nascent junction,[187] while the yield dramatically decreases to below 50% for non-glycyl couplings because of an aza-Wittig reaction.[185,188] However, by slightly tuning the electron density on phosphorus, replacing generally used (diphenylphosphino)methanethiol with (di-*p*-methoxyphenylphosphino)methanethiol, the reaction yield is increased to 80% for non-glycine conjugation.[189] Careful selection of the phosphine for Staudinger ligation is very important in order to obtain the target molecule in high yield. Generally, the reaction kinetics is not high for Staudinger ligation, taking 1-2 days to finish, with a typical of second-order reaction constant in the range of 0.1-7.7 × 10^{-3} M^{-1} s^{-1}. And using a proline-based phosphine template, the reaction kinetics can be accelerated 1000 fold with a second-order reaction constant of 1.12 M^{-1} s^{-1}.[190]

Peptide-conjugates synthesized by Staudinger ligation

After Staudinger ligation was developed, it was soon applied to conjugate peptides together, because the newly formed amide bond is the functional group that commonly links amino acids together in native peptides and proteins. Demonstrations of successfully linking two short peptides (dimers or trimers) together with a traceless Staudinger ligation was reported by Raines and coworkers,[186,191] which showed that Staudinger ligation is a good general method to synthesize protein-conjugates from peptide fragments. The total synthesis of ribonuclease A (containing 124 amino acids) was implemented by a combination of techniques including solid phase peptide synthesis, *m*RNA translation, native chemical ligation, and solid-phase Staudinger ligation.[192] The solid-phase Staudinger ligation which is the reaction between a peptide linked onto a resin with another peptide in solution, lessened the number of purification steps, and the final yield is high (61%).[193] The conjugation of a glycopeptide with another glycolpeptide *via* Staudinger ligation has also been investigated. It is shown that the isolation yield of final glycopeptide depends on the position of the carbohydrate along the peptide chain, with a general yield about 60-70%.[194] In addition to the peptide-peptide conjugates, a cyclic peptide has also been synthesized from a peptide bearing an azide in one end and a borane-protected phosphinothiol on the other through Staudinger ligation with a modest yield (20-36%).[195] Staudinger ligation has also served as a post-functionalization method to decorate polymers with bioactive peptides. For instance, an azide-functionalized poly(lactide)-graft-poly(ethylene glycol) was reacted with an RGD peptide tethered with a phosphine handle in an effort to afford a

biodegradable comb-shaped peptide-polymer conjugate, and the peptide-grafting ratio was well-controlled by the feed ratio.[196] Phosphane-modified substrates have been developed for site-specific immobilization of azido-compounds onto surface through the Staudinger ligation. Waldmann et al. treated carboxylic acid-derivatized glass slides with 2-(diphenylphosphanyl) phenol. Through esterification, substrates presenting phosphane were fabricated, which were subjected to react with azide-bearing molecules. Amide bond formed which covalently linked the small molecule onto surface *via* Staudinger ligation and drug arrays on substrates were generated.[197] Analogous to this strategy, the Raines group carried out site-specific immobilization of protein on substrates based on phosphinothiolester-bounded substrates. After Staudinger ligation, the azide-bearing S-peptide was effective immobilized onto the surface within 1 min. S-protein, which binds with S-peptide, was followed to treat with the surface equipped with S-peptide, and S-protein assay on substrate was achieved. The total conjugation yield of S-protein was 67%. And the enzyme activity was reported to be 92% of their expected activity, which is very impressing for the fabrication of microassays of functional peptides and proteins.[198]

3.8 Native chemical ligation (NCL)

NCL is a reaction that happens between a thiolester and an N-terminal cysteine and results in the formation of an amide bond with a cysteine in the junction part. The reaction proceeds through a reversible transthiolesterification step, and followed by the intramolecular *S, N*-acyl shift generating an amide bond. Generally, the reaction results in high yield at room temperature in aqueous condition near neutral pH after more than 20 hours. Due to its high efficiency, no need of metal catalyst, and formation of a native peptide bond, NCL has been applied as a protocol ligation method for the conjugation of peptide fragments together for protein total synthesis since 1994 after the first demonstration of a successful protein synthesis *via* NCL by Kent and coworkers.[199] Several good reviews on NCL, including reaction mechanisms, parameters that influence reaction kinetics, non-cysteine conjugation and so on, already exist,[39,200,201] so this review will only focus on progress made using NCL after 2008.

Parameters that influence NCL reaction efficiency

The parameters that influence the reaction kinetics and conversion was well summarized by Hackenberger and Schwarzer in their review.[39] Briefly speaking, there are three major parameters, the activity of thiolester, the amino acid located near the thiolester and the reaction medium (pH and buffer system). Alkyl thiolesters are less active than aryl thiolesters, however, the former is less susceptible to hydrolysis and is also easier to handle. Thiolphenol has been used as an additive to catalyze the transthiolesterification and keep the cysteine side chain in a reduced form.[202,203] The impact of the amino acid located near the thiolester is a result of steric hindrance. Reactions with Gly-thiolester proceed much faster than other amino acids.[204] To overcome this problem, the direct oxo-ester peptide ligation method has been developed by using an activated C-terminal para-nitrophenyl ester instead of a thiolester. In this way, NCL between cysteine and bulky C-terminal amino acids can be implemented with high reaction efficiency.[205] The reaction medium also plays a role in the final conversion. Usually a pH ranging from 7.4-8.5 is adequate for the reaction to proceed, and it is reported that different buffer systems also influcence the reaction kinetics.[39,206]

Peptide-conjugates synthesized by NCL

As a very important ligation reaction used to conjugate peptide fragments together, NCL has been applied in the total synthesis of many proteins.[207-210] In this review, we will introduce newer examples of synthesizing peptide-conjugates with other biomolecules, such as carbohydrates, oligonucleotides, polymers and so on. Two new strategies of synthesizing glycopeptides have been developed based on NCL. Wong and coworkers developed sugar-assisted peptide ligation for the convergent construction of glycopeptides, as shown in Fig. 12A.[211] In their study, the thiol group was attached to the glucose moiety in the N-terminal of glycopeptide, which reacted with the thiolester of the C-terminal peptide through transthiolesterification, followed by *S, N*-acyl shift to couple two peptides together. Unlike traditional native peptide ligation, in which the amino acid located near the thiolester of the C-terminal peptide influences the reaction efficiency, in this so called "sugar-assisted peptide ligation", the located position of the thiol group in the glucose and the N-terminal amino acid affects the reaction efficiency, while the C-terminal amino acid near the thiolester does not. The isolation yield was up to 70% for optimized reaction conditions. Scanlan and coworker utilized the cysteine in the ligation junction for thiol-ene reaction with alkene-derivatized glucose and realized the sequential "click" reaction to synthesize glycopeptides.[212] The synthesis of hydrolysis-resistant 3'-peptidyl-RNA conjugates has been implemented through the NCL of a 3'-cysteinyl-RNA and a highly soluble peptide thiolester with isolation yield up to 72%, as shown in Fig. 12B.[213] Peptide-DNA conjugates has also been synthesized *via* NCL, which was proven to maintain the ability to bind with its complementary DNA strand.[214] Multivalent peptide-functionalized dendrons synthesized through NCL was reported by Meijer and coworkers with isolation yields between 40-60%, shown in Fig. 12C, which are potentially useful in disease diagnostics due to its strong and specific binding ability to type I collagen networks in ECM.[15] The *in situ* formation of a hydrogel through NCL without the addition of thiolphenols was not reported until recently by Messersmith, mainly due to the slow reaction kinetics, shown in Fig. 12D.[206] By manipulating the buffer system, reagent concentration and temperature, a PEG-hydrogel was formed within 10 mins and the cysteine residue was subjected to react with a maleimide-bearing peptide in order to obtain a peptide-functionalized hydrogel. Immobilization of peptides onto the surface of thioester-terminated silicon nanowires using NCL was implemented by Coffinier and coworkers, shown in Fig. 12E, which is important for the development of biosensors.[215]

3.9 Other reactions

Beyond the "click" reactions mentioned above, there are other reactions that fulfill the prerequisites of "click" reactions and are also useful in the synthesis of peptide-conjugates, such as the thiol-yne reaction, thiazolidine ligation, and thiol-pyridyl disulfide reaction listed in Table 1. In the thiol-yne reaction, which is very

similar to thiol-ene reaction, an alkyne reacts with two thiols stepwise by anti-Markovnikov addition under UV-irradiation in physiological conditions.[216,217] The advantage of thiol-yne reaction over thiol-ene reaction is that two functional moieties, rather than one, can be introduced into the same site along the peptide chain. Moreover, by carefully controlling the reagent ratio and reaction time, the two functional moieties introduced through thiol-yne reaction can be different, as demonstrated by the Dondoni group who successfully synthesized peptide/protein conjugates with both glucose and fluorophore at the same site *via* thiol-yne reaction.[57,218] Peptide functionalized nanoparticles have also been fabricated with thiol-yne reaction, and further combined with CuAAC for double-click functionalization.[219] The reaction conditions of thiazolidine ligation are very similar to those of oxime ligation, which happens at acidic conditions (pH 4-5), between an aldehyde and N-terminal cysteine, and generates a thiazolidine ring in the conjugated product that is stable between pH 3-9.[142] Applications include synthesis of peptide-functionalized dendrons,[142] peptide-immobilized microchips,[220] and peptide-oligonucleotide conjugates.[143]

4. Orthogonal "click" reactions for construction of multi-functional peptide-conjugates

With so many highly efficient "click" reactions to use, orthogonal "click" reactions can be utilized sequentially or often in one-pot approaches to construct multi-functional peptide-conjugates in an efficient and fast manner for applications in therapeutics, diagnostics, cell behavior studies and tissue engineering. Some pioneering work has been reviewed recently on the use of hetero multi-functional molecular scaffolds to synthesize multi-functional bioconjugates through orthogonal "click" reactions.[221] The advantage of harnessing orthogonal "click" reactions is that multi-functional conjugates can be obtained in a one-pot manner through mild reactions with high yield and without intervening purifications.

Versatile peptide-conjugates synthesized from multi-functional scaffolds through orthogonal "click" reactions were reported (see Table 2). The multi-functional scaffolds or linkages are versatile, from heterofunctional oligoethylene glycol (**14, 15** in Table 2), to biomolecules, such as peptides (**16 - 22** in Table 2) and DNAs (**23** in Table 2) bearing "clickable" tags, to multi-functional NPs and gradient surfaces. Peptide-carbohydrate conjugates have been synthesized through sequential "click" reactions NCL - CuAAC with isolation yield of 49% (**18** in Table 2)[80] and NCL – thiol-ene with isolation yield of 50-60% respectively.[212] Based on a cyclic Lys-rich peptide, the Boturyn and Defrancq groups developed a well-tunable multi-functional scaffold for the synthesis of bioconjugations, including the cRGD/RβAD peptide-tetramer through Oxime – CuAAC with isolation yield of 50-70% (**21** in Table 2),[227] the antiparallel oligonucleotide-tetramer with biotin conjugate through Oxime – CuAAC with isolation yield of 25%.[229] Notably, by adjusting the addition sequence of Lys with "clickable" tags during SPPS, a peptide scaffold (**22** in Table 2) which proceeds triple "click" reactions in a one-pot approach was realized, that is very useful for the synthesis of multi-functional biomacromolecules. Based on molecule **22**, cRGD-tetramer conjugates with biomolecules (peptide/ nucleic acid/ dye) were efficiently synthesized through Oxime – thiol-Michael addition – CuAAC and Oxime – thiol-halogen ligation – CuAAC with isolation yield of 55% and 60% respectively.[228] Peptides bearing two types of "clickable" tags were utilized to fabricate hydrogels with tunable biochemical and biomechanical properties by the Anseth group (**20** in Table 2).[42,60,121,174] In their work, hydrogels formed through one "click" reaction (CuAAC, SPAAC or IEDA reaction) between a 4-arm PEG and multi-functional peptides, which was then subjected to conjugate with other biomolecules *via* thiol-ene reaction. By applying photomasks, patterned and gradient hydrogels were obtained, which is a good platform for the study of cell behaviors in the network with well-defined biochemical and biomechanical properties.

In the Becker group, orthogonal "click" reactions were extensively utilized to fabricate peptide-functionalized tissue engineering scaffolds as well as substrates. In Fig. 13A, a library of amino acid-based poly(ester urea) (PEU) equipped with different "clickable" tags, including alkyne, azide, alkene, tyrosine-phenol and ketone groups, on modified tyrosine amino acids was developed. PEU nanofibers (350-500 nm) were fabricated through electrospinning. The "clickable" functional groups survived the electrospinning process, and are feasible to react with peptides bearing complementary functional groups in "click" reactions. Fluorescein-labeled peptides were conjugated onto the surface of PEU nanofibers in aqueous solution with fast kinetics, through CuAAC, thiol-ene, oxime ligation and ene-type addition, respectively, as shown in Fig. 13A. Because PEU is a degradable polymer with non-toxic degradation products, this facile fabrication of peptide-functionalized nanofiber scaffold shows promise in regenerative medicine applications. A series of poly(caprolactone) (PCL) with multiple "clickable" tags, including ketone, alkyne, azide, and methyl acrylate was developed in the Becker group as well. The synthesis of this multi-functional PCL is quite simple as shown in Fig. 13B. By introducing 2-oxepane-1,5-dione monomer, ketone functional groups were introduced in the PCL, which are subjected to react with aminooxy-bearing small molecules *via* oxime ligation to transfer from ketone to other "clickable" functional groups. Three "click" reactions, thiol-Michael addition – SPAAC – CuAAC took place sequentially between derivatized PCL thin films and peptides, and the process was monitored using quartz crystal microbalance (QCM). From the experimental results, thiol-Michael additions were found to occur prior to SPAAC or CuAAC to achieve total conversion of alkene groups. A two-dimensional orthogonal concentration gradient substrate presenting azide and alkene functional groups were fabricated through vapor deposition method, as shown in Fig. 13C. Metal-free "click" reactions, thiol-ene and SPAAC, was carried out sequentially, to immobilize different peptides onto the substrates. By applying approach to gradient substrates and dual "click" reactions, two types of peptide-functionalized substrates with confined concentration gradient were prepared efficiently and reproducibly. These materials are incredibly helpful in studying the cooperative and synergistic effects between cells and concentration regimes of the respective peptides.

So far, orthogonal "click" reactions have been applied to fabricate multi-functional peptide-conjugates in a more efficient manner, with no need for intervening purification steps, without protecting functional groups, with high reaction yield and in a one-pot approach. Some guidelines should be followed for choosing suitable

reaction types and arranging the sequence of reactions. First, no cross reactions occur between chosen functional groups. In Table 3, reactions between the mostly-used functional groups are summarized. It is critical that the added molecules with certain functional group only react with one of the functional groups in the multi-functional scaffolds. For example, if the molecules with cyclooctyne were added to the system, which is going to react with the azide groups through SPAAC, then the other functional groups in the system shouldn't include thiol and tetrazine, which will also react with cyclooctyne.[100,169] The second concern is the stability of functional groups in the reaction medium. For example, aldehydes are not stable under CuAAC condition,[232] and thiols are oxidized with the presence of copper.[228]

5. Conclusion and Outlook

Proteins serve essential roles in maintaining and developing physiological functions, from supporting tissues and organs like collagen in ECM, to regulating various biological processes, like growth factors that guide the cell adhesion, proliferation, migration, differentiation and apoptosis.[233-235] In most cases, specific peptide subunits of whole proteins contribute specific bioactive functions. Often when cleaved, the functional peptide subunit maintains its bioactivity. Meanwhile, applying the bioactive peptide to a synthetic conjugate instead of the whole protein has several advantages. Automatic microwave-assisted SPPS makes it easy and fast to obtain peptides containing up to 50 amino acids, while whole proteins are typically obtained through recombinant DNA technology.[236] For example the synthesis of thymosin, a 28-mer peptide, with a modern peptide synthesizer only took less than two and half hours, with the purity of crude product up to 60%.[237] The biological role of a defined peptide is specific; in contrast, because of multiple domains contained in a protein, the interactions between proteins and living systems are more complicated. Due to hierarchical structure, proteins are more susceptible to denatureation and consequently lose their bioactivity during conjugation reactions; while the bioactivity of peptides is preserved as long as the primary sequence is not disrupted. Lastly, peptides are feasible to be linked with "clickable" tags at specific locations, while the conjugation of whole proteins with site specificity is harder, because there are so many amino acid residues in the whole protein. In short peptide will continue to play pivotal roles in the development of advanced functional materials with enhanced biological performance.

The toolbox of "click" reactions provides a set of high efficient reactions for bioconjugation stategies. With the exception of thiol groups, the functional groups in the "click" reactions do not exist naturally in biological system, which limits their cross reactivity and results in highly selective products. Due to the difficulties in purification of high molecular mass peptide-conjugates, high conversions within these "click" reactions are essential for the successful synthesis of peptide-bearing macromolecules. As summarized in this paper, CuAAC, SPAAC, thiol-ene, thiol-Michael addition, oxime ligation and other "click" reactions have been employed in construction of peptide-functionalized materials solely in bio-imaging, targeted drug delivery, diagnostics and regenerative medicine. Generally speaking, the "click" reactions are good candidates for the build-up of modular peptide-functionalized materials; however, careful considerations are required due to the restrictions of starting materials, reaction conditions, and reaction rates. For example, CuAAC is not suitable in the synthesis of peptide-bearing QDs or other inorganic compounds, because the interference of Cu(I) with those inorganic starting materials. The *in situ* labeling of specific biomolecules in living beings requires rapid reaction kinetics and low dose-usage, so the inverse-electron-demand Diels-Alder reaction with fast reaction kinetics, high specificity and semi-equimolar dosage is preferred over other "click" reactions. Moreover, each type of "click" reaction comes with its own characteristics which are useful in the construction of different materials. For instance, Cu(I) is the catalyst in CuAAC, which can be generated locally by electrochemistry, and this provides a method to fabricate patterned substrates. The light triggered reactions, such as thiol-ene and thiol-yne reactions, are also convenient methods to prepare substrates and scaffolds with spatial and temperal control. Due to the reversible formation of oxime bond, the oxime ligation is an ideal synthetic methodology for the fabrication of pH-responsive materials. Traceless Staudinger ligation and native chemical ligation both link two modules with a peptide bond, making them favorable in the total synthesis of proteins.

In additional to developing new types of "click" reactions, we believe combinational usage within the already known "click" reaction toolbox, namely, using orthogonal "click" reactions in one-pot or a sequential approach, is important for the construction of multi-functional materials. In this manner, each conjugation step is highly efficient under mild conditions, which results in high total conversion and limited need for intervening purification steps. In the future, the "click" reaction toolbox will continue to serve as the most efficient, convenient and robust synthetic methods for the synthesis of peptide-functionalized materials required for future efforts to mimic the synergistic regulation of biological process in living systems.

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Notes and references

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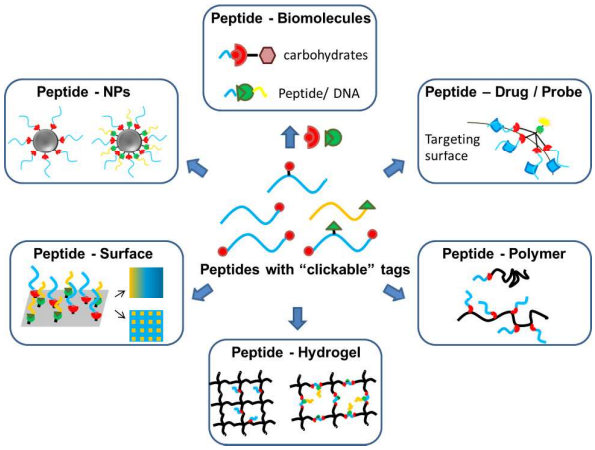
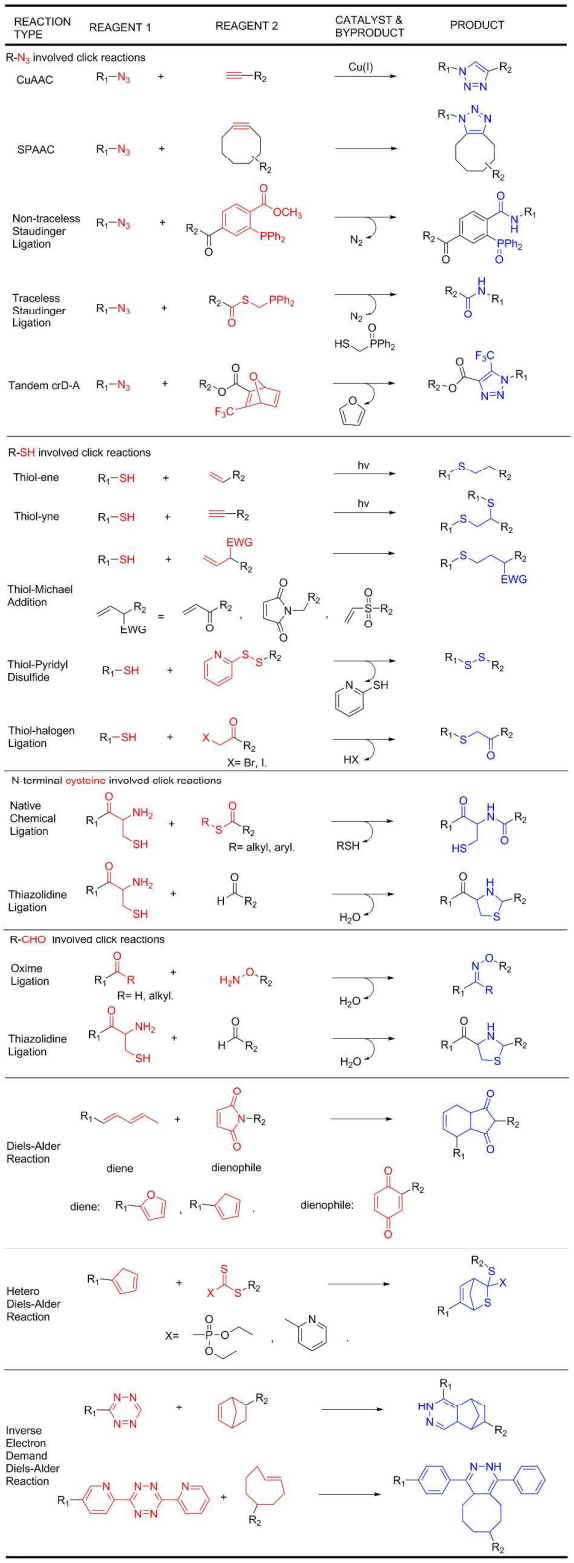


Fig. 1 Versatile types of peptide conjugates with biomolecules, drugs/detective probes, polymers, hydrogels, NPs and 2-D surfaces synthesized by orthogonal "click" reactions.

Table 1 Summary of click reactions that used to synthesize peptide-conjugates.[a,b]



[a] Without further indication, the reaction medium of all those reactions is physiological buffer at ambient temperature.

[b] CuAAC: Cu(I)-catalyzed azide-akylene cyclooaddition ; SPAAC: strain promoted azide-alkene cycloaddition; tandem crD-A: tandem [3+2] cycloaddition-retro-Diels-Alder reaction.[35]

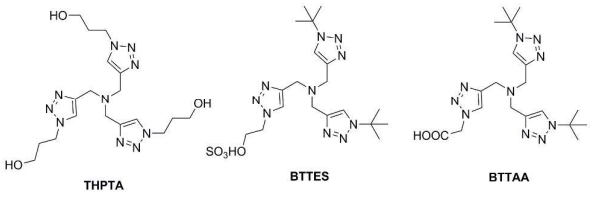


Fig. 2 CuAAC-accelerating ligands used to increase reaction kinetics and conversion. The chemical structure of the respective ligands: tris(3-hydroxypropyltriazolylmethyl)amine (THPTA), 2-[4-{(bis[(1-tert-butyl-1H-1,2,3-triazol-4-yl)methyl]amino)methyl}-1H-1,2,3-triazol-1-yl]acetic acid (BTTAA), and 2-[4-{(bis[(1-tert-butyl-1H-1,2,3-triazol-4-yl)methyl]amino)-methyl}-1H-1,2,3-triazol-1-yl]ethyl hydrogen sulfate (BTTES).

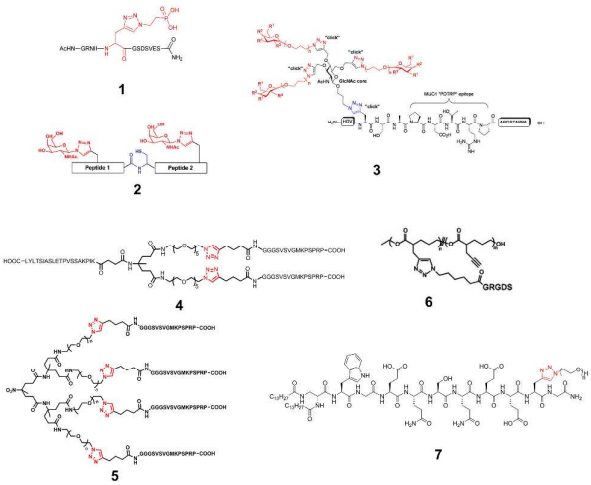


Fig. 3 Peptide-conjugates with biologically relevant small molecules and polymers that were synthesized using CuAAC. **1**: nucleoside diphosphate kinase phosphocarrier domain. Reprinted from Yang *et al.*[79] with permission from the American Chemical Society. Copyright (2011). **2**: neoglycopeptide. Reprinted from Lee *et al.*[80] with permission from the American Chemical Society. Copyright (2009). **3**: multivalent neoglycopeptide conjugate. Reprinted from Lee *et al.*[81] with permission from the American Chemical Society. Copyright (2012). **4**: dendrimeric peptide conjugates containing a BMP-2 peptide (20-mer) and two HA-binding peptides (15-mer). Reprinted from Tang *et al.*[36] with permission from the American Chemical Society. Copyright (2013). **5**: tetrameric HA-binding peptide-functionalized dendron. Reprinted from Tang *et al.*[36] with permission from the American Chemical Society. Copyright (2013). **6**: peptide-grafted aliphatic polyester. Reprinted from Parrish *et al.*[46] with permission from the American Chemical Society. Copyright (2005). **7**: poly(ethylene glycol) (PEG) containing lipopeptide. Reprinted from Jølck *et al.*[82] from the American Chemical Society. Copyright (2013).

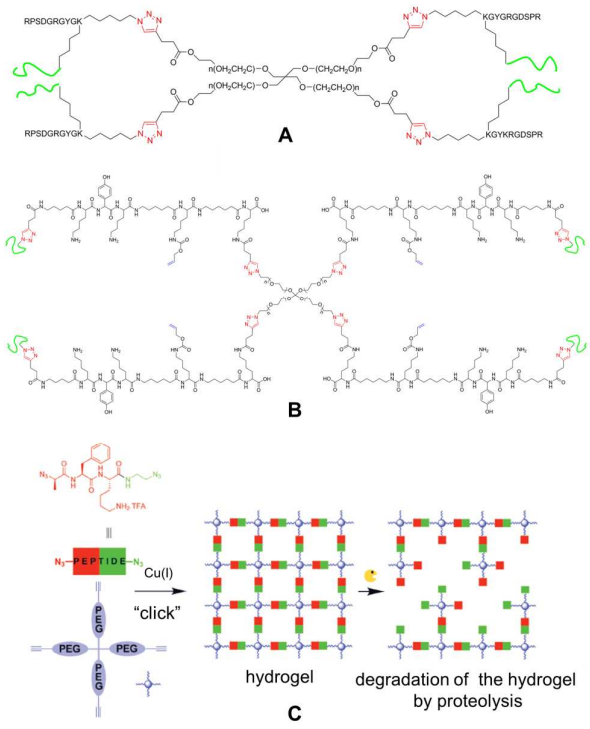


Fig. 4 Peptide-functionalized hydrogels that formed through CuAAC. (A) 4-armed alkyl-PEO reacted with RGD peptide bearing two azide in N-terminus. Adapted from Liu *et al.*[43] with permission from Elsevier. Copyright (2009). (B) 4-armed azido-PEO reacted with peptide bearing two azide in N- and C-terminus. Adapted from Polizzotti *et al.*[37] with permission from the American Chemical Society. Copyright (2008). (C) 4-armed alkyl-PEO reacted with peptide bearing two azide in N- and C-terminus. Adapted from van Dijk *et al.*[83] with permission from American Chemical Society. Copyright (2010).

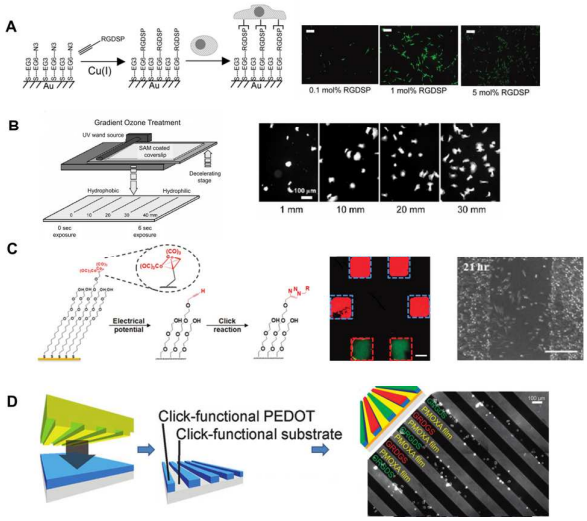


Fig. 5 Peptide-functionalized surfaces for cell behavior study by CuAAC. (A) self-assembly monolayer (SAM) bearing the RGD peptide with tunable surface density (scale bar: 200 mm). Reprinted from Hudalla *et al.*[84] with permission from the American Chemical Society. Copyright (2009). Gallant (B) gradient alkyne bearing surface generated through gradient ozone treatment for the fabrication of concentration gradient the RGD

peptide-functionalized surfaces. Reprinted from Gallant *et al.*[29] with permission from John Wiley and Sons publishing. Copyright (2007). (C) SAMs with on-demand activation of masked alkyne functional groups for the preparation of patterned peptide-modified surface. Left: schematic representations of surface fabrication. Middle: Fluorescence images of surface decorated with two kinds of different fluorescein (scale bar: 25 μm). Right: cell migration study on patterned the RGD peptide-functionalized surface (scale bar: 250μm). Reprinted from Choi *et al.*[85] with permission from the American Chemical Society. Copyright (2011). (D) strip-patterned substrate that selectively functionalized with different peptide sequences. Reprinted from Lind *et al.*[86] with permission from the American Chemical Society. Copyright (2012).

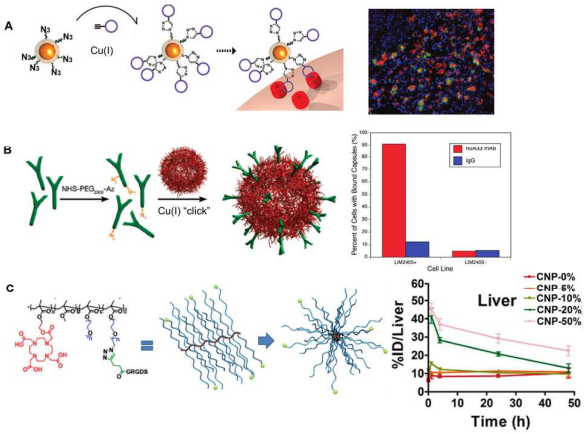


Fig. 6 Peptide-functionalized NPs synthesized using CuAAC. (A) Cyclic LyP-1 targeting peptides-decorated iron oxide NPs. Reprinted from von Maltzahn *et al.*[27] with permission from the American Chemical Society. Copyright (2008). (B) Antibodies-functionalized nanoengineered polymer capsule prepared through CuAAC. Reprinted from von Kamphuis *et al.*[87] with permission from the American Chemical Society. Copyright (2010). (C) Polymer NPs with tunable composition of diagnostic/imaging units and the RGD peptide prepared through block copolymer self-assembly. Reprinted from Shokeen *et al.*[28] with permission from the American Chemical Society. Copyright (2011).

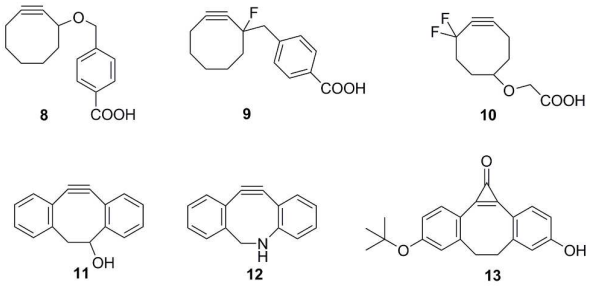


Fig. 7 Chemical structures of cyclooctynes frequently used in SPAAC. The reactivity to azide is **8** < **9** < **10** ≈ **11** < **12**. Under UV irradiation, **13** generates dibenzocyclooctyne which is useful for the fabrication of patterned surface. **8**: cyclooctyne,[88] **9**: fluorinated cyclooctyne,[90] **10**: difluorinated cyclooctyne (DIFO),[89] **11**: dibenzocyclooctyne (DIBO),[91] **12**: Aza-dibenzocyclooctyne (ADIBO),[101,102] **13**: cyclopropenone-masked dibenzocyclooctyne.[103]

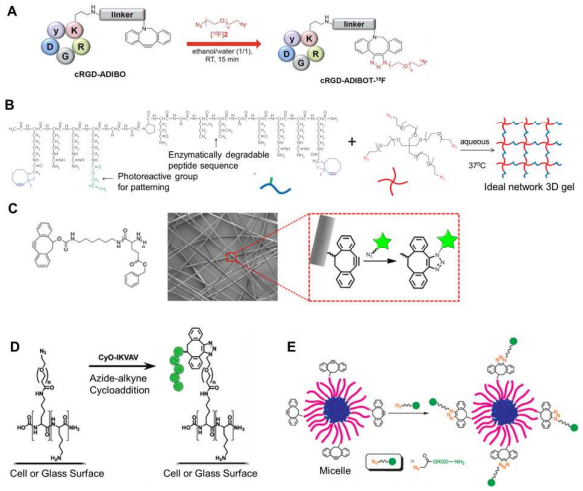


Fig. 8 Peptide-conjugates synthesized using SPAAC. (A) ^{18}F-labeled peptide for PET molecular imaging and diagnosis. Reprinted from Sachin *et al.*[106] with permission from the American Chemical Society. Copyright (2012). (B) Peptide-functionalized hydrogel formed within 1h in aqueous solution. Reprinted from DeForest *et al.*[42] with permission from Nature publishing group. Copyright (2009). (C) DIBO-bearing electrospun nanofibers that is facile to be immobilized with peptides. Reprinted from Zheng *et al.*[94] with permission from the American Chemical Society. Copyright (2012). (D) IKVAV peptide-functionalized cytocompatible polymer for decoration of cell surface and fabrication of peptide microarray. Reprinted from Krishnamurthy *et al.*[108] with permission from the American Chemical Society. Copyright (2010). (E) RGD-functionalized micelles for drug delivery. Reprinted from Guo *et al.*[109] with permission from John Wiley and Sons publishing. Copyright (2010).

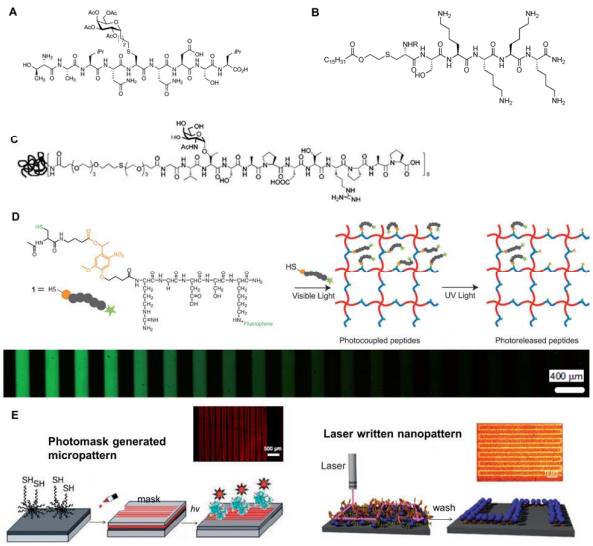


Fig. 9 Peptide-conjugates synthesized using thiol-ene reaction. (A) Glycopeptide. Reprinted from Dondoni *et al.*[115] with permission from John Wiley and Sons publishing. Copyright (2009). (B) Lipopeptide. Reprinted from Wright *et al.*[124] with permission from John Wiley and Sons publishing. Copyright (2013). (C) Multivalent glycopeptide-decorated bovine serum albumin. Reprinted from Wittrock *et al.*[117] with permission from John Wiley and Sons publishing. Copyright (2007). (D) Post-functionalization of hydrogel with photocleavagable peptide and the generation of patterned and gradient hydrogels. Reprinted from DeForest *et al.*[121] with permission from John Wiley and Sons publishing. Copyright (2012). (E) Microarrey of peptide-presenting surface prepared by applying a

photomask (left), and direct writing (right) of nanopatterns by combination of a laser source with confocal microscope (650 nm in width). Reprinted from Jonkheijm *et al.*[123] with permission from John Wiley and Sons publishing. Copyright (2008).

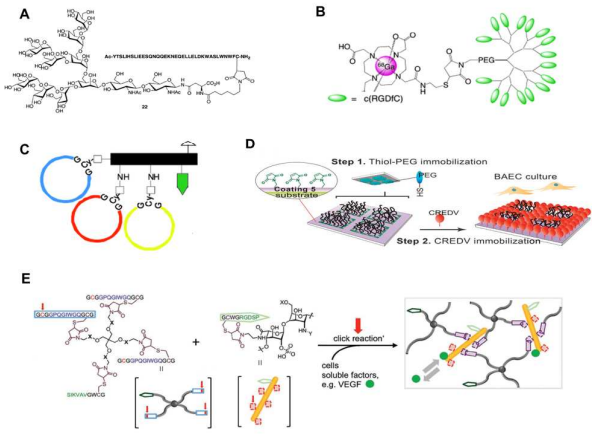


Fig. 10 Peptide-conjugates synthesized using thiol-Michael addition. (A) Glycosylation of a 36-mer peptide that is a potent inhibitor of HIV infection. Reprinted from Ni *et al.*[129] with permission from the American Chemical Society. Copyright (2002). (B) ^{68}Ga-labeled hexadecameric cRGD functionalized dendrimer that acts as PET imaging probe targeting to tumor cells. Reprinted from Wängler *et al.*[130] with permission from John Wiley and Sons publishing. Copyright (2010). (C) A synthetic 23-kDa protein that mimics the ligand-binding extracellular part of a G-protein-coupled receptor. Reprinted from Pritz *et al.*[131] with permission from John Wiley and Sons publishing. Copyright (2008). (D) Peptide-patterned surface formed by reacting PEO-SH and CREDV peptide with maleimide-functionalized poly-*p*-xylylene coating on various substrates that are readily able to manipulate attachments and growth of bovine arterial endothelial cells. Reprinted from Tsai *et al.*[140] with permission from The Royal Society of Chemistry publishing group. Copyright (2012). (E) PEO-peptide and heparin-peptide conjugates obtained via thiol-maleimide reaction and formed cell-instructive hydrogel matrices through thiol-maleimide reaction as well. Reprinted from Tsurkan *et al.*[134] with permission from John Wiley and Sons publishing. Copyright (2013).

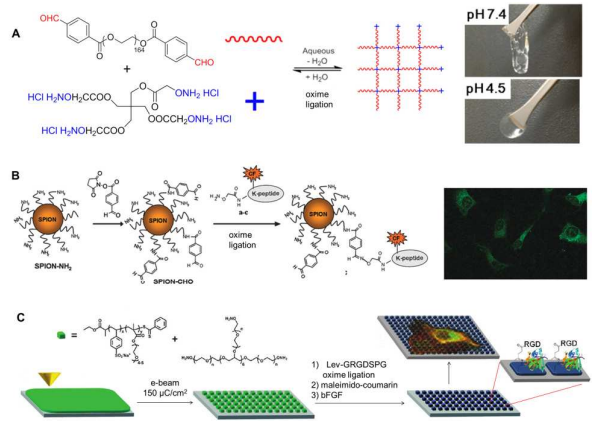


Fig. 11 Peptide-conjugates synthesized via oxime ligation. (A) Hydrogel formation by mixing PEG end-capped with aldehyde with 4-arm aminooxy crosslinker. Reprinted from Lin *et al.*[40] with permission from the American

Chemical Society. Copyright (2013). (B) Modification of superparamagnetic iron oxide nanoparticles (SPIONs) with γ-amino-proline-derived cell penetrating peptides. Reprinted from Cavalli *et al.*[159] with permission from The Royal Society of Chemistry publishing group. Copyright (2012). (C) Patterned peptide-functionalized surfaces fabricated by electron-beam lithography. Reprinted from Kolodziej *et al.*[162] with permission from the American Chemical Society. Copyright (2011).

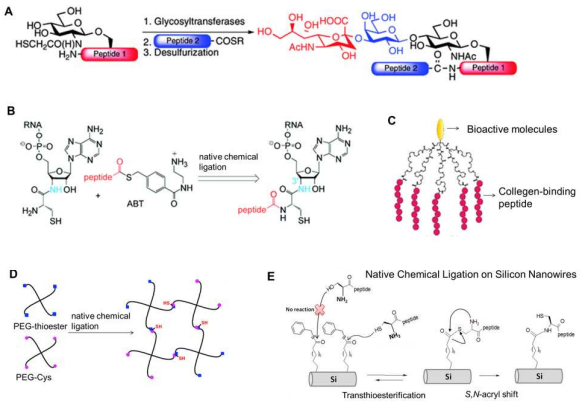
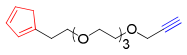
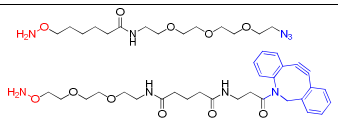
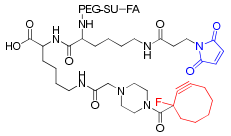
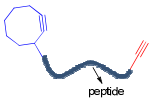
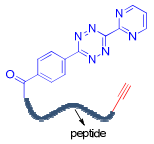
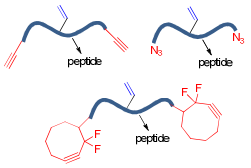
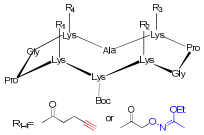
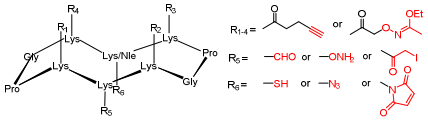
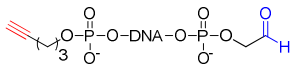


Fig. 12 Peptide-conjugates synthesized using native chemical ligation. (A) Sugar-assistant peptide ligation for the convergent construction of glycopeptide. Reprinted from Bennett *et al.*[211] with permission from the American Chemical Society. Copyright (2008). (B) Hydrolysis-resistant 3'-peptidyl-RNA conjugates. Reprinted from Geiermann *et al.*[213] with permission from the American Chemical Society. Copyright (2011). (C) A pentavalent peptide-functionalized dendron which strongly and specifically binds with collegen type I. Reprinted from Helms *et al.*[15] with permission from the American Chemical Society. Copyright (2009). (D) Formation of hydrogel through NCL. Reprinted from Hu *et al.*[206] with permission from the American Chemical Society. Copyright (2009). (E) Immobilization of peptides onto the surface of thioester-terminated silicon nanowires. Reprinted from Dendane *et al.*[215] with permission from the American Chemical Society. Copyright (2012).

Table 2. Multifunctional scaffolds/linkages for the synthesis of peptide-conjugates by orthogonal "click" reactions.

#	Multifunctional Scaffolds/ Linkages	Sequence of "Click" Reactions	Product	Ref.
14		D-A reaction – CuAAC stepwise	Carbohydrate and protein immobilized surfaces	222
15		Oxime – SPAAC stepwise	A protein-protein conjugate	223
16		Thiol-Michael addition – IEDA reaction Thiol-Michael addition – SPAAC	A protein-drug conjugate	224
17		SPAAC – CuAAC stepwise	A peptide-fluorophore-SiNPs conjugate	225
18		NCL – CuAAC stepwise	A peptide-glucose conjugate	80
19		IEDA reaction – CuAAC stepwise	A peptide-fluorophore conjugate	226
20		SPAAC – Thiol-ene stepwise CuAAC-Thiol-ene stepwise	A peptide-crosslinked hydrogel with patterned peptide functionality	42, 60, 121.
21		Oxime – CuAAC: stepwise and in a one pot approach	A heteropeptide-tetramer conjugates	227
22		Oxime – thiol-Michael addition – CuAAC in a one pot approcach Oxime – thiol-halogen ligation – CuAAC in a one pot approach	A cRGD-tetramer conjugate with biomolecules	228
23		Oxime-CuAAC in a one pot approach	An oligonucleotides-conjugate with biomolecules	152

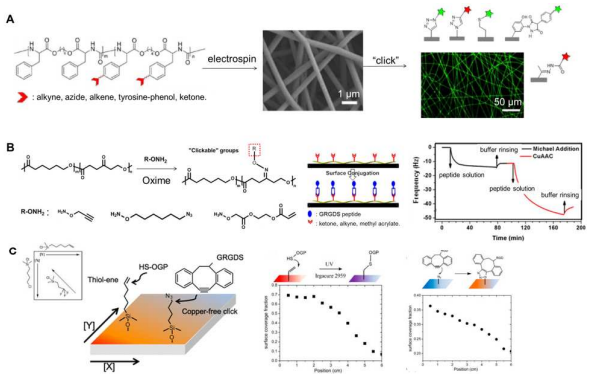


Fig. 13 Multiple peptide-functionalized biomaterials synthesized through orthogonal "click" reactions. (A) Postelectrospinning "click" modification of degradable aminoacid-based poly(ester urea) nanofibers. Reprinted from Lin *et al.*[230] with permission from the American Chemical Society. Copyright (2013). (B) Cascading "triclick" functionalization of poly(caprolactone) thin films quantified *via* a quartz crystal microbalance. Reprinted from Lin *et al.*[38] with permission from the American Chemical Society. Copyright (2013). (C) Fabrication of "dual click" two-dimensional orthogonal peptide concentration gradients. Reprinted from Ma *et al.*[231] with permission from the American Chemical Society. Copyright (2013).

Table 3. "Click" reactions happening between commonly used functional groups.

	$R-N_3$				R−SH		R−CHO	$R-ONH_2$						
$R-N_3$	--	--	Cu(I): CuAAC	SPAAC	--	--	--	--	--	--	--	--	Staudinger Ligation	--
	--	--	--	--	*hv*: Thiol-ene	*hv*: Thiol-ene	--	--	--	--	--	--	--	--
	Cu(I): CuAAC	--	--	--	*hv*: Thiol-yne	*hv*: Thiol-ene	--	--	--	--	--	--	--	--
	SPAAC	--	--	--	Thiol-yne	Thiol-yne	--	--	--	--	--	IEDA Reaction	--	--
R−SH	--	*hv*: Thiol-ene	*hv*: Thiol-yne	Thiol-yne	--	--	--	--	Thiol-Michael Addition	*hv*: Thiol-ene	*hv*: Thiol-ene	--	Thioester Exchange	Thiol-Pyridyl Difulfide
	--	*hv*: Thiol-ene	*hv*: Thiol-yne	Thiol-yne	--	--	pH=4-5: Thiazolidine Ligation	--	Thiol-Michael Addition	*hv*: Thiol-ene	hv: Thiol-ene	--	Thioester Exchange	Thiol-Pyridyl Difulfide
R−CHO	--	--	--	--	--	pH=4-5: Thiazolidine Ligation	--	pH 4.5: Oxime Ligation	--	--	--	--	--	--
$R-ONH_2$	--	--	--	--	--	--	pH 4.5: Oxime Ligation	--	--	--	--	--	--	--
	--	--	--	--	Thiol-Michael Addition	Thiol-Michael Addition	--	--	--	--	D-A Reaction	--	--	--
	--	--	--	--	*hv*: Thiol-ene	*hv*: Thiol-ene	--	--	--	--	--	IEDA Reaction	--	--
	--	--	--	--	*hv*: Thiol-ene	*hv*: Thiol-ene	--	--	D-A Reaction	--	--	--	--	--
	--	--	--	IEDA Reaction	--	--	--	--	--	IEDA Reaction	--	--	--	--
	Staudinger Ligation	--	--	--	Thioester Exchange	Thioester Exchange	--	--	--	--	--	--	--	--
	--	--	--	--	Thiol-Pyridyl Difulfide	Thiol-Pyridyl Difulfide	--	--	--	--	--	--	--	--

[a] unless otherwise mentioned, the reaction condition is able to proceed in physiological condition. The required catalyst or light irradiation is noted before reaction type.

[b] the abbreviation for reactions are as following: Cu-catalyzed azide-alkyl cycloaddition (CuAAC), string-promoted azide-alkyl cycloaddition (SPAAC), Diels-Alder (DA) reaction, and inverse-electron-demand Diels-Alder (IEDA) reaction.