Clicking Biomacromolecules

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Reliable and Efficient Procedures for the Conjugation of Biomolecules through Huisgen Azide–Alkyne Cycloadditions

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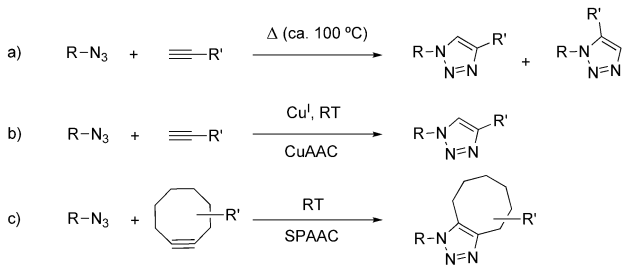
biomacromolecules · click chemistry · synthetic methods

The Cu^I-catalyzed azide–alkyne cycloaddition (CuAAC) has been established as a powerful coupling technology for the conjugation of proteins, nucleic acids, and polysaccharides. Nevertheless, several shortcomings related to the presence of Cu, mainly oxidative degradation by reactive oxygen species and sample contamination by Cu, have been pointed out. This Minireview discusses key aspects found in the development of the efficient and benign functionalization of biomacromolecules through CuAAC, as well as the Cu-free strain-promoted azide–alkyne cycloaddition (SPAAC).

1. Introduction

In 2001, Kolb, Finn, and Sharpless introduced the concept of click chemistry in the field of drug discovery as a series of remarkable thermodynamic and orthogonal processes for the efficient transformation of highly energetic building blocks into easily accessible drug candidates.[1] Actually, behind the concept of click chemistry lies a group of mild, high-yielding, reliable, and clean transformations of broad scope that usually require simple or no purification. Since that seminal report, the click concept has been rapidly applied in many other areas of research that rely on easy and efficient coupling technologies.

The Huisgen 1,3-dipolar azide–alkyne cycloaddition (AAC, Scheme 1) has been recognized as the greatest exponent among the entire collection of click reactions currently proposed.[2] In its classical thermal version (Scheme 1a), this chemical transformation is characterized by high reliability and broad tolerance to diverse reaction conditions and functional groups. In addition, as neither azides nor alkynes are generally present in nature, the AAC is characterized by a unique bioorthogonality. Nevertheless, it was not until the discovery of the Cu^I-catalyzed variant of this reaction by the groups of Meldal,[3] and Fokin and Sharpless[4] (CuAAC, Scheme 1b) that the actual potential of this coupling technology was unveiled. Cu^I catalysis not only significantly reduces the activation barrier of the cycloaddition with terminal alkynes (enabling the reaction to proceed with high rate at room temperature), but it also leads to 1,4-disubstituted 1,2,3-triazoles exclusively.[5] As a result of this discovery, applications of AAC now extend far beyond organic synthesis to further challenging goals in chemistry, polymer science, and biology.[6]



Scheme 1. Thermal (a), Cu^I-catalyzed (b), and strain-promoted (c) azide–alkyne cycloadditions.

However, the application of CuAAC to bioconjugation has not been straightforward. The required Cu^I catalyst can induce severe structural damage to biomolecules, while CuAAC reactions are often too slow at the low micromolar concentrations typically required for bioconjugation purposes. The aim of this Minireview is to analyze the difficulties encountered and the proposed solutions for the efficient and benign in vitro functionalization of biomacromolecules by CuAAC. Special attention will be also paid to the more recently developed strain-promoted azide–alkyne cycloaddi-

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tion (SPAAC, Scheme 1c), a convenient Cu-free AAC originally proposed by Bertozzi and co-workers.[7]

2. Efficient and Benign CuAAC Bioconjugation Procedures Developed for Proteins and Bionanoparticles

Some of the limitations of CuAAC for the functionalization of biomacromolecules were soon pointed out by Fokin, Sharpless, Finn and co-workers, who reported that cowpea mosaic virus (CPMV) either degraded or aggregated under the originally reported CuAAC conditions ($CuSO_4$/sodium ascorbate or $CuSO_4$/Cu wire).[8] The disassembly of the viral capsid was ascribed to coordination of the newly formed triazole linkages to Cu^{II} ions. Thus, incubation of a triazole-containing CPMV (obtained by AAC without a catalyst) with Cu^{II} (or Cu^{I} under aerobic conditions) led to virus degradation, whereas wild-type CPMV remained intact under identical conditions. CPMV also degraded in the presence of reducing agents such as ascorbate or *p*-hydroquinone employed for the in situ reduction of Cu^{II}. In addition, the use of Cu^{II}/Cu as a catalytic system led to high levels of virus aggregation, which was also attributed to the presence of Cu^{II}, since aggregates were broken up after addition of ethylenediamine tetraacetic acid.

The contribution of Cu to oxidative stress in biomacromolecules is well-known.[9] Cu ions readily promote the generation of reactive oxygen species (ROS), which are ultimately responsible for biological damage. The production of the hydroxyl radical (•OH), the most destructive of these ROS, is mediated by a Fenton reaction involving the reduced form of a transition metal, Cu^{I} in the case of CuAAC, and H_2O_2 [Eq. (1)].[10,11] The required H_2O_2 is formed in situ by

$$Cu^{I} + H_2O_2 \rightarrow Cu^{II} + {}^{\bullet}OH + OH^{-} \quad (1)$$

two possible mechanisms: in the presence of ascorbate ($AscH_2$), O_2 can be reduced to H_2O_2 in a process catalyzed by traces of Cu^{II} or other transition-metal ions [Eq. (2)];[10,12]

$$AscH_2 + O_2 \rightarrow Asc + H_2O_2 \quad (2)$$

alternatively, in the absence of ascorbate or other reducing agents, Cu^{I} itself can reduce O_2 to H_2O_2 through a two-step process involving the superoxide anion radical ($O_2^{\bullet-}$) as an intermediate [Eqs. (3) and (4)].[13] The presence of ROS

$$Cu^{I} + O_2 \rightarrow O_2^{\bullet-} + Cu^{II} \quad (3)$$

$$Cu^{I} + O_2^{\bullet-} + 2H^{+} \rightarrow Cu^{II} + H_2O_2 \quad (4)$$

during the functionalization of biomolecules such as proteins, nucleic acids, polysaccharides, and lipids is a major concern, as the structural and functional integrity of these substrates might be severely compromised. In the case of proteins ROS are known to induce degradation of amino acids and cleavage of the polypeptide chain;[14] this has also been observed under CuAAC conditions.[15]

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Ricardo Riguera received his PhD in Chemistry from USC in 1973 under the supervison of Prof. I Ribas, and carried out postdoctoral studies at University College London with Prof. P. Garratt (1974). He was appointed Lecturer in 1978, and in 1990 he became Full Professor of Chemistry at USC. His research interests include bioactive natural products, medicinal chemistry, and NMR methods for determination of absolute configuration. He is currently interested in polymeric nanostructures for bioapplications as well as stimuli-responsive dynamic polymers.

Eduardo Fernandez-Megia completed a PhD in Chemistry in 1995 at USC under the supervision of Prof. F. J. Sardina. After a postdoctoral stay with Prof. Steven V. Ley at the University of Cambridge (1997–1999), he returned to USC as a Marie Curie Fellow and Prof. Asociado. Thereafter he became a Ramon y Cajal Fellow (2003), was installed as Prof. Contratado Doctor (2008), and was appointed Profesor Titular (2009) at USC. His research focuses on the interface between organic and polymer chemistry with emphasis on the preparation of well-defined polymeric nanostructures for biomedical applications and the development of NMR tools for their characterization.

The formation of diacetylenes through Cu^{I}-catalyzed Glaser homocoupling of terminal alkynes is another side reaction that has been observed under CuAAC conditions.[16] Altogether, these secondary processes diminish the efficiency of the conjugation, and are particularly undesired in the functionalization of macromolecular platforms, as purifications are problematic or impossible.

In a general sense, the limitations of CuAAC can be efficiently overcome with the proper selection of the catalytic system. This usually requires the use of Cu^{I}-chelating ligands that are able to: 1) stabilize the oxidation state of Cu^{I}; 2) accelerate the cycloaddition reaction; 3) prevent the formation of undesired by-products; and 4) sequester Cu ions to prevent biomolecule damage and facilitate removal.[17–20] Different types of ligands have been proposed, and investigations on this topic are ongoing. To date, tris(benzyltriazolylmethyl)amine (TBTA),[17] tris(hydroxypropyltriazolylmethyl)amine (THPTA),[17] and bathophenanthroline disulfonate disodium salt (BPDS)[18] have been those mostly

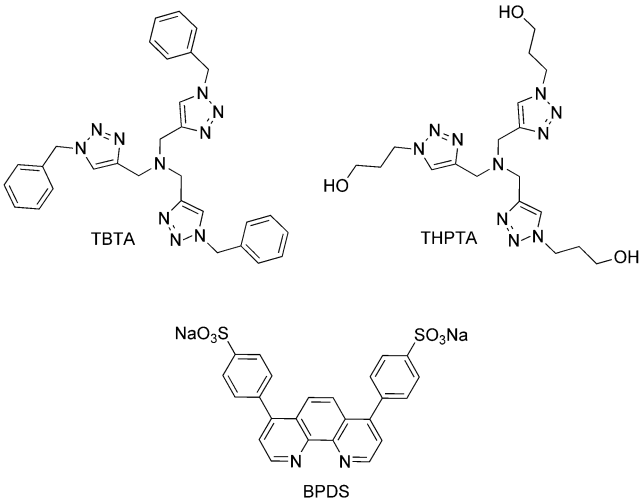


Figure 1. Cu^{I}-chelating ligands commonly used in CuAAC bioconjugations: TBTA, THPTA, and BPDS.

employed (Figure 1). With these ligands under anaerobic conditions, a balance can be achieved between the stabilization of the Cu^{I} oxidation state and the CuAAC rate enhancement.[15]

The tetradentate binding ability of tris(triazolylmethyl)amine ligands leads to the formation of stable Cu^{I} chelates, which are associated with an increase of roughly 300 mV in the redox potential of Cu^{I}/Cu^{II}.[17] TBTA was the first member of this family to be identified. Owing to the superior protection conferred by TBTA on Cu^{I} towards oxidation, usually it is not necessary to exclude O_2 from the reaction medium, even at very low catalyst loadings. A great shortcoming of TBTA is, nevertheless, its poor solubility in water; a small amount of an organic cosolvent (ca. 10%) is required to solubilize TBTA. This has fueled the development of water-soluble tris(triazolylmethyl)amine ligands such as THPTA. In addition, partly because of its poor solubility in water, the typical rate enhancements attributed to TBTA are low, with kinetics comparable to that of other classical bioconjugation reactions such as cysteine–maleimide coupling, amine acylation, and disulfide formation from 2-thiopyridylsulfide precursors.[8]

BPDS represents an attractive alternative to tris(triazolylmethyl)amine ligands. It exhibits high solubility in water and excellent catalytic activity, even under high dilution conditions or with a small excess of coupling probes.[21] However, the BPDS/Cu^{I} salt catalytic system is very sensitive to air oxidation, and unless a sacrificial reducing agent is added, rigorous exclusion of O_2 from the reaction medium is required to avoid oxidation of Cu^{I}. The superior kinetic efficiency of BPDS over TBTA in CuAAC and other classical bioconjugation techniques was elegantly demonstrated by Finn and co-workers in the functionalization of CPMV with proteins and peptides, polymers, and other low-molecular-weight probes (Figure 2).[21] Under the reported anaerobic conditions, $[Cu(MeCN)_4][OTf]$ was employed as a Cu^{I} source. Similar conditions were used to functionalize CPMV with Gd complexes for magnetic resonance imaging,[22] glycopolymers for polyvalent binding to cell-surface lectins,[23] and various

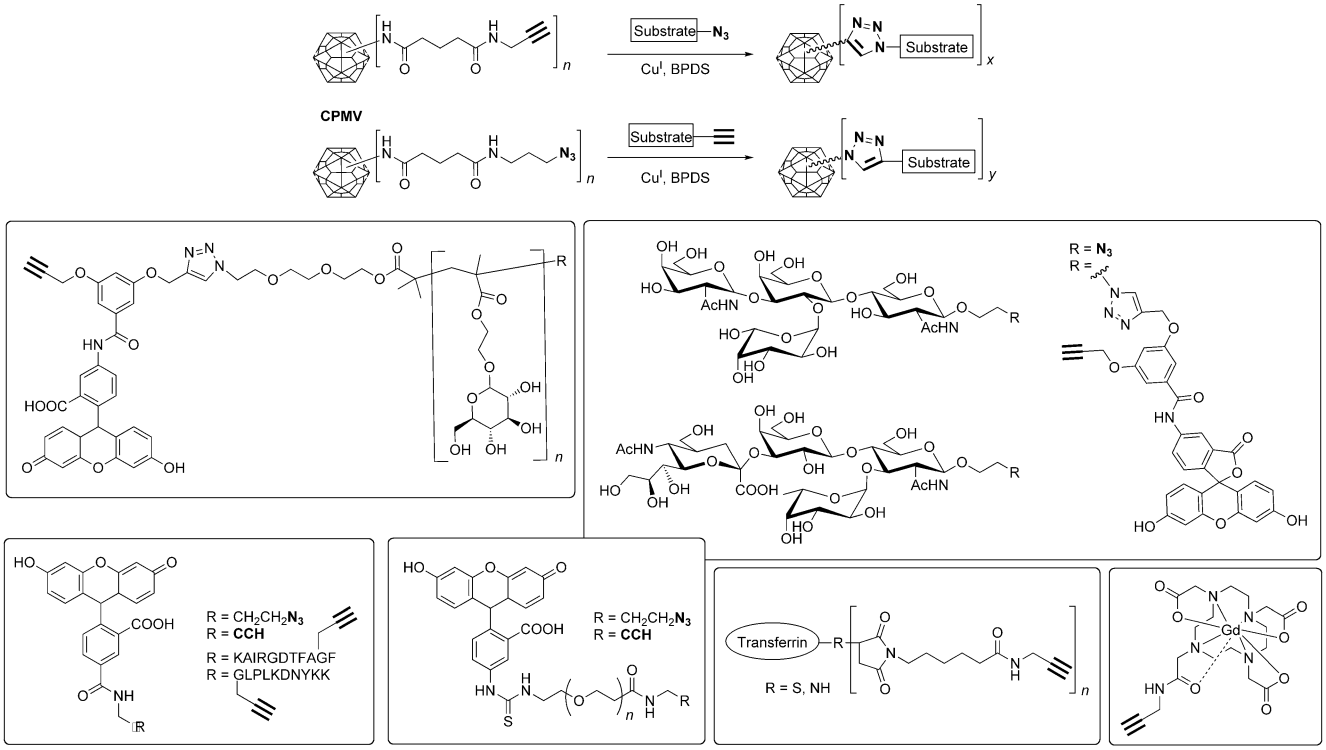


Figure 2. Diverse functionality (polymers, oligosaccharides, peptides, proteins, fluorescent probes, and imaging agents) incorporated on the surface of CPMV by means of CuAAC.

carbohydrates to elicit anticarbohydrate antibodies.[24] The group of Carrico has also relied on the BPDS/CuBr system for the chemoselective modification of a human adenovirus type 5 (hAd5, metabolically labeled with azido sugars) with a FLAG peptide, fluorophores, and the cancer-selective ligand folate. The folate-decorated hAd5 has demonstrated a significant increase in transgene delivery to murine breast cancer cells.[25]

A survey of the literature indicates that with the correct selection of the catalytic system, CuAAC is a safe and efficient tool for the bioconjugation of proteins and bionanoparticles. For instance, Wang and co-workers have reported that $CuSO_4$/ascorbic acid and $CuSO_4$/tris(2-carboxyethyl)-phosphine (TCEP) induced structural damage to the protein cage horse spleen apoferritin (apo-HSF), while intact particles resulted when CuBr/BPDS was used under anaerobic conditions (Figure 3).[26] The integrity of the modified apo-

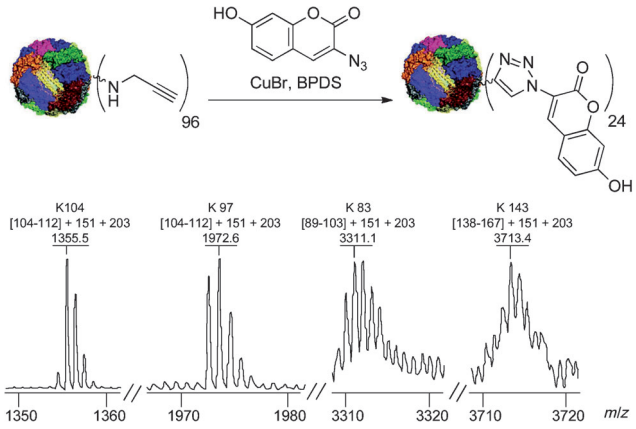


Figure 3. Fluorogenic labeling of protein cage apo-HSF and analysis by MALDI MS after enzymatic digestion (modified from Ref. [26] with permission).

HSF particles was confirmed by size-exclusion fast protein liquid chromatography and transmission electron microscopy. In addition, MALDI MS analysis of the enzymatic digestion of the protein after CuAAC revealed *m*/*z* peaks corresponding to intact triazole-containing fragments.

Nevertheless, even with the use of Cu^I ligands, in some instances a decrease in biological activity has been reported for proteins after CuAAC bioconjugation. In this regard, Bertozzi and co-workers observed a qualitative decrease in the immunoreactivity of an antibody used to stain a glycoprotein GlyCAM-Ig previously labeled by means of CuAAC in the presence of TBTA ($CuSO_4$, TCEP).[7] In another example, van Hest and co-workers reported the CuAAC functionalization ($CuSO_4$, sodium ascorbate, BPDS) of the enzyme *Candida antarctica* lipase B (CalB) containing one solvent-accessible azidohomoalanine residue (AHA-CalB);[27] a loss in enzymatic activity of 31% was reported after labeling with an alkynated dansyl probe (Figure 4). Noteworthy, significant loss in enzymatic activity was also observed when AHA-CalB and wild-type CalB (Met-CalB)

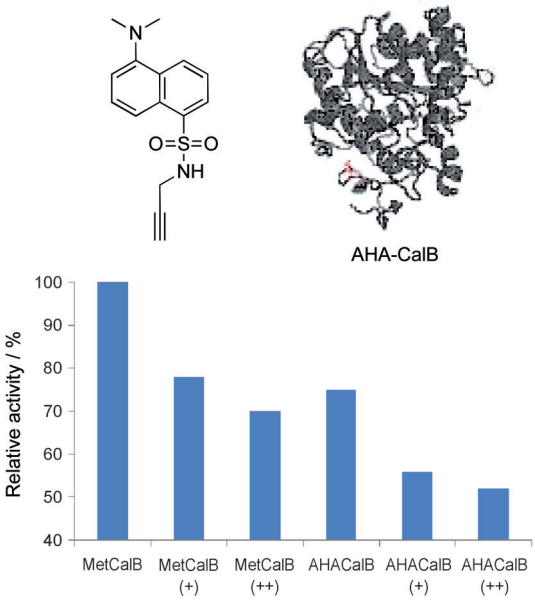
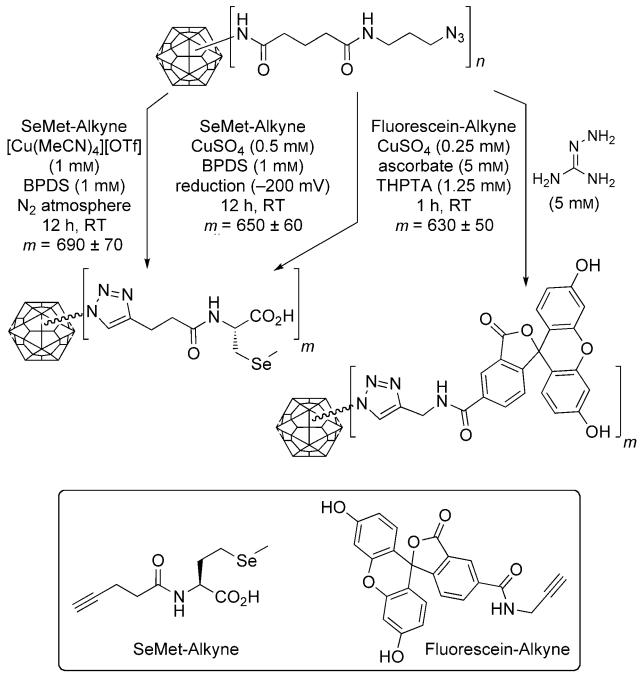


Figure 4. Effect of CuAAC functionalization and reaction conditions on the hydrolytic activity of AHA-CalB and Met-CalB. (+) after incubation with $CuSO_4$, sodium ascorbate, and BPDS; (+ +) after reaction with $CuSO_4$, sodium ascorbate, BPDS, and an alkynated dansyl probe (modified from Ref. [27] with permission).

were incubated in a mixture of $CuSO_4$, ascorbic acid, and BPDS.

This loss of protein bioactivity indicated that more-convenient CuAAC protocols are necessary for bioconjugation. Collman, Chidsey, and co-workers have recently developed a selective CuAAC functionalization of independently addressable electrodes by electrochemical activation and deactivation of the Cu^I catalyst.[28] This strategy has been adapted by the group of Finn for solution-phase bioconjugation.[29] In their approach, Cu^{II} is electrochemically reduced to Cu^I in the presence of the coupling substrates and the desired accelerating ligand. The oxidation state of Cu^I is therefore continuously maintained with no need for a sacrificial reducing agent; this is particularly relevant when one considers the protein degradation and/or precipitation associated with ascorbate and other reducing agents.[8] In addition, the oxidative degradation of substrates by ROS is drastically diminished, as the O_2 present in the reaction medium is reduced to H_2O ($O_2 + 4H^+ + 4e^- \rightarrow 2H_2O$). Finn et al. demonstrated the efficiency of this methodology by conjugating up to 650 copies of a seleniomethionine–alkyne derivative to an azide-modified bacteriophage Qβ in 12 h at ambient temperature, a result comparable to that obtained under O_2-free conditions (Scheme 2).[29]

More recently, Finn and co-workers have proposed an optimized bioconjugation protocol that simplifies the application of CuAAC as a general tool for the functionalization of biomacromolecules.[30] This approach relies on the simplicity and reliability of the Cu^{II}/ascorbate system, and on the use of THPTA as a water-soluble tris(triazolylmethyl)amine ligand. Besides the expected ligand-accelerated catalysis, THPTA was also found to inhibit protein degradation by ROS (by strongly accelerating the decomposition of H_2O_2 formed in



Scheme 2. Functionalization of bacteriophage Qβ by means of different CuAAC bioconjugation protocols.

the course of the reaction, and by acting as a radical scavenger). In agreement with previous observations by the group of Brown on the functionalization of nucleic acids (see Section 3),[31] Finn et al. recommended a fivefold molar excess of THPTA relative to Cu^{I} in order to minimize oxidative degradation. Under these conditions, the adverse effects of ascorbate (and other by-products derived from ascorbate oxidation) on the stability of proteins can also be avoided by addition of the carbonyl-capturing reagent aminoguanidine in a 1:1 molar ratio relative to ascorbate.[32] In this way, the efficient functionalization of azide-modified bacteriophage Qβ with an alkyne–fluorescein derivative was accomplished without degradation or aggregation of the virus (Scheme 2).[30] In related work, Qβ was functionalized with transferrin[33] and with a PEG-C_{60} conjugate.[34] Finn, Park, and co-workers have taken advantage of a Qβ decorated under similar reaction conditions (N_2 atmosphere) with an oligodeoxynucleotide (ODN) for the creation of a non-compact lattice by DNA-programmed crystallization with complementary ODN on gold nanoparticles.[35] The group of Douglas has expanded this CuAAC bioconjugation protocol for the preparation of coordination polymers inside the mutant protein cage Hsp G41C (Figure 5) with higher protein recoveries than when the $[Cu(CH_3CN)_4][OTf]$/BPDS system was used.[36] More recently, the Cu^{II}/ascorbate/THPTA/aminoguanidine system has been also employed by the group of Finn for the labeling of cell-surface glycans on mammalian cells.[37] The use of very short reaction times (5 min at 4 °C) and low Cu concentrations (50 μM) ensure effective labeling without significant loss of cell viability.

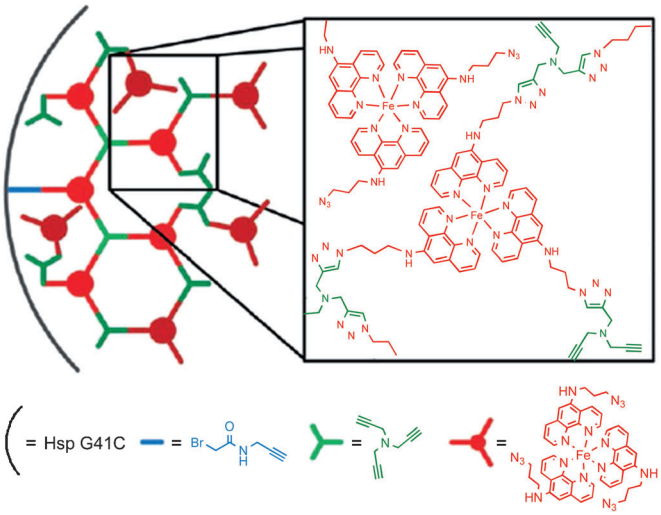


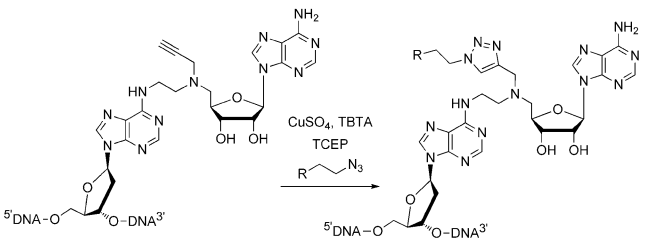
Figure 5. Schematic representation of coordination polymers constructed by means of CuAAC inside the mutant protein cage Hsp G41C (modified from Ref. [36] with permission).

3. Bioconjugation of Nucleic Acids through CuAAC

Nucleic acids represent another highly demanding platform for bioconjugation by means of CuAAC.[38] The thermal version of the Huisgen AAC has been applied for the fluorescent labeling of oligodeoxynucleotides (ODNs)[39] and their template-mediated immobilization on glass surfaces.[40] However, as this approach usually entails undesired heating or the use of activated coupling reagents,[41] CuAAC was soon envisaged as a panacea for the functionalization of nucleic acids. Unfortunately, this development was initially hampered by the deleterious effects associated with Cu. Thus, similar to the degradation of proteins, Cu-mediated production of ROS leads to strand scission of nucleic acids by both metal-assisted and free radical mechanisms.[42] Not surprisingly, in the first synthetic application of CuAAC to nucleic acids (oligonucleotide templates used for reaction discovery), roughly 50 % degradation was observed by Liu and co-workers after only 10 min at room temperature.[43]

Attempts to minimize oligonucleotide degradation by accelerating the CuAAC by means of microwave irradiation have been made in solution and on solid support, leading to the preparation of conjugates in shorter reaction times and in higher purities than under classical conditions.[44] Nevertheless, it is worth mentioning that these protocols still result in partial degradation, and compulsory exclusion of O_2 has been recommended.[45]

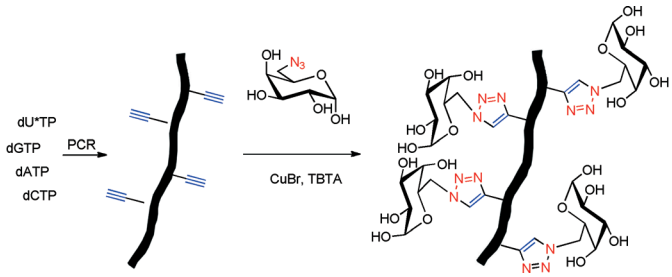
As an alternative approach, the use of Cu^{I}-stabilizing ligands to reduce the oxidative degradation of nucleic acids has been also investigated. Pioneering work on the usefulness of TBTA in the nucleic acid arena was reported by the groups of Rajski[46] and Carell,[47] who described the successful modification of ODN and long DNA chains by means of CuAAC (Scheme 3). In the presence of TBTA, integrity of the substrates was confirmed by denaturing polyacrylamide gel electrophoresis (DPAGE) analysis, whereas severe depolymerization resulted in its absence. Similarly, in the presence



Scheme 3. CuAAC functionalization of an alkynated DNA (modified from Ref. [46] with permission).

of TBTA, the successful CuAAC coupling of ODN to self-assembled monolayers was reported by Collman, Kool, Chidsey, and co-workers.[48]

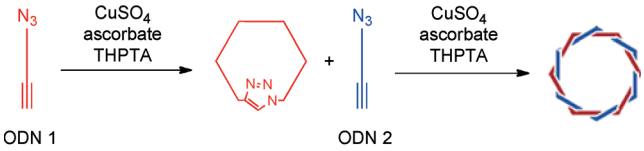
These successful reports on the beneficial effects of TBTA paved the way for CuAAC as a reliable tool for nucleic acid modification. In this way, Carell and co-workers have continued investigating the high-density functionalization of long DNA chains by means of a polymerase chain reaction (PCR)-CuAAC protocol.[49] To this end, DNA containing up to 2000 base pairs and 800 alkynes per chain was prepared by means of PCR by incorporating synthetic alkyne-modified nucleotide triphosphates into selected genes. CuAAC functionalization of the resulting alkyne-modified DNA with saccharides proceeded in excellent yields in the presence of TBTA, with no sign of DNA degradation (Scheme 4). In the



Scheme 4. Schematic representation of a PCR–CuAAC protocol for the preparation of functionalized DNA chains. dATP: deoxyadenosine triphosphate, dCTP: deoxycytidine triphosphate, dGTP: deoxyguanosine triphosphate, dU*TP: alkyne pyrimidine triphosphate (modified from Ref. [49a] with permission).

analysis of the enzymatic digestion of the triazole-containing DNA chains by HPLC–MS the chromatograms were very clean, and the molecular weights of the resulting species matched those of triazole-containing or unreacted alkyne-modified bases. The resulting carbohydrate-modified DNAs were selectively metalized at the anomeric positions with Ag^0 after a limited exposure to the Tollens' reagent, resulting in the preparation of DNA nanowires with potential conductive properties.[50] In a similar fashion, bimetallic Ag–Au DNA-based nanowires[51] and chainlike assemblies of Au nanoparticles on artificial DNA have been obtained.[52] The same research group has more recently reported a combination of PCR with CuAAC and nitrile oxide/alkene cycloaddition for the selective dual functionalization of long DNA chains.[53] In collaborative work, Carell, Bein, and co-workers also applied the CuAAC assembly of double-stranded ODN on mesoporous colloidal silica nanoparticles for the preparation of DNA-based molecular valves with thermoresponsive release behavior.[54]

The group led by Brown has proposed the use of THPTA as an alternative to TBTA for the functionalization of nucleic acids.[31,55–57] Its adequate solubility in water obviates the need for organic co-solvents, which are otherwise necessary with TBTA. As for proteins, an at least fivefold molar excess of ligand relative to Cu^I has been reported to minimize oxidative degradation by ROS. In this way, efficient CuAAC conditions for the template-mediated intermolecular ligation of ODN strands[31] and the efficient cross-linking of DNA strands have been developed.[56] Also, these researchers have reported the preparation of cyclic DNA duplexes from hairpin ODN precursors,[57] and a template-free intramolecular circularization of a single-stranded ODN which was subsequently used as template in the preparation of a double-stranded DNA catenane (Scheme 5).[31] Other research groups have also



Scheme 5. Formation of a double-stranded DNA catenane by sequential direct and template-mediated circularization of ODN (modified from Ref. [31] with permission).

taken advantage of the reliability of THPTA for the efficient surface decoration of superparamagnetic iron oxide nanoparticles with ODN,[58] the preparation of six-membered DNA circularized nanoconstructs,[59] and the DNA-templated coupling of dendrimers.[60]

Quite in contrast to the broad application of tris(triazolylmethyl)amine ligands, the use of BPDS in the CuAAC functionalization of nucleic acids has been only scarcely explored probably because of the higher sensitivity of the BPDS/Cu^I catalytic system towards O_2. In this regard, it is worth mentioning the results by Gothelf and co-workers on the small-molecule-controlled CuAAC cross-linking of ODN strands into DNA duplexes and triplexes.[61] These authors found BPDS to show little capability to protect ODN from degradation (as determined by DPAGE and HPLC), while cross-linking in the presence of THPTA proceeded in very good yields (80–90%) with only minor ODN degradation.

4. Bioconjugation of Polysaccharides through CuAAC

CuAAC has also demonstrated a wide versatility in the selective functionalization of cyclodextrins and oligo- and polysaccharides for applications aimed at the preparation of hydrogels,[62] films,[63] MRI contrast agents,[64] and nanostructures for drug and gene delivery.[65] Functionalized polysaccharides have also been proposed as supported catalysts for CuAAC.[66] Despite this wide use, little attention has been

paid to the possible deleterious effects of Cu on the polysaccharide backbone. This is surprising as the damaging effect of transition-metal ions on polysaccharides has been thoroughly reviewed[10] and even exploited for their controlled depolymerization.[67] In addition, the use of polysaccharides as radical scavengers for the protection of DNA against oxidative damage is well known.[68]

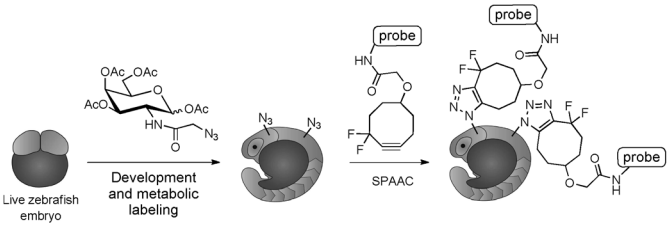
The first report on the depolymerization of polysaccharides under CuAAC conditions came from the group of Fernandez-Megia and Riguera. In their program towards the development of PEG-grafted chitosan (CS-*g*-PEG) as a drug carrier across the blood–brain barrier, these authors pointed out the limitations of CuAAC for the functionalization of CS-*g*-PEG-N_3 incorporating azide groups at the terminal ends of PEG.[69] Thus, quantitative conversions were accompanied by severe depolymerization of the CS backbone as revealed by size-exclusion chromatography. This depolymerization has been rationalized as resulting from $^{\bullet}OH$ radicals (which were detected in the reaction medium); they promote the scission of the glycosidic bonds, which ultimately results in a drastic reduction of the molecular weight. Interestingly, the study of this phenomenon in H_2O with various polysaccharides revealed the general scope of this process, with molecular weight losses that paralleled the Cu-complexing ability of the polysaccharides [polysaccharide (% decrease in M_w): mannan (3%), dextran (38%), CS (95%), hyaluronic acid (>99%)]. This is in agreement with the stronger deleterious effect of the short-lived $^{\bullet}OH$ radical when it is produced closer to the polymer backbone. The great tendency of this depolymerization to proceed in the case of CS, along with the high content of cytotoxic Cu in the final conjugates (severely compromising their biomedical applications), led these authors to turn their attention to SPAAC as an efficient Cu-free alternative for the functionalization of CS-*g*-PEG nanoparticles (see Section 5).[69]

More recently, similar observations have been made by the groups of Makuska and Li in the CuAAC grafting of CS to PEG (CS-*g*-PEG),[70] and poly[(2-dimethylamino)ethyl methacrylate] and poly(*N*-isopropylacrylamide) [CS-(*g*-PDMAEMA)-*g*-PNIPAM][71]. In agreement with the aforementioned relationship between Cu complexation and degradation, lower depolymerizations were found in reactions of CS samples with lower degrees of amination. Also, Seppala and co-workers have reported the depolymerization of dextran-*g*-PEG prepared by CuAAC.[72] In this case, attempts to improve the degree of grafting by longer reaction times (24 h) led to reduced hydrodynamic size of the copolymers, which was interpreted as resulting from oxidative degradation. As was reported for proteins and nucleic acids, it is expected that the development of optimized catalytic protocols could alleviate the oxidative damage of polysaccharides under CuAAC conditions.

5. SPAAC as a Cu-Free Click Technology for the Functionalization of Biomacromolecules

Despite the demonstrated reliability of CuAAC for the efficient functionalization of biomacromolecules in vitro, the negative effects of the Cu catalyst have greatly limited its use for in vivo applications. Thus, although CuAAC has been used to label bacterial[73] and mammalian cells,[74] the presence of Cu has often been found to be detrimental to living cells, which has stimulated the development of CuAAC bioconjugation protocols specifically designed for minimal damage.[37,20]

Alternatively, benign Cu-free AAC strategies, not requiring cytotoxic metals and additives, have also appeared. The group of Bertozzi has taken advantage of the inherent ring strain of cyclooctynes as an effective way for lowering the activation barrier of AAC and an alternative to the use of metal catalysts (Scheme 1c).[7] This strain-promoted AAC variant, SPAAC, has been exploited by the Bertozzi group in the context of the bioorthogonal chemical reporter strategy for the fluorogenic labeling of proteins and cell-surface glycans in living cells and organisms, including zebrafish and mice (Scheme 6).[7,75,76] Thanks to its simplicity and great



Scheme 6. Metabolic labeling and SPAAC for the non-invasive imaging of cell-surface glycans during zebrafish development (modified from Ref. [75c] with permission).

orthogonality,[77] SPAAC has been rapidly adopted by many other groups not only for the study of dynamic processes of biomolecules in living systems, but also as a powerful coupling technology in nanotechnology and in materials and polymer science.[78,79]

Optimization of the reactivity of cyclooctyne reagents and the development of more-efficient synthetic routes for their preparation have been objectives pursued by the groups of Bertozzi [difluorocyclooctynes (DIFO) and biarylazacyclooctynones (BARAC)],[75,80] Boons [dibenzocyclooctynes (DIBO)],[81] and van Hest and van Delft [dibenzoazacyclooctynes (DIBAC) and bicyclo[6.1.0]nonynes (BCN)][82,83] (Fig-

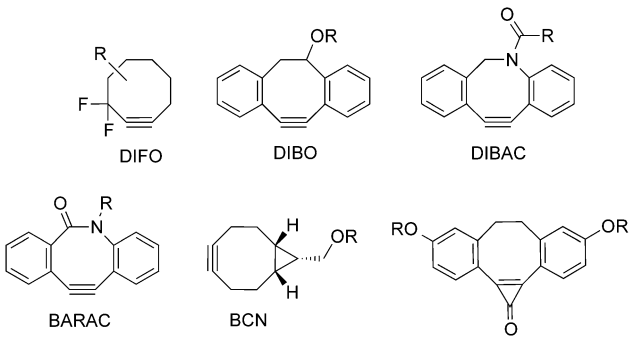


Figure 6. Activated cyclooctyne derivatives used in SPAAC bioconjugations.

ure 6). The improvement in reactivity of the resulting activated reagents compared to unfunctionalized cyclooctyne is related to the presence of electron-withdrawing groups and increased ring strain, which have afforded reaction rates comparable to those of ligand-less CuAAC. Interestingly, a cyclooctyne precursor of the reactive DIBO in which the triple bond is masked as cyclopropenone (Figure 6) has been developed by Boons, Popik, and co-workers for the photo-triggering of SPAAC under UV irradiation (ca. 350 nm).[84]

Indeed, SPAAC has proved to be an efficient tool for bioconjugation in vitro in cases where the presence of Cu prevented the use of CuAAC. The group of Fernandez-Megia and Riguera has described the use of SPAAC as an alternative to CuAAC for the orthogonal functionalization of polysaccharides and polysaccharide-based nanostructures which avoids the depolymerization and contamination associated with Cu (see Section 4).[69] In this way, the functionalization of cross-linked CS-*g*-PEG-N_3 nanoparticles with a cyclooctyne-derived anti-BSA immunoglobulin G (IgG) proceeded quantitatively under physiological conditions, in what represents a step forward in the development of environmentally friendly bioconjugation technologies for the preparation of immunonanoparticles (Figure 7).

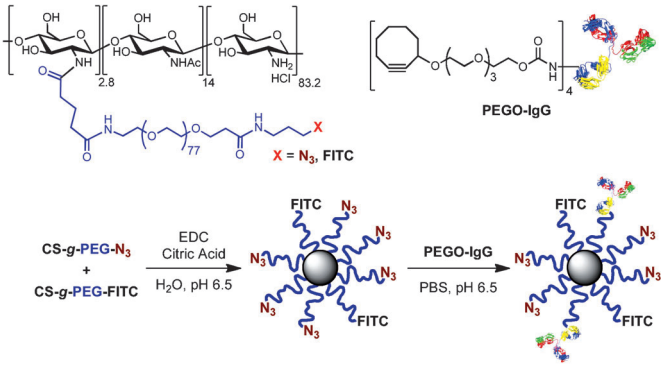
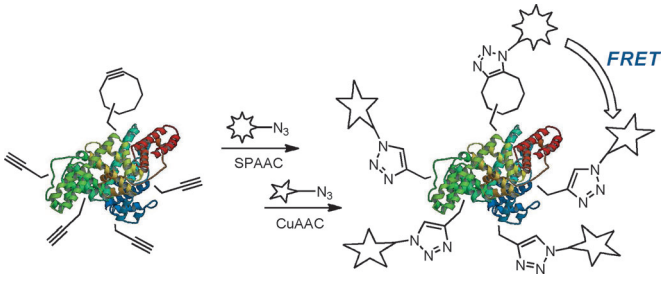


Figure 7. Preparation of CS-*g*-PEG immunonanoparticles by SPAAC (modified from Ref. [69] with permission).

Several examples illustrating the advantages of SPAAC for the functionalization of proteins and bionanoparticles have recently appeared. For instance, the Yin group has relied on SPAAC for the labeling of *Escherichia coli* bacteriophage M13, as the infectivity of the virus drastically decreased after it had been exposed to micromolar concentrations of Cu.[85] In another example, van Hest, van Delft, and co-workers have reported the use of DIBAC for the PEGylation of proteins.[82] The solvent-accessible azidohomoalanine residue in AHA-CalB was quantitatively PEGylated by incubating the protein with DIBAC-PEG for 3 h under physiological conditions; this is in contrast to the incomplete functionalization by CuAAC.[27] Other interesting examples illustrating the advantages of SPAAC in bioconjugation include nucleic acids[86] and quantum dots (QDs).[87] While the deleterious effect of Cu on nucleic acids is well known, it has been reported recently that the luminescence properties of QDs are dramatically altered in the presence of Cu ions.

Interestingly, the versatility of SPAAC spreads beyond bioconjugation to applications where the removal of metal catalysts and additives is difficult, or their use not recommended.[88] For example, Turro and co-workers have relied on SPAAC for the in situ cross-linking of azide-terminated photodegradable star polymers, which otherwise required extensive washing after CuAAC.[89] Similarly, Anseth and co-workers have used SPAAC for the preparation of enzymatically degradable hydrogels as three-dimensional platforms for cell cultures.[90] To this end, a PEG-based polymer network was constructed in the presence of 3T3 fibroblasts by cross-linking a star-shaped PEG tetraazide with a biodegradable difunctional DIFO-containing RGD peptide. Alkene functionalities were additionally incorporated into the peptide backbone, allowing the spatially controlled photopatterning of the hydrogel through a thiol–ene addition.

Finally, some interesting applications have been developed by taking advantage of the different reactivity of CuAAC and SPAAC. Kele, Wolfbeis, and co-workers have reported the sequential and orthogonal, dual labeling of proteins (BSA) and silica nanoparticles for the preparation of fluorescence resonance energy transfer (FRET) nanoprobes (Scheme 7).[91] This concept was applied in the preparation of



Scheme 7. Schematic representation of sequential, dual SPAAC–CuAAC labeling of nanoprobes.

fluorescently doped silica nanoparticles to which a FRET-based enzyme substrate had been conjugated through sequential SPAAC and CuAAC reactions; these nanoparticles were used to determine nanomolar concentrations of matrix metalloproteinase II, an enzyme considered a major tumor marker.[92]

6. Summary and Outlook

Over the past few years, the Cu^I-catalyzed azide–alkyne cycloaddition (CuAAC) has been shown to be a powerful coupling technology for the bioconjugation of proteins, nucleic acids, and polysaccharides. Owing to the reliability of the process and experimental simplicity for nonspecialists, CuAAC has been adopted as a universal coupling process in many areas of research. Nevertheless, several shortcomings of CuAAC related to the presence of Cu have been pointed out. Indeed, Cu is known to play a role in oxidative stress in biomacromolecules by promoting the generation of the reactive oxygen species responsible for structural damage. In addition, contamination by Cu in the final conjugates can

be detrimental in some biological applications. With the aim of overcoming these limitations, extensive efforts have been devoted to the development of catalytic systems incorporating Cu^{I}-chelating ligands for increased kinetics and stabilization of the Cu^{I} oxidation state. Alternatively, the strain-promoted SPAAC, developed initially for the study of dynamic processes of biomolecules in living systems, has proved to be an effective bioconjugation tool for in vitro applications where Cu catalysts cannot be used. Interestingly, the different reactivity of CuAAC and SPAAC can be used in applications where the two processes can be implemented sequentially for orthogonal and dual-labeling purposes.

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