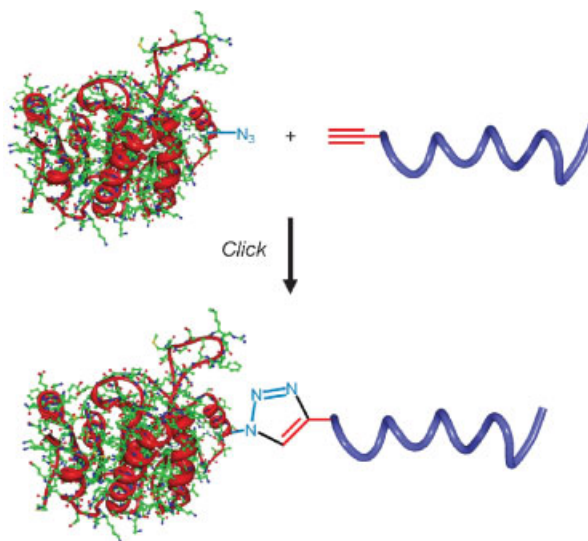


# Click Chemistry: A Powerful Tool to Create Polymer-Based Macromolecular Chimeras

Benjamin Le Droumaguet, Kelly Velonia\*

The combination of polymeric with biological materials, to create biohybrid macromolecules that merge the properties of both the natural and synthetic components, is a flourishing area in both life sciences and biotechnology. The click chemistry philosophy has recently provided a powerful tool in this direction, leading to a plethora of novel, tailor-made biomacromolecules with unprecedented structural characteristics and properties. The different synthetic strategies, using the alkyne–azide click cycloadditions to bioorthogonally achieve the coupling of synthetic polymers with nucleic acids, peptides, sugars, proteins or even viruses and cells is described. The review covers the latest developments in this very dynamic and rapidly expanding field.



## Introduction

The elegance and efficiency of the structures and function observed in nature, has long directed scientists' efforts both on understanding how nature functions through observation and mimicry and on creating novel, synthetic biomaterials merging the optimized by evolution properties of biomolecular structures with that of synthetic components. Taking into account the multifunctional and, most of the times, chemically fragile nature of biological

entities, the major challenge into this direction is a chemical one: the need to proceed through bioorthogonal reactions and under the benign reaction conditions that would preserve and respect the multifunctionality and structure of biologically derived components.

The introduction of the "click" concept in 2001 by Sharpless and coworkers<sup>[1]</sup> has certainly had an enormous impact on the chemical philosophy driving the design of such synthetic biomaterials. Inspired by nature, the "click" chemical philosophy states that "all searches must be restricted to molecules that are easy to make," highlighting therefore a chemistry tailored to efficiently generate substances by exploring reactions that are inherently efficient at linking—clicking—two components, instead of focusing on the tedious construction of difficult bonds. The stringent set of criteria that a process must meet to be useful in this context was also described by Sharpless and coworkers,<sup>[1]</sup> as they described that "click" reactions

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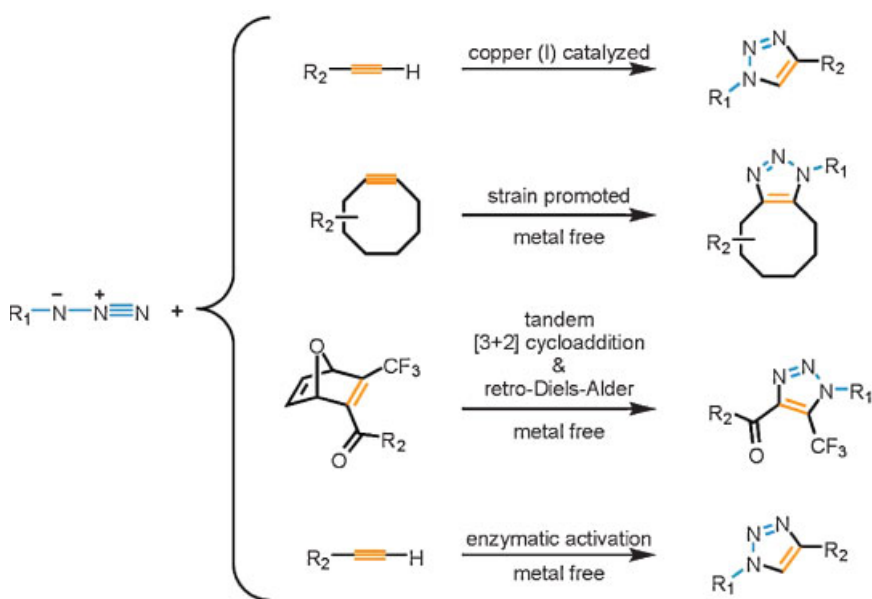
**Kelly Velonia** joined the faculty of the Department of Materials Science and Technology of the University of Crete in 2008, after 4 years in the Department of Organic Chemistry of the University of Geneva. She has been involved in the past in mechanistic studies of enzymatic reactions, the development of protein–polymer conjugates and nanoreactors and single molecule photophysics and is now focusing in the development of well-defined, multifunctional biohybrid nanomaterials for application in polymer, material and life sciences and nanotechnology. In 2002, she received the Europa Medal and Prize Award.

should be “modular, wide in scope, high yielding, stereospecific, simple to perform creating only inoffensive by-products (that can be removed without chromatography) and requiring benign or easily removed solvents, preferably water.” Although meeting these requirements is a tall order, several processes such as the nucleophilic ring opening reactions, the non-aldol carbonyl chemistry, the additions to carbon–carbon multiple bonds, and the cycloaddition reactions, were identified to step up to the mark. Among these, the copper (I) catalyzed variant<sup>[2,3]</sup> of the Huisgen 1,3-dipolar cycloaddition<sup>[4–6]</sup> (CuAAC, Scheme 1) has certainly been the “cream of the crop” of click reactions, so much that it is now often referred to as the “click chemistry reaction.” Several reports have confirmed the wealth of applications of this practical and sensible chemical approach in the areas of bioconjugation,<sup>[7–10]</sup> polymer and materials sciences<sup>[11–14]</sup> and drug discovery.<sup>[15]</sup>

The present review aims at summarizing the latest discoveries in the area of polymer bioconjugation using the azide–alkyne “click” chemistry (Scheme 1). The conjugation of biomolecules with synthetic polymers for the preparation of biohybrid

macromolecules (often also referred to as “macromolecular chimeras”) that combine the properties of both components, has been a flourishing area in pharmaceutical chemistry for many decades,<sup>[16–18]</sup> and more recently one of the holy grails of nanobiotechnology.<sup>[9,19–21]</sup> Since the foundations of click reactions were laid, there has been an explosive growth in the area. A major contribution to this direction was certainly given by the breakthrough study of Bertozzi and Saxon in 2000,<sup>[22]</sup> in which the azide functionality was introduced for the first time to biomolecules with the aim to serve as a handle for the purposes of promoting bioorthogonal bioconjugation reactions. Furthermore, the introduction of the robust and flexible living radical polymerization techniques has revolutionized the area of polymer synthesis in the recent years, and allowed for the preparation of an unprecedented variety of multifunctional, complex polymer architectures.<sup>[23]</sup> These tailor-made designer polymers are now available for numerous applications among which, impressive examples have already been seen in the area of bioconjugation.<sup>[24]</sup> The designation of the azide function as the group possessing the appropriate bioorthogonality, the major advances recently seen in the area of polymerization, together with the introduction of the click chemistry philosophy, influenced tremendously the area of bioconjugation where nowadays, the click azide–alkyne bioorthogonal approaches involve the reactions that are summarized in Scheme 1.

In this review, an overview of the application of the azide–alkyne click chemistry into the conjugation of polymers with biological entities will be given. The different synthetic strategies to achieve such polymer



■ Scheme 1. General scheme of “click” chemistry cycloaddition reactions.

bioconjugates with nucleic acids, peptides, sugars, proteins, viruses, and cells will be described together with the strategies giving rise to biomolecular polymeric structures and, in the cases of lack of examples describing the above-mentioned approaches, the appropriate molecular examples will be highlighted with the aim to introduce the possibilities in the area.

## Oligonucleotides and Nucleic Acids

The unparalleled biological functions of nucleic acids (deoxyribonucleic acid, DNA or ribonucleic acid, RNA) as for instance in recognition, heredity, and protein expression have captivated the interest of scientists in the last decades. As a result, research in the area of nucleic acid mimics and derivatization for the creation of novel materials with applications in the bio and nanosciences has flourished.<sup>[25–29]</sup> Several elegant examples demonstrate the applicability and bioorthogonality of the click chemistry philosophy in this arena.<sup>[30–32]</sup> Following their work on the site specific labeling of DNA using Staudinger ligation,<sup>[33]</sup> Ju and coworkers<sup>[32]</sup> reported on the selective coupling of fluorophores to DNA molecules using the click chemistry 1,3-dipolar cycloaddition reaction between alkynyl 6-carboxyfluorescein (FAM) and an azido-labeled single-stranded (ss) DNA molecule. The FAM-labeled ssDNA was produced in quantitative yields, characterized by high stability, and successfully used without any further purification as a primer to produce DNA sequencing products with single base resolution in a capillary electrophoresis DNA sequencer using laser-induced fluorescence detection. This click chemistry-based methodology could be further utilized to produce large numbers of primers useful in genomics, especially for DNA sequencing. Nevertheless, this early approach proceeded in a non-regioselective manner, leading to both the 1,3- and 1,4-regioisomers of the FAM-labeled ssDNA.

A very elegant and highly versatile approach on the synthesis of modified oligodeoxyribonucleotides (ODNs) bearing alkyne reporter groups in high density was reported by the group of Carell.<sup>[34]</sup> The synthesis of the alkyne side chain ODNs relied on the incorporation of unnatural side alkyne bearing nucleobases into the DNA-strand by solid phase chemical synthesis. The subsequent development of a click reaction protocol utilizing the classical Cu(I)/tris(benzyltriazolylmethyl)amine (TBTA) catalytic system enabled the post-synthetic loading of the DNA strands with a variety of molecular labels such as dyes and/or carbohydrates, in high yields. The presence of the TBTA ligand was judged to be necessary as studies on oligonucleotides showed that standard CuAAC conditions lead to strands degradation.<sup>[34,35]</sup> Further studies performed by the same research

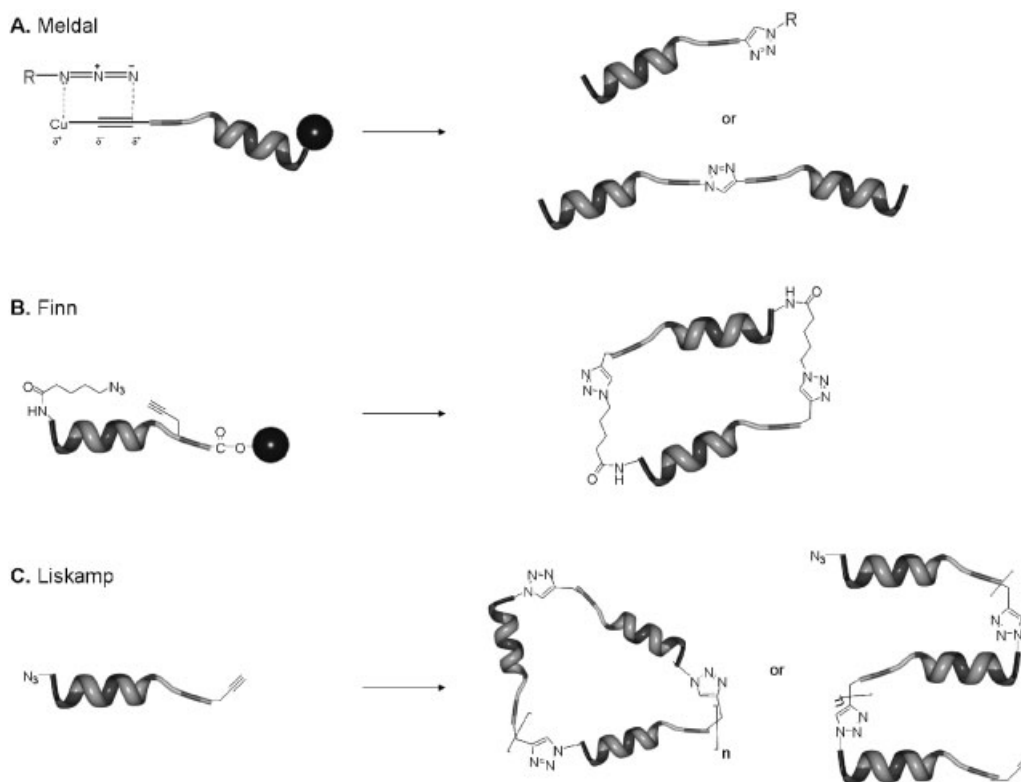
group, used the click chemistry reaction on such acetylene bearing DNA polymers to develop a selective and efficient method for the deposition of Ag(0).<sup>[35]</sup> During the course of their studies, the incorporation of acetylene-containing nucleotide triphosphates was performed enzymatically using DNA polymerases. The click chemistry reaction which introduced single or multiple – dendritic – aldehyde functionalities onto the nucleic acid analog backbone was found to be necessary for the metal deposition.

## Peptides

The use of peptides as building blocks for the synthesis of biopolymers is an area of growing interest since these polymers can find application as drug delivery systems, scaffolds for tissue engineering and repair, novel biomaterials, even protein mimics.<sup>[36–39]</sup> Bearing multifunctional building blocks and labile structural functionalities, such biopolymers have greatly benefited from click chemistry. In fact, the pioneering study by Meldal and coworkers<sup>[2,3]</sup> demonstrating the applicability of click chemistry in peptide synthesis appeared almost concomitantly to the introduction of the click chemical philosophy.<sup>[1]</sup> It was during this study that the regioselective copper(I)-catalyzed variant of the 1,3-dipolar Huisgen cycloaddition was introduced for peptidic terminal alkynes and azides using solid-phase synthesis (Figure 1A). By either the incorporation of the alkyne functionality to the peptidic backbone or the direct alkyne functionalization of the N-terminus, the authors demonstrated the applicability and efficiency of the copper catalyzed 1,3-dipolar cycloaddition, and more importantly highlighted the reaction as an excellent candidate for further applications in peptide synthesis as it proceeds in a mild and efficient fashion with conditions fully compatible with solid-phase peptide synthesis, all the amino acids, and their protecting groups. Furthermore, the ability of the produced [1,2,3] peptidotriazoles to create and sustain hydrogen bonds in conjunction with their aromaticity and rigidity enhances the potential of such products into the evolving area of peptidomimics.

A plethora of strategies in which click chemistry was the key step in peptide functionalization evolved subsequent to this publication.<sup>[11,12]</sup> For the scope of this review however, we focus on either the production of peptidic biomacromolecules or their functionalization with polymers for the production of biologically relevant biopolymers and polymer amphiphiles.

Many examples stemming from the de novo cyclic oligopeptide design field validated the applicability of the click chemistry orthogonal approach.<sup>[40–42]</sup> In one such ground-breaking investigation by the Finn laboratories,



**Figure 1.** General synthetic scheme for the (A) copper(I)-catalyzed 1,3-dipolar cycloaddition of alkynes to azides affording peptidotriazoles or *N*-substituted histidine analogs as introduced by Meldal and coworkers.<sup>[2]</sup> Reaction conditions: (i) R-N<sub>3</sub>, DIPEA, Cu(I); (ii) 20% piperidine/DMF; (iii) 0.1 M NaOH (aq). (B) RGD peptide cyclization by azide-alkyne cycloaddition as introduced by the Finn laboratories.<sup>[40]</sup> Reaction conditions: Room temperature, (i) Cu(I), Na ascorbate, 2,6-lutidine, CH<sub>3</sub>CN/DMSO/H<sub>2</sub>O (8:2:1), (ii) CF<sub>3</sub>CO<sub>2</sub>H/H<sub>2</sub>O or ethanedithiol/SiH(iPr)<sub>3</sub>. (C) Liskamp and coworkers<sup>[44]</sup> approach to linear and cyclic polypeptides. Reaction using CuSO<sub>4</sub>/Na ascorbate or Cu(OAc) led predominantly to linear polymerization, while with lower monomer ratio and Cu(OAc), cyclic products were obtained.

the selective production of large cyclic dimers of an 11-mer and a 19-mer Arg–Gly–Asp (RGD) peptide containing sequence that was taken from an adenovirus serotype which binds several  $\alpha_v$  integrins, was reported (Figure 1B).<sup>[40]</sup> The dimer synthesis was performed using precursor resin-tethered peptides containing azide and alkyne functions at the appropriate linking ends. Due to the chemoselectivity of the click chemistry reaction itself and the inertness of the azide or alkyne functionalities, the functional “handles” for the final cycloaddition reaction could be installed at early stages of the synthesis and ignored until the appropriate time for their connection. The selectivity of this reaction proved to be a striking consequence of the mechanistic constraints posed by the 1,3-copper(I) catalyzed cycloaddition itself.

Following the bibliography reports indicating that most of the peptide-based biopolymers derive their structural, mechanical, and biological properties from sequential repetitions on their backbone (e.g., collagens, elastin and spider silk, antifreeze proteins, mussel glue, and reflectins),<sup>[43]</sup> in a study aimed to mimic such natural peptide biopolymers with repetitive sequences, Liskamp and co-

workers<sup>[44]</sup> developed a method to prepare high molecular weight linear or medium-sized cyclic polypeptides based on click chemistry (Figure 1C). Their work relied on the polymerization of azido-phenylalanyl-alanyl-propargyl amides by a microwave-assisted 1,3-dipolar polycycloaddition reaction. As proven in their comparative studies, the model azido-phenylalanyl-alanyl-propargyl amide which was utilized as a monomer, could be efficiently converted into high molecular weight biopolymers (up to 45 kDa) by a microwave-assisted 1,3-dipolar cycloaddition reaction.<sup>[45]</sup> Depending on the reaction conditions, it was also found that the outcome of the click reaction could be directed either to these large linear polymers (up to 300 amino acid residues) or to medium-sized peptide macrocycles (4–20 amino acid residues). It was for example proven that microwave heating was in general superior to conventional heating for direction of the reaction outcome in such high molecular weight polymers. Following the same approach, Angell and Burgess<sup>[46]</sup> achieved the synthesis of SH2 domain-binding peptides and their open-chain analogs by reacting with monomers containing a single azide or alkyne group each<sup>[46]</sup> while van

Maarseveen and coworkers<sup>[47]</sup> synthesized triazole-containing analogs of the naturally occurring tyrosinase inhibitor *cyclo*-[Pro-Val-Pro-Tyr] and showed that the analogs retain enzyme inhibitory activity, demonstrating the effectiveness of a 1,4-connected 1,2,3-triazole as a *trans* peptide bond isostere.

Since <sup>18</sup>F is readily available from most small medical cyclotrons, has almost 100% positron efficiency and physical half-life well-suited for routine clinical use and matched to the biological half-life of peptides, <sup>18</sup>F-labeled target-specific peptides are becoming widely used as *in vivo* imaging agents, a few of which have entered early phase clinical trials.<sup>[48]</sup> To surpass the intrinsic problems connected with the synthesis of such <sup>18</sup>F-labeled peptides, the groups of Sutcliffe<sup>[49]</sup> and Arstad<sup>[50]</sup> published at the same time on the application of the Cu(I) mediated 1,3-dipolar cycloaddition for the formation of a series of <sup>18</sup>F-labeled peptides. In fact, in the first case the conjugation of  $\omega$ -[<sup>18</sup>F]fluoroalkynes to peptides decorated with 3-azidopropionic acid, provided a series of <sup>18</sup>F-labeled peptides in 10 min with yields of 54–99% and excellent radiochemical purity (81–99%).<sup>[49]</sup> In the later case, the labeling agent 2-[<sup>18</sup>F]-fluoro-ethyl-azide was coupled with small terminal alkynes and a model alkyne-1-appended peptide to form the corresponding 2-[<sup>18</sup>F]-fluoroethyl-1,2,3-triazoles excellent yields within a short time and under mild conditions.<sup>[50]</sup> However, it was only very recently that Chen and coworkers<sup>[51]</sup> reported the first *in vivo* PET study on <sup>18</sup>F-labeled tracers synthesized by click chemistry. During the course of this study, the Cu(I)-catalyzed Huisgen cycloaddition was employed to label dimeric RGD peptides with <sup>18</sup>F by forming the <sup>18</sup>F-fluoro-PEG-[1,2,3]-triazoles-RGD<sub>2</sub> with high radiochemical yield. This tracer was then used to study the tumor targeting efficacy, *in vivo* kinetics, and metabolic stability of this tracer in tumor-bearing mice and found to exhibit good tumor-targeting efficacy, relatively good metabolic stability as well as favorable *in vivo* pharmacokinetics.

A microwave assisted variation of the Huisgen 1,3-dipolar cycloaddition reaction was utilized by the group of Liskamp<sup>[45]</sup> to synthesize multivalent dendrimeric peptides. Upon the synthesis of the dendrons bearing terminal alkynes a wide variety of biologically relevant azido-functionalized peptides were clicked onto their terminal functionalities with yields ranging from 46 to 96%. The fact that by multivalency the biological activity can be significantly enhanced makes these multivalent dendrimeric peptides potentially very useful in the preparation of synthetic vaccines or for example in the diagnosis and treatment of infections and possibly protein mimics.

The click cycloaddition reaction of short biologically active peptides such as RGD—a cell adhesion sequence—or TAT—a protein transduction domain—to well-defined

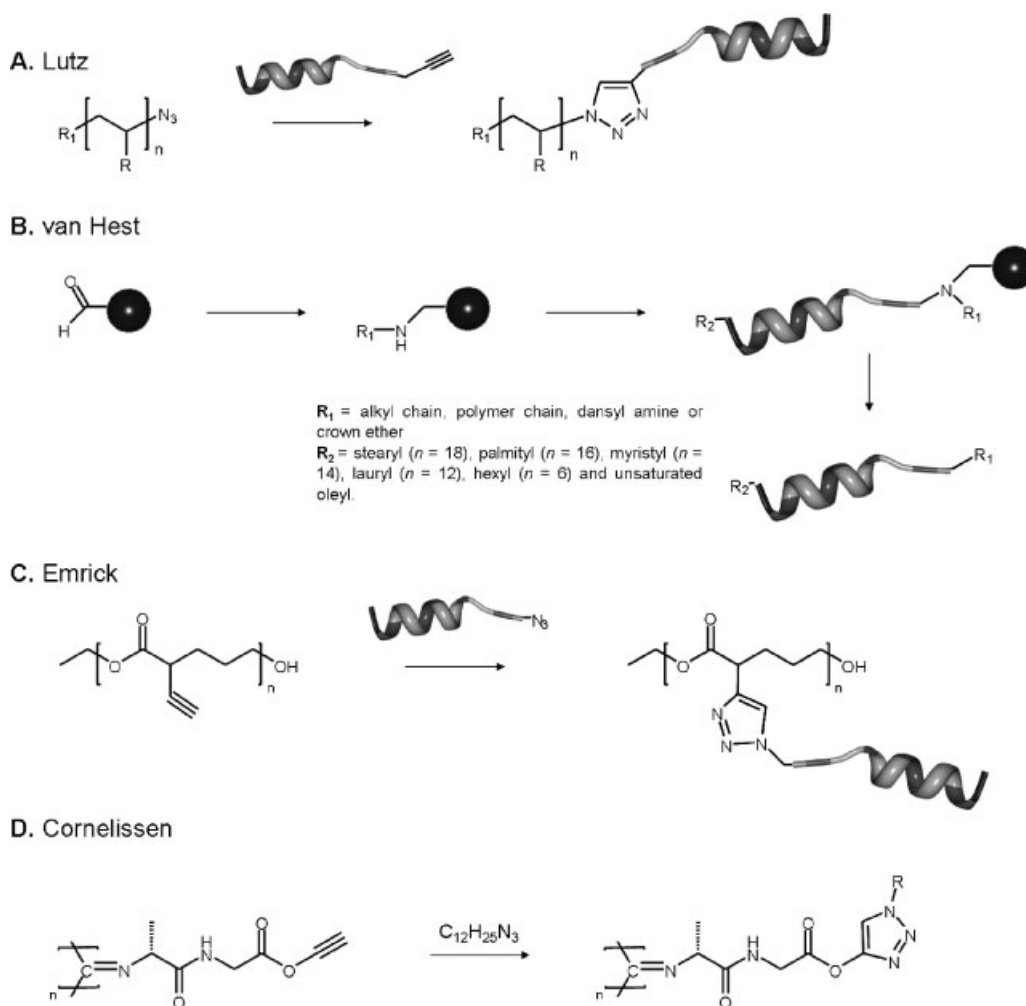
synthetic polymers that were synthesized by atom transfer radical polymerization (ATRP) was reported by Lutz and coworkers (Figure 2A).<sup>[52–54]</sup> Following their approach, the  $\omega$ -bromine chain-ends of ATRP synthesized polymers were initially transformed into azides and subsequently reacted with alkyne-functionalized peptides.<sup>[53,54]</sup> During the course of their studies, the amino acid protecting side-groups were preserved even upon the completion of the solid-phase synthesis to enhance solubility of the peptide segment in organic solvents and therefore allow the SEC chromatographic analyses of the polymer bioconjugates. Such precaution is theoretically not necessary as other studies indicated that unprotected peptides can be directly used in click chemistry coupling reactions, as also expected from the high chemoselectivity of CuAAC.<sup>[40,55]</sup>

In their studies, van Hest and coworkers<sup>[56]</sup> utilized a conceptually different approach to synthesize peptidic biopolymers (Figure 2B). Following a straightforward experimental protocol, the labeling of the C-terminus of peptides with any desired moiety bearing a primary amine (such as polymers and long alkyl chains) could be achieved. The protocol involved an initial step attaching—via reductive amination—the amine appended molecule to an aldehyde-functionalized resin, which was followed by the coupling of an amino acid sequence to the secondary amine via a standard solid-phase peptide synthesis protocol. Cleavage from the resin, afforded a variety of peptide hybrids.

Recently, Emrick and coworkers<sup>[57]</sup> described the synthesis of novel aliphatic polyesters with pendent acetylene groups and graft density tailored by the controlled ring-opening copolymerization of *R*-propargyl- $\delta$ -valerolactone with  $\epsilon$ -caprolactone utilized during the synthesis. The functionalized aliphatic polyesters proved to be biocompatible by *in vitro* cytotoxicity evaluations, suggesting their suitability for a range of biomaterial applications. Indeed, the subsequent copper mediated clicking of azide-terminated PEGs and oligopeptides proceeded successfully under click chemistry conditions without appreciable polyester degradation and led to the desired amphiphilic and biotailored graft peptidic copolymers that are expected to be useful in several arenas including that of polymer-based delivery applications (Figure 2C).

Following their work on the synthesis of polyisocyanopeptides,<sup>[58]</sup> Cornelissen and coworkers<sup>[59]</sup> recently reported on the synthesis of alkyne-functionalized rigid rod polyisocyanopeptides containing acetylene groups on the side arms as scaffolds that can be multifunctionalized by the copper-catalyzed click reaction as it was demonstrated using aliphatic tails functionalized with azide moieties such as dodecyl azide (Figure 2D).

The creation of combinatorial libraries of assembled and scaffolded peptides is undoubtedly one of the emerging areas in biomedicine and biotechnology. Using



**Figure 2.** Schematic representation of various click chemistry directed synthetic approaches toward peptide-polymer bioconjugates. (A) Click functionalization of POEGA as reported by Lutz et al.<sup>[52]</sup> Reaction conditions: Cu(I)Br, bipyridine, *N*-methyl pyrrolidone under dry argon atmosphere. (B) Solid-phase synthesis of C-terminally modified peptides as proposed by van Hest and coworkers.<sup>[56]</sup> (C) Synthesis of polyester-graft-GRGDS as reported by Emrick and coworkers<sup>[57]</sup> using CuSO<sub>4</sub>/Na ascorbate click catalytic system at 100 °C. (D) Click functionalization of an alkyne-functionalized rigid rod poly(isocyanopeptide) scaffold as reported by Cornelissen and coworkers<sup>[59]</sup> using the CuBr/pentamethylene diethyltriamine (PMDETA) complex as catalyst.

copper(I)-catalyzed 1,3-dipolar cycloaddition, Eichler and coworkers<sup>[60]</sup> efficiently generated model assembled and scaffolded peptides from peptide and scaffold precursors, which were N-terminally modified with azido and alkyne moieties, respectively (Figure 3). For example, three peptidic azides were sequentially 'clicked' onto a cyclic peptide, by incorporating orthogonal protection at the appropriate points on the oligomer, followed by selective deprotection, functionalization with alkyne functionality and finally the click reaction to the desired product. Their approach, proved to be compatible with several types of peptide functionalities.

As expected, click chemistry has also found application in the field of peptidomimetic oligomers. A series of highly functionalized peptidoid oligomers was generated by the Kirshenbaum group utilizing a novel sequential click

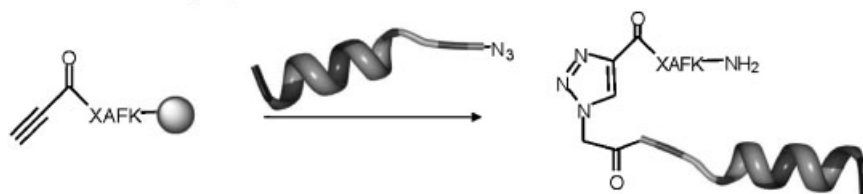
chemistry protocol.<sup>[61]</sup> During the course of their studies, they demonstrated the ability to precisely and efficiently position a variety of functional groups along a peptidomimetic scaffold. The sequential click chemistry scheme that was introduced, demonstrated that 1,2,3-triazole linkages are compatible with multiple rounds of peptid chain elongation on solid-phase support, a scheme that one can easily envision applied for the sequential bioconjugation of polypeptides immobilized on a solid phase and may be amenable to automation.

## Glycoconjugates

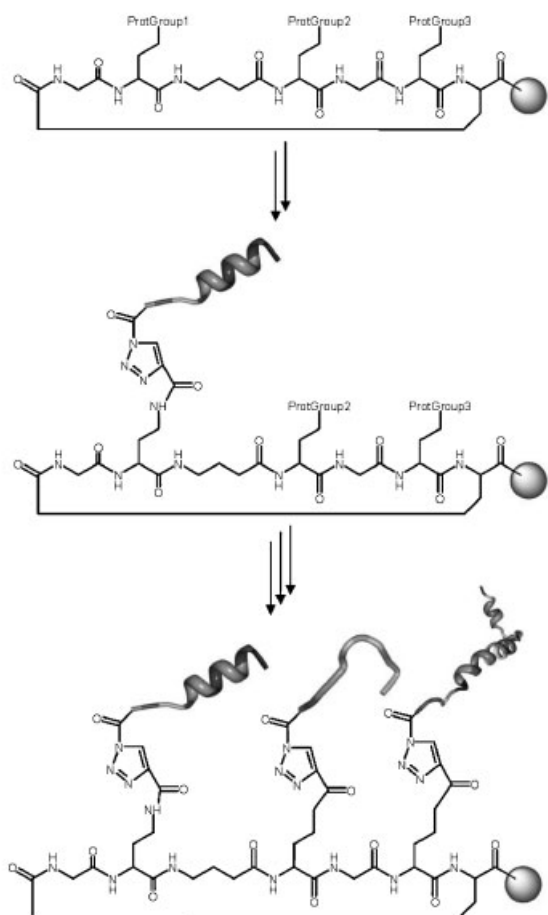
Owing their essential role to their ability to act as energy sources and structural materials as well as to their



## A. Assembled peptides



## B. Scaffolded peptides



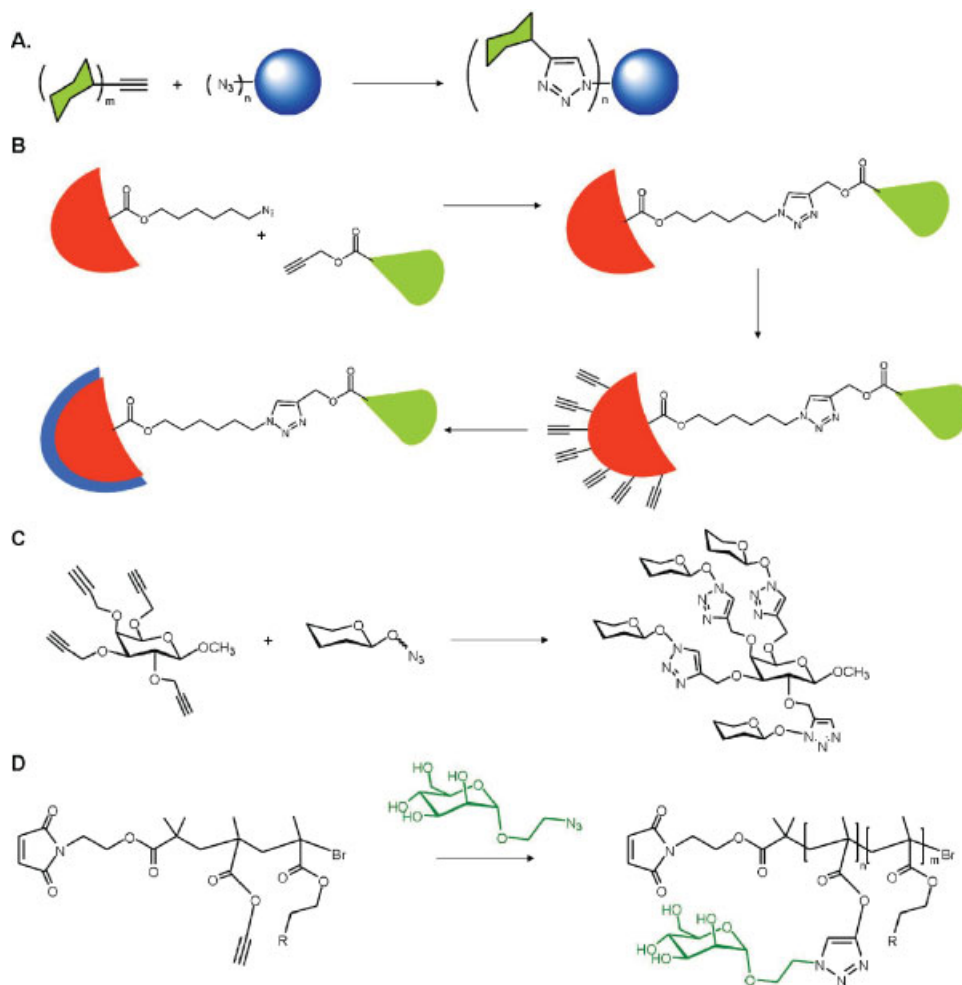
**Figure 3.** Schematic representation of the construction of assembled (A) and scaffolded (B) peptides from peptide and scaffold precursors N-terminally modified with azido and alkyne moieties, respectively, by Eichler and coworkers.<sup>[60]</sup>

different geometries, endowing them the significant complexity and the higher level of structural, functional, and informational sophistication and flexibility which is important for their diverse physiological functions. As a result, their synthesis is far from trivial due to the presence of a large variety of functional groups and the need for chemospecific approaches to control chemo- and stereo selectivity. Furthermore, carbohydrates are often attached to other polyfunctional biomolecules, augmenting the necessity for orthogonal coupling reactions under mild conditions. Click glycochemistry has therefore attracted the attention of many research groups in the area and provided numerous examples for the construction of—otherwise impossible to obtain—glycoconjugates within the last few years. Several examples of click glycochemistry dealing with the synthesis of low molecular weight saccharides and glycol conjugates have been published and reviewed recently.<sup>[67]</sup> Among these, several studies involve the click chemistry mediated functionalization and ligation of protected or unprotected sugar building-blocks (i.e., mono- and disaccharides),<sup>[68–70]</sup> for the formation of hybrid structures such as defined glycopeptides.<sup>[71–75]</sup> Furthermore, high molecular weight polysaccharides such as cellulose, curdlan, or proteoglycans have also been directly functionalized using click chemistry.<sup>[76–79]</sup>

As a direct logical approach, many applications involve the conjugation of clickable sugar moieties onto appropriately functionalized macromolecular materials such as dendrimers and polymers. Following this approach, the construction of a series of precisely defined multivalent 1,4-disubstituted 1,2,3-triazole neoglycoconjugates, was reported by the group of Santoyo-Gonzalez using cycloaddition reactions either in the absence of a metal catalyst<sup>[80]</sup> or catalyzed by organic soluble copper complexes such as  $(\text{Ph}_3\text{P})_3 \cdot \text{CuBr}$  and  $(\text{EtO})_3\text{P} \cdot \text{Cu(I)}$  (Figure 4A).<sup>[81]</sup> During the later study, it was found that microwave irradiation shortens the reactions' times considerably. In a similar approach

involvement in intermolecular and intercellular communication in almost all biological/physiological processes, oligosaccharides have received significant attention in the last decade in the effort to decipher and define the complexity of multicellular life.<sup>[62–66]</sup> Unlike nucleic acids and proteins, oligosaccharides possess polyvalent repeat units and can therefore polymerize in either a branched or a linear fashion at a number of linkage positions and with

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**Figure 4.** Schematic representation of click chemistry mediated approaches for the construction of multivalent bioconjugates. (A) The Santoyo-González approach using organic-soluble copper complexes  $(\text{Ph}_3\text{P})\cdot\text{CuBr}$  and  $(\text{EtO})_3\text{P}\cdot\text{Cu(I)}$  and simultaneous microwave irradiation.<sup>[80,81]</sup> (B) Synthesis of multivalent, asymmetrical dendrimers as reported by the group of Hawker.<sup>[82]</sup> (C) Glyoclusters preparation using a carbohydrate structure as the core unit by Lee and coworkers.<sup>[85]</sup> (D) Synthesis of neoglycopolymers through a combination of ATRP polymerization and the click chemistry reaction by Haddleton and coworkers.<sup>[90,91]</sup>

published by Sharpless and Hawker,<sup>[82]</sup> the copper(I)-catalyzed azide-alkyne cycloaddition proved to be efficient for the preparation of unsymmetrical dendrimers containing both mannose binding units and coumarin fluorescent units and for the efficient differentiation of the dendritic chain end groups leading to a library of dual purpose recognition/detection agents for the inhibition of pathological conditions such as hemagglutination conditions via multivalent interactions (Figure 4B). During this study, dendrons with unique acetylenic and azide groups at the focal point were synthesized and subsequently coupled with high efficiencies through the formation of a stable [1,2,3]-triazole linkage allowing the sequential differentiation of the chain ends by the introduction of mannose and coumarin unit derivatives to the periphery of individual blocks. Liskamp and coworkers<sup>[83]</sup> outfitted a

series of alkyne-terminated dendrimers consisting of the 3,5-di-(2-aminoethoxy)-benzoic acid repeating unit or its close relative 3,4,5-tris(3-aminopropoxy)benzoic acid with azidosugars. Their approach provided another general foundation for the preparation of multivalent biomolecular constructs since the basis dendrimers utilized are easily variable, contain significant rigidity, and considerable distances between the alkyne containing end groups. Even larger glycodendrimers were prepared by Riguera and coworkers<sup>[84]</sup> by placing the azido functionality of the dendrimer core and click coupling with sugars (mono- and disaccharides) glycosylated with propargyl alcohol.

On a different approach, Lee and coworkers<sup>[85]</sup> prepared glyoclusters using a carbohydrate structure, a galactose, as the core unit (Figure 4C). By introducing four individual alkyne groups on methyl  $\beta$ -D-galactopyranoside they



obtained a tetraalkyne scaffold which was subsequently reacted with azidolinked lactose or *N*-acetyl lactosamine (LacNAc) derivatives to yield, glycoclusters with a sugar core. The resulting tetravalent lactosides were found to be good inhibitors of the RCA120 lectin with two binding sites per dimer and were 400-fold more potent than the free lactose. Later studies have also reported on similar divalent<sup>[73,86–88]</sup> or trivalent systems.<sup>[30,89]</sup>

The main chain of linear polymers has also been extensively utilized to construct carbohydrate derivatives. In one such approach, Haddleton and coworkers<sup>[90]</sup> reported on the synthesis of neoglycopolymers through a combination of ATRP polymerization and the click chemistry reaction. Through the polymerization or copolymerization [with either methyl methacrylate (MMA) or methoxy(poly(ethylene glycol))<sub>300</sub> methacrylate (mPEG<sub>300</sub>MA)] of a trimethylsilyl protected alkyne methacrylate monomer in the presence of a Cu(I)Br/*N*-(*n*-ethyl)-2-pyridylmethanimine catalyst,<sup>[91]</sup> a series of hydrophilic/hydrophobic homopolymers/copolymers was obtained in good polydispersities even after 80% monomer conversion (Figure 4D). The subsequent deprotection of the alkyne functionalities was realized in mild conditions using TBAF in THF and acetic acid as a buffering agent<sup>[92]</sup> and was followed by the co-clicking of azido  $\alpha$ -mannoside and  $\beta$ -galactoside sugar derivatives in different ratios. This protocol allowed the synthesis of a wide diversity of glycopolymers with control over the polymer's epitope sugar content. The bioaffinity experiments that were performed in the presence of concanavaline A (ConA) by turbidimetry assays demonstrated that the epitope density had a dramatic influence on the rate of glycopolymer–ConA aggregation. In the case of a glycopolymer containing 100% of mannose derivatives on its backbone, the aggregation in the presence of ConA was observed to be dramatically faster to that of glycopolymers containing lower mannose epitope ratios.

In another approach, the disaccharide trehalose, Glc( $\alpha$ 1– $\alpha$ 1)Glc was decorated at the two 6 positions with an azido group and subsequently click polymerized with various diynes.<sup>[93]</sup> The resulting polycationic polymers had a molecular weight ranging from 34 to 40 kDa and were evaluated as DNA delivery agents. Due to its large hydrated volume, the disaccharide induced a reduction in the aggregation of the DNA–polymer complex with serum proteins along with a high gene delivery efficacy.

Multivalent carbohydrates have also been linked to silica gel with the aim to produce matrixes for the analysis or purification of appropriate (protein) mixtures. In such a study, click chemistry was employed to prepare trivalent unprotected mannosides which were then attached via another click reaction to the core silica unit to afford the matrix.<sup>[94]</sup> The sugar modified silica gel was evaluated for affinity chromatography and, as the trivalent mannoside

is a good ligand for the lectin ConA, the later was efficiently isolated from a plant root mixture.

It is worth mentioning that one-pot variants of the click chemistry mediated synthesis of the basic multivalent glycoconjugate precursors were very recently reported by the research groups of Wittmann<sup>[95]</sup> and Wang.<sup>[68]</sup> Both approaches allow surpassing the intermediate isolation step of the poly azido systems and are expected to have an important impact in the development of new synthetic approaches toward glycoconjugates. Furthermore, fluorescent and non-fluorescent multivalent neoglycoconjugates which were recently prepared by means of Cu(I) catalyzed azide–alkyne 1,3-dipolar cycloaddition were subjected to cellular assays using U-937 and RAW 264.7 monocyte/macrophage cells and showed to possess the ability to act as synthetic activators mimicking the lipopolysaccharide (LPS) effects.<sup>[96]</sup> The click compounds proved to promote cell adhesion and stimulation of monocytes, as measured from increase in the amount of TNF $\alpha$ , facilitating their differentiation to macrophages. Considering the flexibility and efficiency of “click chemistry,” the authors suggest that these well-defined and custom-made click multivalent neoglycoconjugates will be valuable compounds in applications not limited to the activation of monocytes/macrophages but also with potential for the development of therapeutics.

Perhaps the ultimate demonstration of the efficiency of click chemistry in glycoconjugation is the rapid and specific covalent labeling of cellular glycans that was recently developed.<sup>[76,97]</sup> In their studies, Wong and coworkers report on the biosynthetic incorporation of 5-azido-fucose into cell surface glycoproteins. A subsequent reaction with alkynylated fluorophores led to the development of an *in vivo* method for imaging fucosylated glycans. This methodology was later improved by the incorporation of ligands (such as polytriazolylamines) for the stabilization of the Cu(I) catalyst, allowing for the reaction to be fully compatible with intact cells as it efficiently proceeds in a selective and mild fashion at room temperature.<sup>[98]</sup>

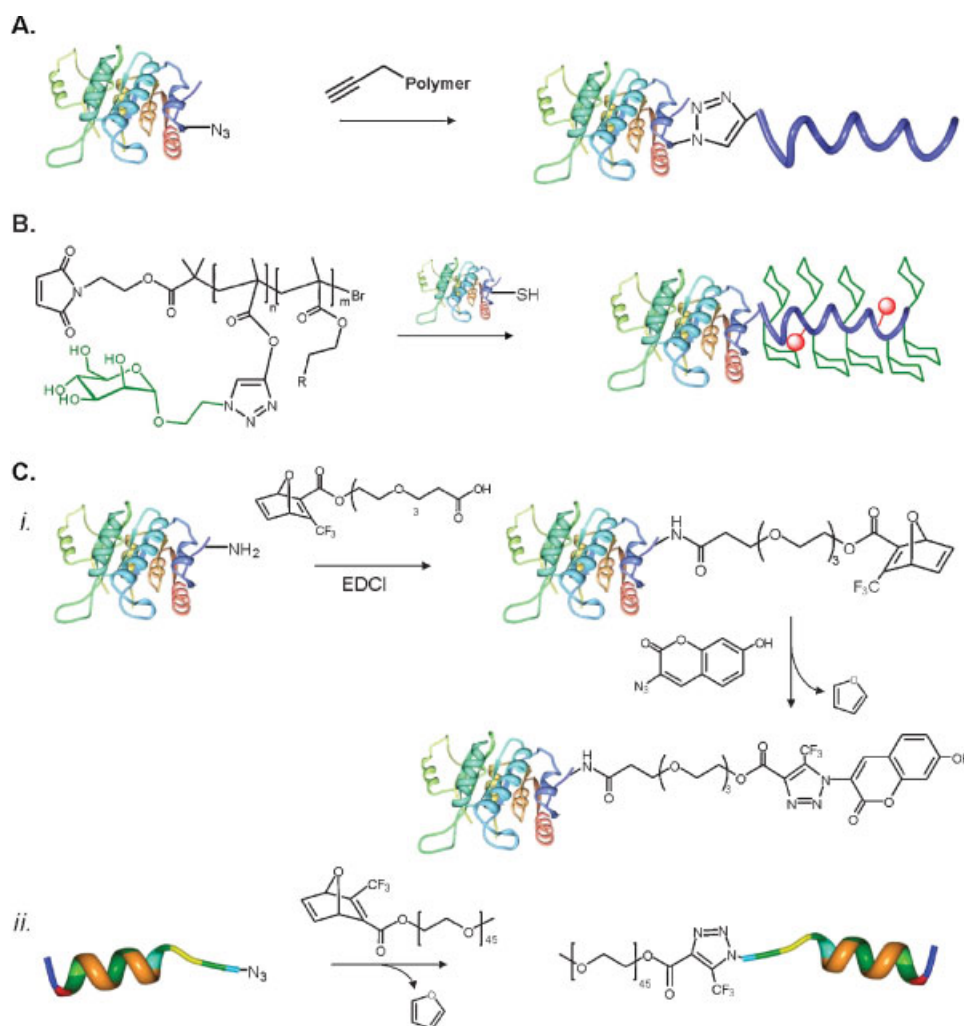
## Proteins

Structurally, proteins are linear biopolymers biosynthesized through templated processes with unparalleled control of monomer assembly, sequence, and molecular weight. Functionally, they offer some ‘high-end’ activities such as recognition, catalysis, and information processing. The unique combination of structure and functionality of proteins makes them ideal candidates as scaffolds or bioactive components for biophysical, medicinal, and biotechnological applications. To this end, the mild, biocompatible conditions of click chemistry reactions have

directed an explosion of studies in the area of protein bioconjugation to either natural or synthetic molecular and/or polymer components. Several precautions to preserve the structural and biological integrity of proteins are though necessary as for example, several proteins possibly chelate the catalytic copper ions and can therefore be either denaturated or loose their function(s). For the purposes of this review we only focus on the click chemistry induced conjugation of proteins to either natural or synthetic polymers.

The enhanced physical and pharmacological properties of PEGylated proteins have driven PEGylation to become probably the most studied area of protein bioconjugation.<sup>[99,100]</sup> Schultz and coworkers<sup>[101]</sup> compared the PEGylation of a genetically modified superoxide dismutase (SOD) with either an alkyne-appended PEG and click

chemistry or the corresponding conventionally utilized PEG NHS-activated esters (Figure 5A). For this reason, the Trp33 residue, which was known to be highly exposed on the exterior of the protein, was genetically replaced by a non-natural amino-acid similar to Tyr, bearing an azide instead of the hydroxy group. The mutated SOD was then reacted with terminal alkyne bearing PEGs in the presence of CuSO<sub>4</sub>, TBTA ligand, Cu wire, and reducing agent TCEP overnight at 37 °C. SDS-PAGE revealed 70–85% yield and a molecular weight corresponding to the specific addition of one PEG chain per protein molecule.<sup>[102]</sup> No PEGylation was observed when using the wild-type SOD under the same reaction conditions. In contrast to the orthogonal click reaction, the NHS-activated PEG chains were non-specifically coupled on the wild-type SOD, affording a variety of products.



**Figure 5.** General synthetic schemes applied for the synthesis of hydrophilic protein–polymer bioconjugates following the bioorthogonal click chemistry philosophy. (A) Direct [1,3] Huisgen cycloaddition of azidated PEG to an alkyne bearing, genetically modified SOD by the group of Schultz.<sup>[101]</sup> (B) Glycoprotein mimics using ATRP and click chemistry by Haddleton and coworkers.<sup>[99,104]</sup> (C) Copper free tandem [3 + 2] cycloaddition-retro-Diels–Alder reactions reported for the protein HEWL (i) and the peptide sequence GGRGDG (ii) by the group of Nolte.<sup>[105]</sup>

Following their previous studies on the synthesis of neoglycopolymers using a combination of ATRP and “click chemistry” reaction,<sup>[90,103]</sup> Haddleton and coworkers<sup>[104]</sup> applied the same method in glycoprotein mimics (Figure 5B). A series of  $\alpha$ -maleimido functionalized glycopolymers was synthesized in a controlled fashion using different synthetic pathways. The first consisted in the polymerization of trimethylsilyl protected alkyne monomers on a  $\alpha$ -maleimido functionalized ATRP initiator using *N*-(ethyl)-2-pyridylmethanimide/Cu(I)Br in the presence of rhodamine B methacrylate (fluorescent comonomer)<sup>[103]</sup> and was followed by the clicking of azidosugar (especially mannose) derivatives. In the second pathway followed during this study, the deprotected alkyne monomer was first clicked with the azidosugar derivative to give a sugar monomer for the polymerization, which was in the second step polymerized on the same initiator. The resulting glycopolymers were then selectively coupled to bovine serum albumin (BSA) through the 1,4-Michael type addition between the maleimide moiety of the polymer and free cysteine residue of BSA at cysteine 34. Studies on glycoprotein mimics led to the observation of the induction of an immunological response via the lectin pathway when compared to either BSA (blank) or mannan, a plant polysaccharide constituted of repeating mannose. It is important to mention that BSA was found to conserve its native conformation after bioconjugation, as verified through Circular Dichroism measurements, and its pseudoesterase enzymatic activity. This is a promising area of research as it would allow combining the properties of proteins (enzymatic activity, ligand affinity, and ability of acting as source of specific amino acid sequences with those of glycopolymers) with those of glycopolymers.

The Nolte group recently reported on a novel, copper free tandem 3 + 2 cycloaddition – retro-Diels–Alder reaction involving oxanorbornadienes which affords stable 1,2,3-triazole-linked compounds (Figure 5C).<sup>[105]</sup> The reaction was successfully applied to the decoration of proteins or small peptides with fluorescent tags. For example, hen egg white lysozyme (HEWL) was functionalized with a heterobifunctional linker bearing at one extremity an oxanorbornadiene moiety and a carboxylic acid in the other (Figure 5Ci). Initially the carboxylic moiety was coupled to the exposed lysine residues of HEWL. Subsequently, the derivatized protein was incubated with azido-7-hydroxycoumarin and gently shaken for 36 h at 25 °C. Achievement of the coupling was confirmed by SDS-PAGE gel electrophoresis through a clear band, visible by both UV light (at 366 nm) and Coomassie Blue staining, corresponding to the biohybrid products. Since this novel reaction releases the toxic product furan, the scope of this reaction might be limited for bioconjugation and *in vivo* applications. The potential of this approach in bioconjugation lies in the increased reactivity of the trifluoro-methyl

substituted oxanorbornadiene derivatives as compared to the electron deficient alkynes, leading to mild reaction conditions, even in the absence of transition metal catalysts, as copper. This potential was also demonstrated with the preparation of the PEGylated oligopeptide GGRGDG (Figure 5Cii).

In 2006, the Chaikof group reported on a protocol using sequentially the two click chemistry methods that have been shown to be exceedingly useful for bioconjugation reactions, i.e., Diels–Alder and the azide–alkyne [3 + 2] cycloaddition reaction, to immobilize different biological materials on solid supports (proteins, biotin, sugars).<sup>[106]</sup> For this purpose, a maleimide-derivatized glass slide was modified in the first step using a heterobifunctional  $\alpha,\omega$ -PEG linker through a Diels–Alder reaction with its cycloaddition extremity. Its other terminus, consisting of a propargyl motif, was in turn clicked with azido-derivatized compounds. An example of site-specific and regiospecific protein immobilization to the alkyne-derivatized surface was provided through studies on a recombinant thrombomodulin bearing a non-natural azide-containing amino acid at the C-terminus together with an N-terminal S-Tag sequence. The efficiency of the coupling was in this case demonstrated by means of fluorescence microscopy. To this end, an S-Tag specific binding protein-FITC was incubated with the *r*TM-N<sub>3</sub> protein-functionalized glass slides and studied by fluorescence confocal microscope which revealed S-Tag binding and therefore the production of surface grafted at a high density with *r*TM-N<sub>3</sub> protein.

In 2007, Choi and coworkers<sup>[107]</sup> reported on the synthesis of poly(oligo-(ethylene glycol) methyl ether methacrylate (pOEGMA) nanobrushes polymerized on SAM. After derivatizing the exposed polymer termini with azide groups, a final click chemistry coupling step was achieved using various alkyne derivatives among which the model biological compound, biotin. The specific interaction of biotin with streptavidin (SA) was confirmed by surface plasmon resonance (SPR) and was compared with that of other model proteins such as BSA, fibrinogen, lysozyme, and RNase A. Furthermore, a series of studies of sugar immobilization on SAM using click chemistry reaction revealed their potential in protein recognition.<sup>[108]</sup>

Recently, Shia et al.<sup>[109]</sup> took advantage of the copolymerization of 5-methyl-5-propargyloxycarbonyl-1,3-dioxan-2-one (MPC) and L-lactide (LA) monomers to create biodegradable electrospun polymer fibers that can immobilize azido-derivatized proteins through [3 + 2] Huisgen dipolar cycloaddition. Briefly, a copolymer was prepared by copolymerization of MPC and LA by ring-opening copolymerization using ZnEt<sub>2</sub> as a catalyst. The copolymer p(LA-co-MPC) was electrospun into continuous fibers with a regular average diameter of ca 2–4  $\mu$ m. In parallel, Testis-specific protease 50 (TSP50), a breast cancer sensitive protease, was non-specifically functionalized with azide

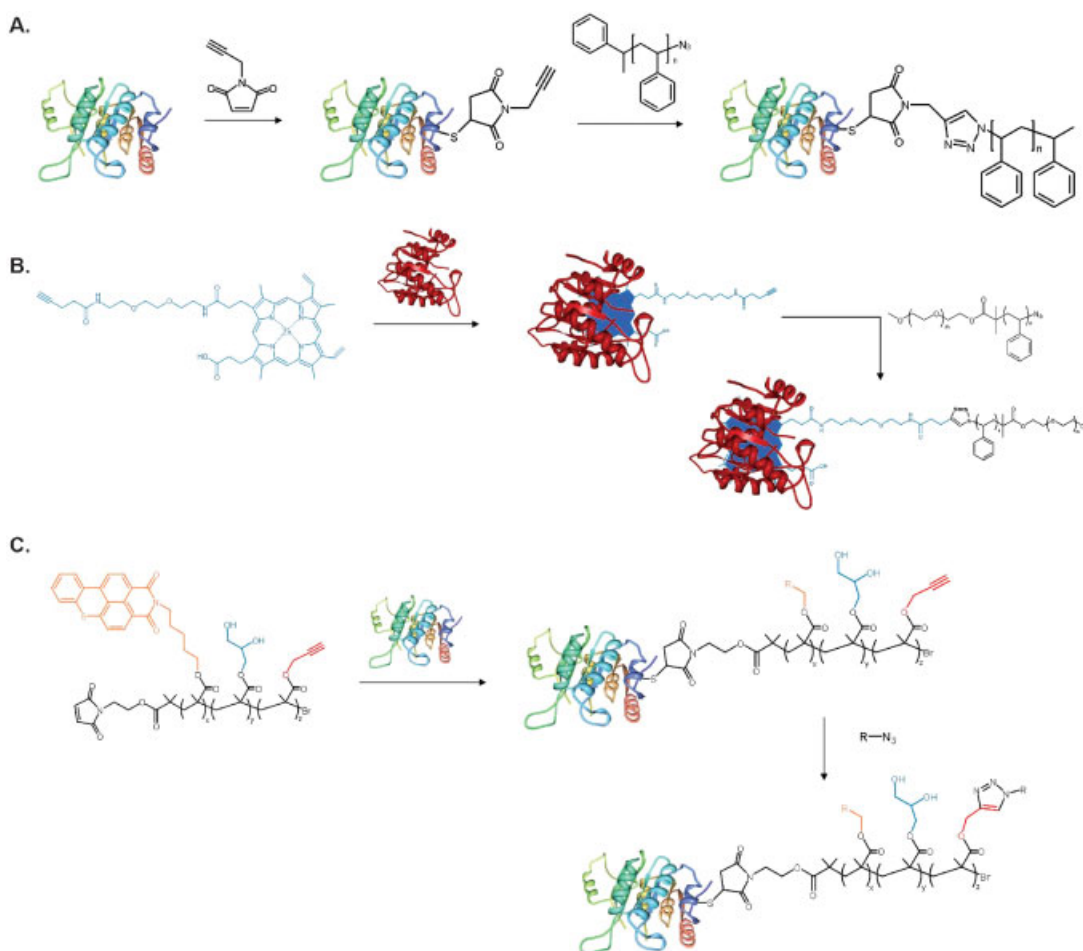
groups<sup>[110–112]</sup> and subjected to a copper (I) catalyzed click chemistry cycloaddition with the p(LA-co-MPC) copolymer. The TSP50-immobilized fibers were successively incubated with anti-TSP50 antigen and then with HRP-Ig. ELISA analysis proved the immobilization of proteins onto the polymeric support. The immobilized anti-TSP50 could be recovered by glycine-HCl elution. This technique displays enormous potential for the selective detection, separation, and purification of any pair antigen/antibody that can be derivatized by azide functions.

A general method for the site-specific covalent conjugation of proteins onto agarose gels was reported by Distefano and coworkers.<sup>[111]</sup> This approach employed initially an enzymatic post-translational modification method to site-specifically label a target protein with an azide, which was followed by a bioorthogonal Cu(I)-catalyzed cycloaddition reaction to covalently attach the protein to agarose and beads bearing alkyne functionalities. It is important to note that in this study it was also

demonstrated that the click capture step could be efficiently performed on both purified and complex protein mixtures.

With the use of the copper(I)-catalyzed [3 + 2] azide-alkyne cycloaddition, Frechet and coworkers<sup>[113]</sup> prepared stationary phases carrying long alkyl chains or a soybean trypsin inhibitor for use in HPLC separations in the reversed-phase and affinity modes, respectively. The ligands were attached via a triazole ring to size monodisperse porous beads containing either alkyne or azide pendant functionalities. When the soybean trypsin inhibitor was functionalized with *N*-(4-pentynoyloxy)succinimide to carry alkyne groups and subsequently allowed to react with the azide-containing beads, an affinity sorbent for trypsin was produced as demonstrated with the HPLC separations of a variety of peptides and proteins.

The click chemical philosophy has also been successful in the synthesis of a particular subclass of polymer-protein bioconjugates called *Giant Amphiphiles*, which



**Figure 6.** General synthetic schemes applied for the synthesis of *Giant Amphiphiles*, i.e., amphiphilic protein-polymer bioconjugates, following the bioorthogonal click chemistry philosophy. (A) Direct [1,3] Huisgen cycloaddition of azidated polystyrene to a BSA bioconjugate bearing one alkyne.<sup>[114]</sup> (B) Click chemistry mediated, cofactor reconstitution approach on HRP and myoglobin.<sup>[115]</sup> (C) Post-functionalization, multiclick chemistry approach yielding BSA *Giant Amphiphiles* with various pendant functionalities.<sup>[116]</sup>

differ from other protein–polymer bioconjugates in the sense that the hydrophobicity of the polymer conveys an overall amphiphilic character to the resulting biomacromolecules. In 2005, the Nolte group reported on the direct synthesis of BSA-polystyrene *Giant Amphiphiles* using click chemistry (Figure 6A).<sup>[114]</sup> A hetero-bifunctional linker bearing an alkyne and a maleimide moiety in the ends was initially coupled to BSA through a 1,4-Michael type addition step. The resulting alkyne-functionalized BSA bioconjugate was subjected to a copper catalyzed Huisgen 1,3-dipolar cycloaddition reaction with an azide-terminated polymer. The resulting *Giant Amphiphiles* were analyzed by FPLC and the aggregation was studied by transmission electron microscope (TEM). Nolte and coworkers<sup>[115]</sup> also recently reported on the cofactor reconstitution approach using well-defined PEG-*b*-PS diblock copolymers and heme proteins (Figure 6B). The polymers were initially linked to the heme cofactor via an azide/alkyne cycloaddition reaction, and subsequently reconstituted with either myoglobin or horse radish peroxidase. Since the click chemistry step is performed independently, this method elegantly allows for the reconstitution to proceed in the absence of the copper catalyst which is known to interact with several protein structures. In 2007, Velonia and coworkers<sup>[116]</sup> developed the so-called post-functionalization approach for the synthesis of *Giant Amphiphiles* (Figure 6C). This approach differs from the previously employed synthetic pathways in the sense that the protein is initially coupled to a hydrophilic polymer leading to a water soluble bioconjugate which can be easily isolated, surpassing therefore the intrinsic purification problems associated with the amphiphilic character of such biomacromolecules. For this purpose, a hydrophilic polymer containing a terminal maleimide moiety, a gradient of glycerol and propargyl repeat units and Hostasol Yellow as fluorescent tracer, was synthesized using ATRP polymerization in low polydispersity. The coupling to the carrier protein BSA proceeded via a Michael addition in good yields and the resulting BSA–polymer bioconjugates were isolated using standard protein purification techniques. The hydrophobicity, responsible for the amphiphilic character of the final product, was introduced in a subsequent step, the post-functionalization step, by a single multiclicking reaction between grafted alkyne moieties present on the polymer backbone and a variety of hydrophobic azides. It is important to note that this method allowed for the first time to observe a difference in the aggregation behavior of the *Giant Amphiphiles*, depending on the nature of the hydrophobic monomer which introduced amphiphilicity.

Finally, click chemistry has also contributed in the creation of protein–protein dimers. In a study published by the Nolte group, a heterodimer of thermomyces lanuginosa lipase (TLL) and BSA was synthesized as part of

studies aiming to specifically position enzymes on surfaces.<sup>[117]</sup> For this reason, BSA was initially functionalized with an azide moiety through a Michael addition on the exposed Cys 34 residue, and subsequently conjugated to a monoalkyne bearing lipase, which was constructed by derivatization of a single accessible lysine residue. The heterodimer could be isolated in approximately 80% yield and possessed improved adsorption as compared to the non-functionalized lipase.

## Viruses and Cells

The azide/alkyne click chemistry has also found application in the case of multimeric and complex biological entities such as the viruses, the bacteria, and the cells. Taking into account their extremely increased degree of complexity, these highly organized structures represent a challenging target when compared to the simpler biological entities that were described in the previous paragraphs. Further precautions are necessary to avoid disassembly and/or loss of activity, while purification and isolation entail further difficulties. The majority therefore of published studies deals with the click chemistry mediated conjugation with small molecular compounds such as dyes.

The pioneering example demonstrating the applicability and versatility of click chemistry in such complex structures was given by Sharpless and Finn already in 2003.<sup>[118–120]</sup> Cowpea mosaic virus (CPMV), the biomolecular scaffold utilized in this study, is a structurally rigid assembly which is composed of 60 identical subunits. In their study, a virus-derivative displaying 60 azido, or acetylenic groups pendant to the external viral surface was quantitatively coupled with appropriately functionalized rhodamine B dyes. As initial results indicated virus decomposition in the presence of copper (II), the disassembly was avoided by using tris(triazolyl)amine (TBTA) as a ligand. Later studies by the same group reported on the attachment of a wide variety of substrates on CPMV using click chemistry.<sup>[121]</sup> Interestingly, during these studies bathophenanthroline disulfonic acid disodium salt was also reported to be successful when applied in conjunction with Cu(I) salts for the decoration of the CPMV virus capsid.

More recently, the same group reported on the formation of virus–glycopolymer fluorescent bioconjugates by an elegant combination of ATRP and click chemistry.<sup>[122]</sup> For this reason a  $\alpha$ -azido-capped glycopolymer of good polydispersity, was synthesized by ATRP polymerization of methacryloxyethyl glucoside. The glycopolymer was subsequently clicked to a dialkynyl functionalized fluorescein molecule, yielding the corresponding fluorescent alkynyl-terminated glycopolymer as



the major product. The fluorescent alkyne-functionalized glycopolymer was clicked to an azido-derivatized CPMV capsid using Cu(I) triflate and sulfonated bathophenanthroline ligand.<sup>[123]</sup> The average number of glycopolymer chains covalently bound to CPMV capsid was calculated to roughly  $125 \pm 12$ , which closely corresponds to the 150 azido groups present on the exterior of the virus shell after reaction with the NHS heterobifunctional linker.

Due to cytotoxicity of the mandatory copper catalyst required for the catalytic version of cycloadditions toward both bacterial and mammalian cells and also toward the catalytic activity of numerous enzymes, catalyst-free Huisgen cycloadditions have also been developed during the last few years. Bertozzi and coworkers<sup>[124]</sup> developed a method that relies on the strain promoted [3 + 2] cycloaddition between a strained cycloalkyne and an azido-derivatized biomolecule. Using this approach, the selective modification of biomolecules in living systems was achieved. More specifically, the [3 + 2] cycloaddition was performed at physiological conditions using cyclooctyne derivatives bearing a biotin moiety and a modified glycoprotein GlyCAM-Ig. These linkers proved to be stable in mild acidic and basic conditions and toward biological nucleophiles such as thiols for prolonged times and the reaction proceeded without any apparent toxicity. Western Blots confirmed the orthogonality of the reaction. In a more recent work, the same group successfully applied this newly copper-free click chemistry reaction to the imaging of dynamic processes in living cells.<sup>[125]</sup> As Staudinger ligation reaction is too slow to follow rapid biological processes and the click chemistry reaction toxic toward living systems, the copper-free version of click chemistry was utilized to follow labeling of proteoglycans of cells within a short time scale. A cyclooctyne derivative capped with an Alexa Fluor 488 dye was utilized to achieve the selective labeling of glycans at the surface of CHO cells. More importantly, the *in vivo* trafficking of labeled glycans within the cells into cell compartments (e.g., Golgi, endosome, lysosome) could be followed within the 1 min time scale of the experiments.

In another ground-breaking example, Tirrell and coworkers<sup>[126]</sup> reported that click chemistry can be utilized for the selective labeling of the cell surfaces of *E. coli* bacteria. In their work, azidohomoalanine, a methionine surrogate, was metabolically incorporated into porin C (OmpC), a protein that is abundant in the outer membrane of *E. coli* bacteria. A copper mediated [3 + 2] azide-alkyne cycloaddition realized in the presence of CuSO<sub>4</sub>, TCEP and TBTA was used to functionalize OmpC with a biotin. The biotin decorated cells could in fact be subsequently stained with avidin and therefore discriminated from cells lacking the unnatural amino acid. In a more recent study, the same experiments were reproduced, this time with three different methionine surrogates and this method was

significantly improved by the substitution of the CuSO<sub>4</sub>/TCEP catalyst with highly pure CuBr leading to an impressive ca. 10-fold more extensive cell surface labeling.<sup>[127]</sup> This method found already practical application in the discrimination of recent from old proteins in mammalian cells.<sup>[128]</sup>

## CuAAC: Precautions and Limitations

Despite the numerous advantages that made the copper catalyzed, azide-alkyne click reaction a universal tool for orthogonal conjugations, several limitations, and precautions should always be taken into account.

Sodium azide, a common reagent for the preparation of azide derivatives, is highly toxic through inhalation in aerosols, ingestion, or skin absorption. While it is not explosive under normal conditions, it does impose explosion risks when shocked or heated to very high temperatures and can form dangerous explosive heavy metal azides if not cautiously utilized in a chemical laboratory.<sup>[129,130]</sup> Furthermore, although high molecular weight organic azides are generally safe compounds, those of low molecular weight are unstable and, therefore, have to be handled with extreme precaution. It is generally advised to consider all low molecular weight azide derivatives as potentially dangerous, not to store them in their purest form and to avoid preparing them in large scales.

The principal shortfall precluding the *in vivo* use of CuAAC in living systems is its requirement of the toxic copper catalyst. As demonstrated in several cases within this review, the adverse interaction of the toxic copper catalyst with several biomolecules, such as proteins and DNA, can introduce undesirable structural and biological modifications. To circumvent such problems, the copper-free variants of click chemistry should be considered when biological systems are involved and focus should be placed on the development of novel, metal-free, and traceless techniques.

## Conclusion

The examples given in this review prove that click chemistry, and especially the copper (I) catalyzed variant of the Huisgen 1,3-dipolar cycloaddition, is an excellent tool for the formation of polymer bioconjugates. Within the last few years, a wealth of applications of this practical and sensible chemical approach has appeared in the area of polymer and material sciences as well as in the areas of pharmaceuticals and drug discovery.

A broad range of polymers bearing biological entities as their backbone monomeric unit has been synthesized using click chemistry. Even more impressively, representatives of all major discrete categories of biological entities (varying from low molecular weight materials, to biomacromolecules or even the cell) were specifically coupled to polymers, giving rise to previously inaccessible biohybrids. However, as the demand for multifunctional, defined polymer bioconjugates increases in the area of bionanotechnology, one has to bear in mind that when dealing with biological entities – and especially living systems – some of the variations of the azide–alkyne “click” chemistry have serious limitations mainly caused by either metal or by-product poisoning. For this reason further focus will certainly be placed in the near future in the development of metal-free, traceless techniques. Nevertheless, since the biological and chemical properties of the hitherto derived bioconjugates have certainly been impressive and unprecedented, these macromolecular chimeras will have without doubt a very exciting future in polymer sciences, nanotechnology, and therapeutics.

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