10

Click Chemistry in the Preparation of Biohybrid Materials

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10.1 Introduction

From a relatively small reservoir of molecules, Nature is able to construct an incredibly wide range of materials that have highly specific functions. Scientists have made extensive use of many natural building blocks to construct materials for applications in medicine and nanotechnology. Molecules, such as peptides, proteins, carbohydrates and lipids, can be assembled into large arrays or can be covalently attached to other biomolecules or synthetic scaffolds, such as polymers, viruses or dendrimers, to generate biohybrid materials with a diverse selection of new and improved properties. The formation of biohybrid materials is motivated by numerous structural and functional reasons and involves the exploitation of biological systems at various levels of their natural hierarchical organization, these being monomeric, oligomeric, polymeric or supramolecular. Building blocks, such as nucleobases, oligonucleotides and oligopeptides, are coupled to scaffolds because of their exceptional capability to self-organize – a desirable property for the creation of highly ordered synthetic nanomaterials – whereas those of higher complexity, such as proteins or enzymes, are used more for their biological properties.[1]

The synthesis of biohybrid materials requires methods that are both selective and biocompatible to ensure the primary properties of the building blocks are retained in the final structure. Numerous approaches have been developed with varying degrees of success. The most critical aspect for the synthesis of biohybrid materials is that the reaction is orthogonal in nature, that is, that it only occurs at the desired site with no side reactions. The copper-catalyzed azide–acetylene cycloaddition reaction (CuAAC) has been found to

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be ideal for this purpose.[2–4] This reaction is efficient at linking two components, avoiding the construction of large and complicated structures that would require the formation of difficult bonds. The CuAAC reaction is high yielding, highly specific, regioselective and has a high tolerance to other functional groups present within the components. It also allows reactions to proceed in many different solvents, including aqueous media, without the loss of efficiency, and is easy to work up. The versatility and scope of the CuAAC reaction has given enormous potential for the construction of new biohybrid materials as the relative unreactivity of azides and alkynes towards most functional groups ensures that bioconjugation occurs only at the desired positions.[5] This reaction has, therefore, been used extensively for biohybridization in the fields of materials and medicinal sciences in recent years and has been widely reviewed.[1,5–16]

10.2 Polymer-containing Biohybrid Materials

The use of polymers of both natural origin (optimized by billions of years of evolutionary changes) and synthetic design (simpler, but chemically more diverse) is an appealing method for the preparation of biohybrid molecules as they generate materials with interesting and diverse properties. Polymers of various chemical and topological compositions (linear, branched, stars) can be prepared allowing a great deal of freedom to material scientists. The combination of biomacromolecules with synthetic polymers is an attractive method for increasing the *in vivo* and *in vitro* applications of these compounds. The conjugation of polymers with proteins alters the solubility and surface properties of the protein and therefore affects its stability, activity and biocompatibility. The applications for these types of biohybrid compounds include the areas of biosensors,[17,18] artificial enzymes,[19] biometrics,[17,18] photonics[20] and nano-electronic devices.[21,22] The appendage of polymers with an alkyne or an azide moiety allows for their post modification by click chemistry with various materials, such as biomacromolecules (proteins, nucleic acids, polysaccharides, etc.), and opens the door to a vast range of possible biohybrid materials. The decorating of polymers with alkyne and azide functionalities in preparation for clicking can occur by different 'controlled' approaches. End-functionalized polymers can be obtained by the polymerization of a monomer using a functional initiator or by the conversion of an existing functional group of a polymer into an azide or alkyne. Side arm appended clickable polymers can be prepared by the polymerization of azide or (protected) alkyne containing monomers using controlled polymerization techniques to generate a variety of well-defined homo- and block copolymers.[6]

10.2.1 Polymers from Controlled Techniques

One of the first reports, in which controlled polymerization techniques were used in conjunction with click chemistry for the construction of biohybrid materials, was given by Opsteen and Van Hest.[23] Using atom transfer radical polymerization (ATRP), a bromide functionalized polystyrene (PS) chain was obtained, which was then converted into the corresponding azide terminated molecule [Figure 10.1(a)]. The azide reacted under CuAAC conditions with several alkyne-functionalized polymers including poly(ethylene glycol) (PEG), PS and poly(methyl methacrylate) (PMMA) to generate the desired block copolymers in a

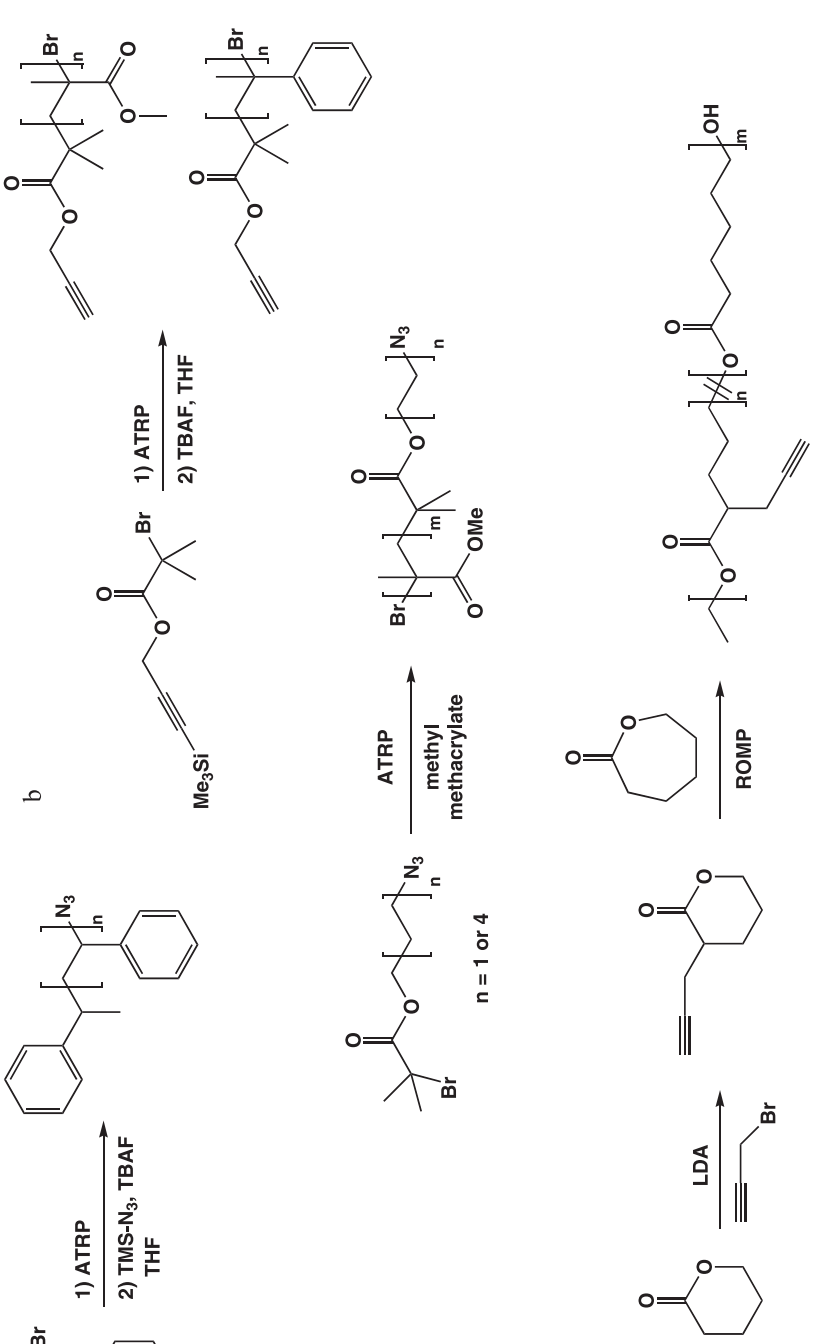


Figure 10.1 *Examples of end- and side-functionalized polymers formed by controlled polymerization techniques for use in the CuAAC reaction from the work of (a, b) Opsteen and van Hest,[23] (c) Haddleton[25] and (d) Emrick.[26]*

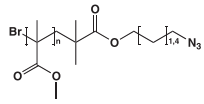
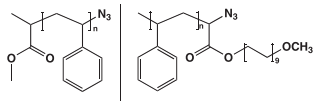
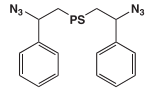
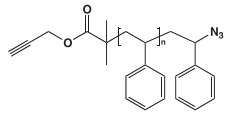
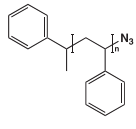
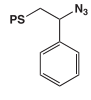
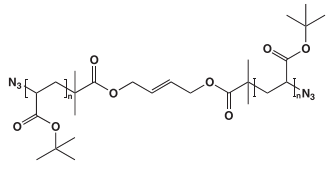
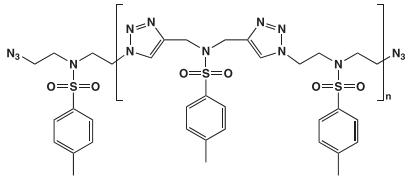
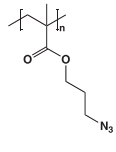
modular fashion and in high yields. Alkyne-appended PS and PPMA blocks were also prepared by ATRP, but in this case a functional initiator, a trimethylsilane-protected acetylene, was used [Figure 10.1(b)]. Matyjaszewski and coworkers have, however, reported a case, in which an unprotected acetylene initiator was successfully used.[24] Azide-functionalized initiators are less commonly employed, but have been used by Haddleton and coworkers to prepare azide terminated PMMA [Figure 10.1(c)].[25] After the ATRP was complete, the azide moiety was further functionalized by click chemistry in one-pot setup using the same catalyst for both processes. The group of Emrick, using controlled anionic ring-opening polymerization, prepared side-arm functionalized aliphatic polyesters [Figure 10.1(d)].[26] The homo- and copolymerization of α-propargyl-δ-valerolactone generated polymers that were clicked to PEG and peptide functional groups. The resulting amphiphilic materials were shown to be biocompatible by *in vitro* cytotoxicity evaluation and have the potential for use in biomaterial delivery applications. Later work by the same authors has resulted in the formation of biocompatible zwitterionic polymers by the clicking of the same homopolymer to phosphorylcholine (PC) moieties.[27]

A large number of azide and alkyne-containing end- and side-functionalized polymers, synthesized by controlled polymerization techniques, are presented in Table 10.1. Further details of some specific examples, with their relevant bioconjugation applications, will be given in later sections.

10.2.2 Bio-inspired Polymers via Click Chemistry

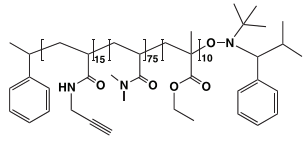
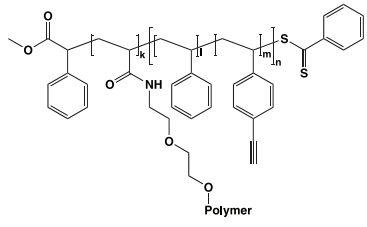
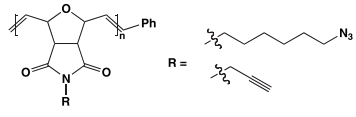
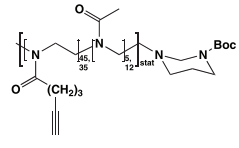
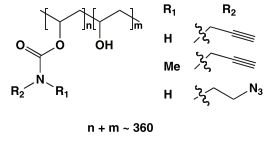
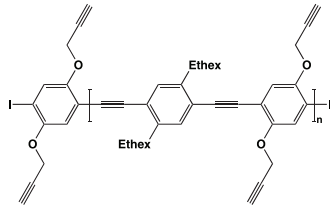
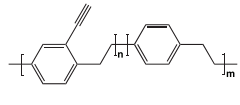
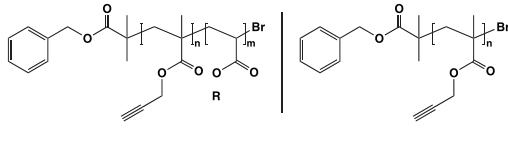
Controlled polymerization techniques can, in some cases, limit the choice of monomers that can be employed for this purpose and consequently limit the diversity of the polymer backbone composition. Other techniques can give access to materials with more bio-inspired structures based on polypeptides or DNA. These materials are of growing interest because of their potential applications as drug delivery systems, scaffolds for tissue engineering and repair, and protein mimics.[46–49] Based on short peptides, the polyisocyanides form well-defined, stable β-helical architectures due to the presence of a hydrogen-bonding chain parallel to the covalent polymer backbone. The hydrogen bonding network rigidifies the array resulting in extremely stiff polymers as seen in the AlaAlaOMe polyisocyanide (L,L-PIAA) shown in Figure 10.2(a).[50,51] The polyisocyanides have a 4_1 helical conformation (i.e. four repeat units per helical turn) with an average spacing between the side chain n and $(n + 4)$ of 4.7 Å [Figure 10.2(b)]. The rigidity, in conjunction with the highly organized arrangement of the side arms makes the polyisocyanides favorable materials as scaffolds for the arrangement of many types of chemical motifs, such as biomolecules or fluorescent markers. The rigid polymers can be readily functionalized with azide or acetylene groups, both at end and side arm positions, which allows for post-modification of the rigid polymers with a wide variety of functional moieties. The groups of Rowan and Nolte prepared end-functionalized polyisocyanides for clicking using functionalized nickel initiators.[52] The use of a functional initiator was previously demonstrated in the formation of poly(styrene)–poly(isocyanide) block copolymers; the isocyanide monomer was polymerized by a poly(styrene)-functionalized nickel initiator.[53] The polymerization of the AlaAlaOMe isocyanide monomer with an azide functionalized nickel initiator resulted in azide end-appended polymers, which on reaction with a coumarin dye were found to emit strong fluorescence [Figure 10.2(c)].

Table 10.1 *Azide and alkyne-containing end- and side-functionalized polymers from controlled polymerization techniques*

Entry	Polymer	Polymerization method	References
1		ATRP	25
2		ATRP	28, 29
3		ATRP	30
4		ATRP	24, 31
5		ATRP	32
6		ATRP	33
7		ATRP	34
9		Polyaddition	35
10		ATRP	36

(*continued*)

Table 10.1 (*Continued*)

Entry	Polymer	Polymerization method	References
12		Nitroxide living-radical polymerisation	37, 38
13		RAFT	38
15		ROMP	39
16		Living cationic ring-opening polymerisation	40, 41
17		Free-radical polymerisation	42
18		polyaddition	43
19		CVD polymerisation	44
20		ATRP	45

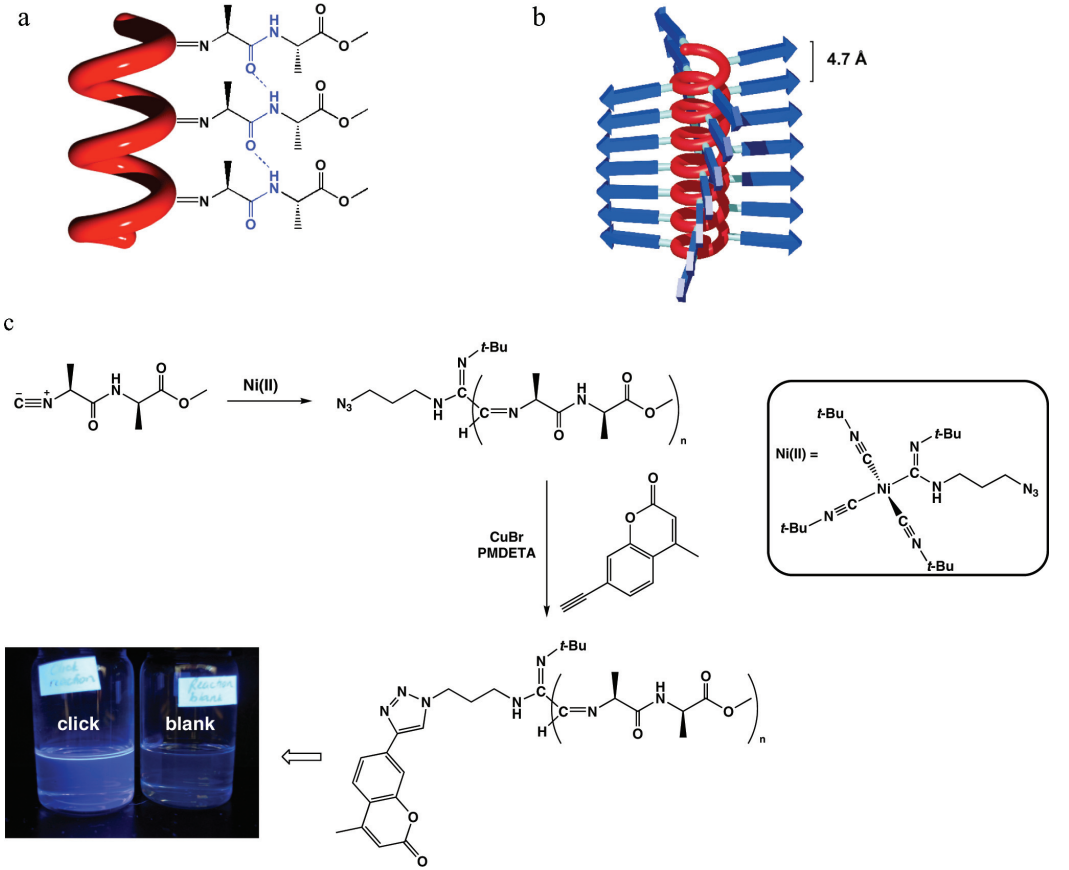


Figure 10.2 *Schematic representations of (a) the hydrogen-bonding network present between the alanine units of the side arms in L,L-PIAA (Reprinted with permission from ref.[50]. Copyright 2001 AAAS) and (b) the 4_1 helical conformation found in polyisocyanides (Reprinted with permission from ref.[51]. Copyright 2002 American Chemical Society). (c) Polymerization of an isocyanide monomer with a functionalized nickel initiator and subsequent clicking with coumarin dye to yield a highly fluorescent polymer.*

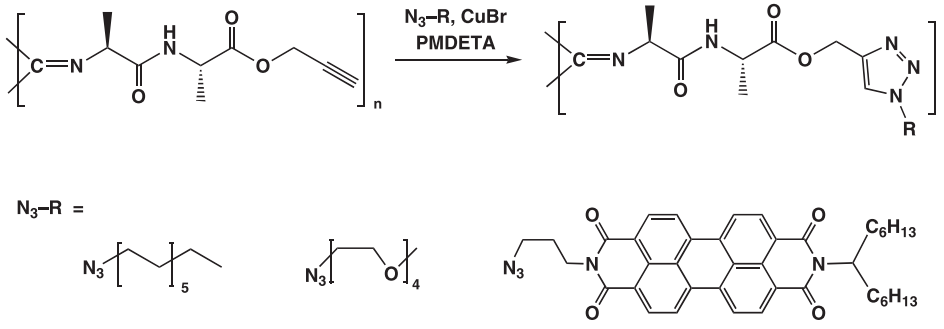


Figure 10.3 *The click reaction of L,L-PIAAPE to form highly functionalized side arm appended polyisocyanides. Reprinted with permission from refs.[54] and [55]. Copyright 2007 and 2008 respectively, Royal Society of Chemistry.*

Rowan, Nolte and coworkers have also shown that isocyano peptide monomers containing acetylene side arms can be effectively polymerized under nickel conditions generating the appended polymers.[54,55] The polymer, poly(L-isocyanoalanyl-L-alanine prop-2-ynol ester) (L,L-PIAAPE) was easily formed and isolated; however, it was found to be only mildly soluble in chlorinated solvents.[54] The solubility was greatly increased on clicking with azide functionalized aliphatic tails, namely dodecyl azide (Figure 10.3). The clicking of the same scaffold was also employed to prepare polyisocyanides decorate with ethylene glycol chains and fluorescent markers based on perylene and coumarin moieties.[55] These reactions resulted in the formation of water-soluble homopolymers from the ethylene glycol azide and fluorescent water-soluble random copolymers by the co-clicking of ethylene glycol and perylene azides. The random clicking of the polyisocyanide scaffold with a mixture of perylene and coumarin azides resulted in polymers from which both absorption and emission from each chromophore could be observed as evidence by a quenched and blue-shifted emission of the coumarin molecules in close proximity to a perylene molecule. The ability to construct, using these scaffolds, water-soluble and modular systems to which other biomacromolecules can be readily attached offers the possibility of many interesting biologically relevant materials.

The need for reliable DNA sequencing and detection methods is important for the diagnosis of pathogenic and genetic disorders and is therefore an area being extensively researched. A variety of methods are available for the sequencing of specific DNA strands,[56] including the incorporation and detection of fluorescently tagged nucleoside building blocks.[57] The enzymatic replacement of each natural building block with a fluorescently tagged analog can, however, be a challenging exercise. An alternative method involves the incorporation of appropriately modified nucleoside building blocks into DNA strands that can then be post functionalized. Following this idea, Carell and coworkers employed solid-phase DNA synthesis to prepare alkyne-functionalized oligodeoxyribonucleotides (ODNs) for CuAAC post functionalization.[58,59] The modified uridine nucleosides (Figure 10.4) were incorporated into a series of 16-mer ODNs and clicked to azido sugars or fluorescent labels under CuAAC conditions The oligonucleotides prepared with the rigid spacer [Figure 10.4(a)] did not result in complete conversion to the triazole due to steric crowding in cases where there were adjacent alkyne-containing nucleotides, whereas those of containing the flexible

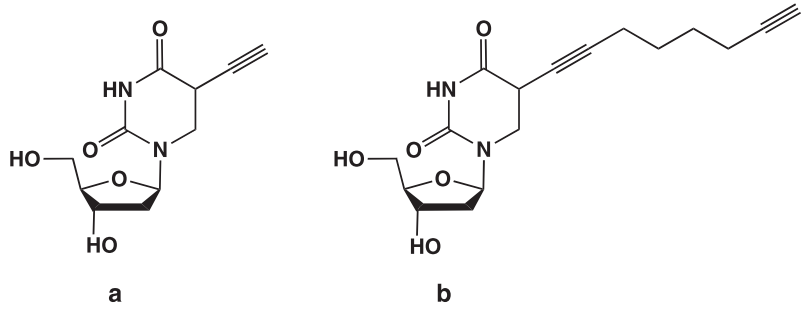


Figure 10.4 *Rigid (a) and flexible (b) alkyne-containing modified uridine nucleosides for DNA incorporation. Reprinted with permission from ref.[58]. Copyright 2006 American Chemical Society.*

spacer [Figure 10.4(b)] gave full conversion. To determine whether this method was viable for longer DNA fragments, primers containing two flexible click sites were synthesized and used in an enzymatic process (the PCR technique) to generate a range of products from two different plasmids. The click reaction of these strands with fluorescein azide gave only a single product in each case, evidenced by gel electrophoresis with no sign of degradation. This methodology was tested on PCR fragments up to 2000 base pairs.

The same authors made use of sugars, which function as hemi-protected aldehydes, for the sequence-selective metal deposition on DNA.[60,61] Using alkyne-decorated PCR products prepared as above, the clicking of galactose azide gave a modified DNA strand that when silver stained gave yellow/brown spots on a gel; natural DNA was stained under the same conditions but was not evidenced. The ability to deposit silver around the aldehyde-modified DNA is a promising result opening the way to a highly sensitive DNA detection method.

Oligonucleotides exhibit an extraordinary range of bioactivities, but their pharmacological properties, such as their ability to transverse the cell membrane, are often poor. Click chemistry has therefore found a role in the modular construction of biomolecules consisting of these components with the hope of improving their use as therapeutic agents. Kirshenbaum and coworkers used solid-phase synthesis to build up *N*-substituted glycine peptoidic oligomers containing azide and alkyne moieties.[62] Repeated chain elongation, followed by click coupling reactions with a range of azide and alkyne-containing compounds, led to highly functionalized bioconjugate materials (Figure 10.5). A water-soluble estradiol–ferrocene peptoid conjugate prepared by this method demonstrated the potential for application in the modular synthesis of biosensors.

Barthélémy *et al*. have clicked a lipid moiety to oligonucleotides (ON) to increase cellular uptake and allow intracellular delivery.[63] A series of lipid ONs were prepared by the attachement of alkyne-modified lipids derived from cholesterol and octadecanol to an azide-appended nucleotide (Figure 10.6). The click reaction generated the triazole intermediates, which were then converted into the phosphoramidites and further coupled onto the ON chain using solid-phase synthesis. The ON sequence chosen for the study was the 17-mer-2′-*O*-methylribonucleotide antisense (ON17mer) of the hepatitis C virus (HCV) RNA, specifically targeting the subdomain IIId of the internal ribosome entry site

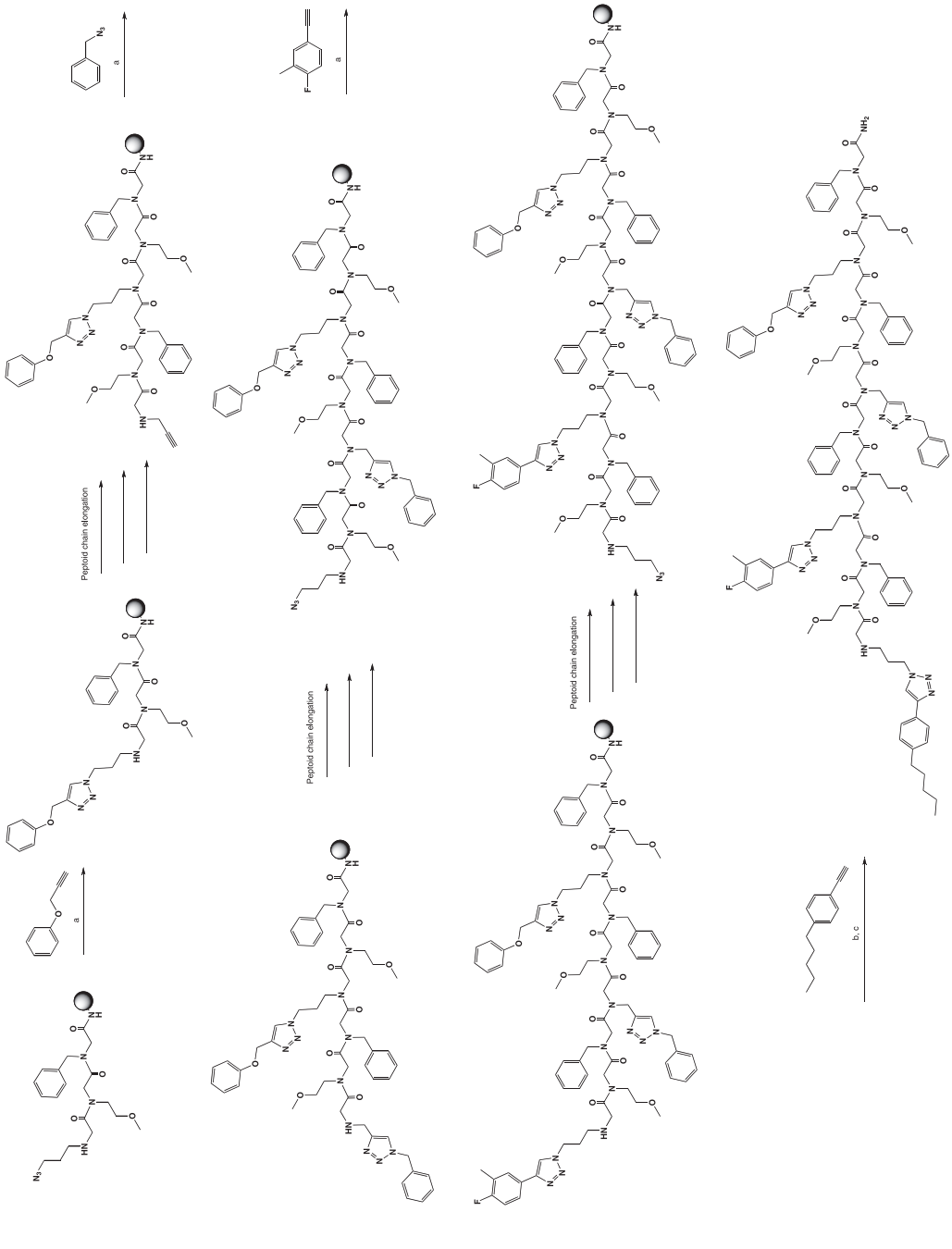


Figure 10.5 *N-substituted glycine peptoidic oligomers formed by the repeated chain elongation and subsequent click reactions to generate highly functional biohybrid materials. Reprinted with permission from ref.[62]. Copyright 2006 Royal Society of Chemistry.*

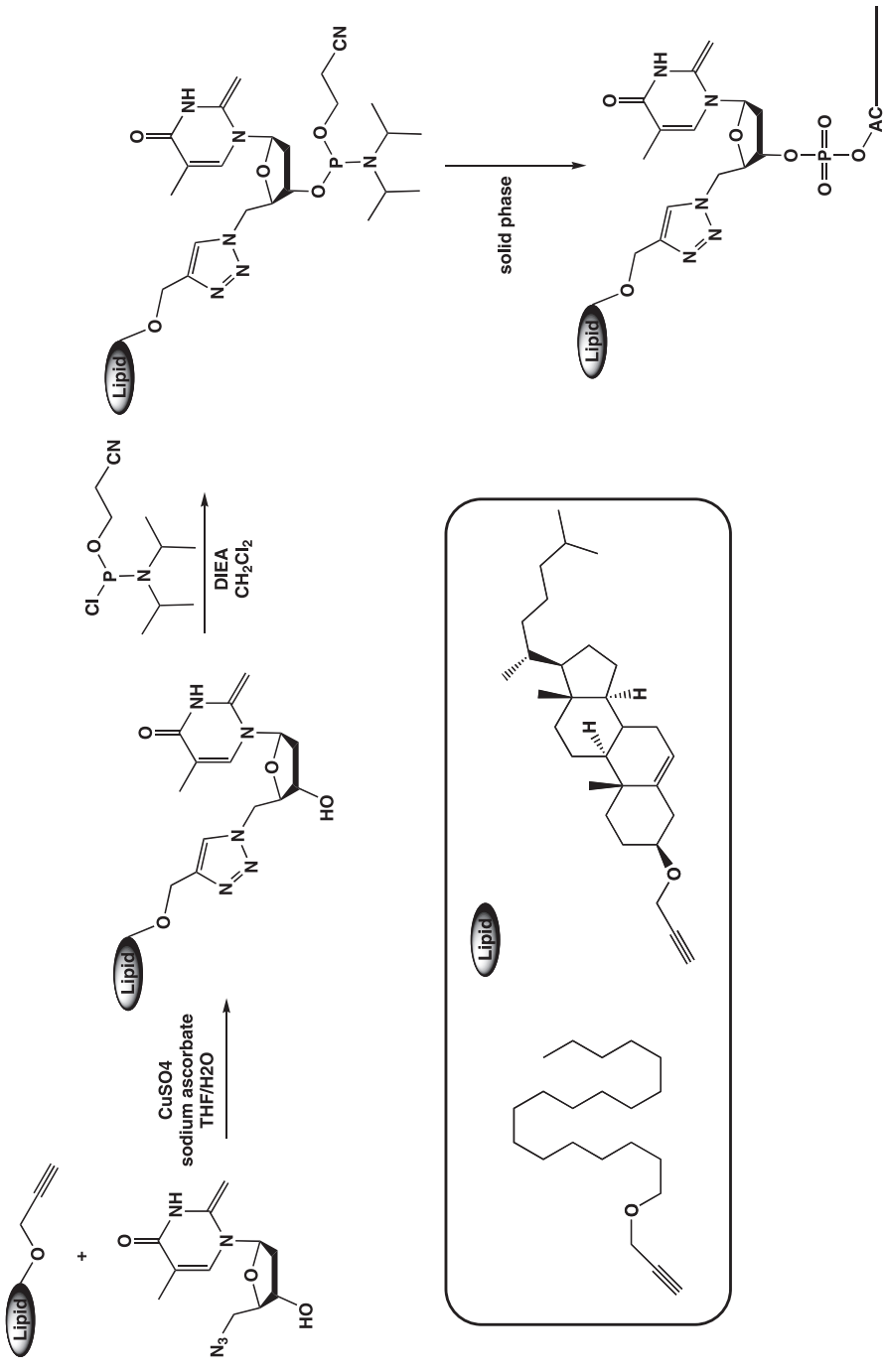


Figure 10.6 *Synthesis of lipid–oligonucleotides. Reprinted with permission from ref.*[63]*. Copyright 2008 American Chemical Society.*

(IRES). The lipid modification was found to significantly increase the lipophilicity of the oligopeptide, allowing cellular uptake and therefore generating an increase in delivery. Both lipid-containing oligonucleotides induced a dose-dependent reduction of the HCV IRES-dependent translation in the human hepatic cells in which they were tested. The toxicity of the lipid–ON conjugates was found to be negligible.

10.3 Biohybrid Structures based on Protein Conjugates

Structurally, proteins are linear biopolymers biosynthesized through templated processes with unparalleled control of monomer assembly, sequence and molecular weight. Functionally, proteins offer numerous desired activities such as recognition, catalysis and information processing.[11] These factors make proteins attractive building blocks for formation of biologically active materials. They can be used either as the bioactive component or as the macromolecular scaffold for the attachment of other bioactive motifs for applications in biophysical, medicinal and biotechnological disciplines.

The conjugation of a protein to poly(ethylene glycol) (PEG; termed PEGylation) often increases the stability and solubility of biomolecules and has therefore become a frequently employed technique in the field of bioconjugation. Peschke *et al*. have elongated human growth hormone (hGH) at its C-terminus by the addition of a Leu–Ala functionality.[64] The C-terminal amino acid could then be converted into an azide, which was then clicked with various sized PEG groups to give PEGylated hGH derivatives [Figure 10.7(a)]. The PEGylated compounds were prepared in the quest to identify long-acting hGH drugs, which would require less frequent injecting, but have the same relative activity as those currently used. The plasma half-life of hGH is increased upon PEGylation, thereby allowing control over its release. The *in vitro* biological activity of these bioconjugate molecules was determined and was governed by both the size and the shape of the PEG group attached. The cases in which branched PEG groups were attached saw a remarkable drop in activity of the coordinated hGH when compared with wild-type hGH, whereas the linear PEG groups showed only a small decrease in activity.

The use of non-natural amino acids fitted with unique reactive groups is also a powerful technique for the site-specific modification of proteins.[65] The group of Schultz has achieved site-specific PEGylation by the incorporation of non-natural amino acids containing azide functionalities into mutant proteins by genetic engineering.[66] In this way, human superoxide dismutase (SOD) was equipped with an azide group at a specific position in the amino acid sequence. Conjugation with alkyne-terminated PEGs of various lengths resulted in enzymes [Figure 10.7(b)], which showed activity similar to that of the native enzyme; a key enzyme in the processes that prevent the formation of reactive oxygen species in cells.

As seen in the previous example, the use of non-natural amino acids can play an important part in protein engineering.[65,67] The effect of replacing an amino acid with a non-natural analog can lead to increased protein stability[68,69] and large spectral shifts in fluorescent proteins.[70] Tirrell and coworkers utilized this approach for the selective labeling on the cell surface of *E. coli* bacteria by the incorporation of an azido functionalized homoalanine moiety into porin C (OmpC; a protein abundant in the outer membrane of *E. coli*).[71,72] Azidohomoalanine, a methionine surrogate, was metabolically incorporated into OmpC and subsequently clicked with a biotin (Figure 10.8). The biotin-decorated cells could then be stained with avidin allowing discrimination between cells containing the natural and

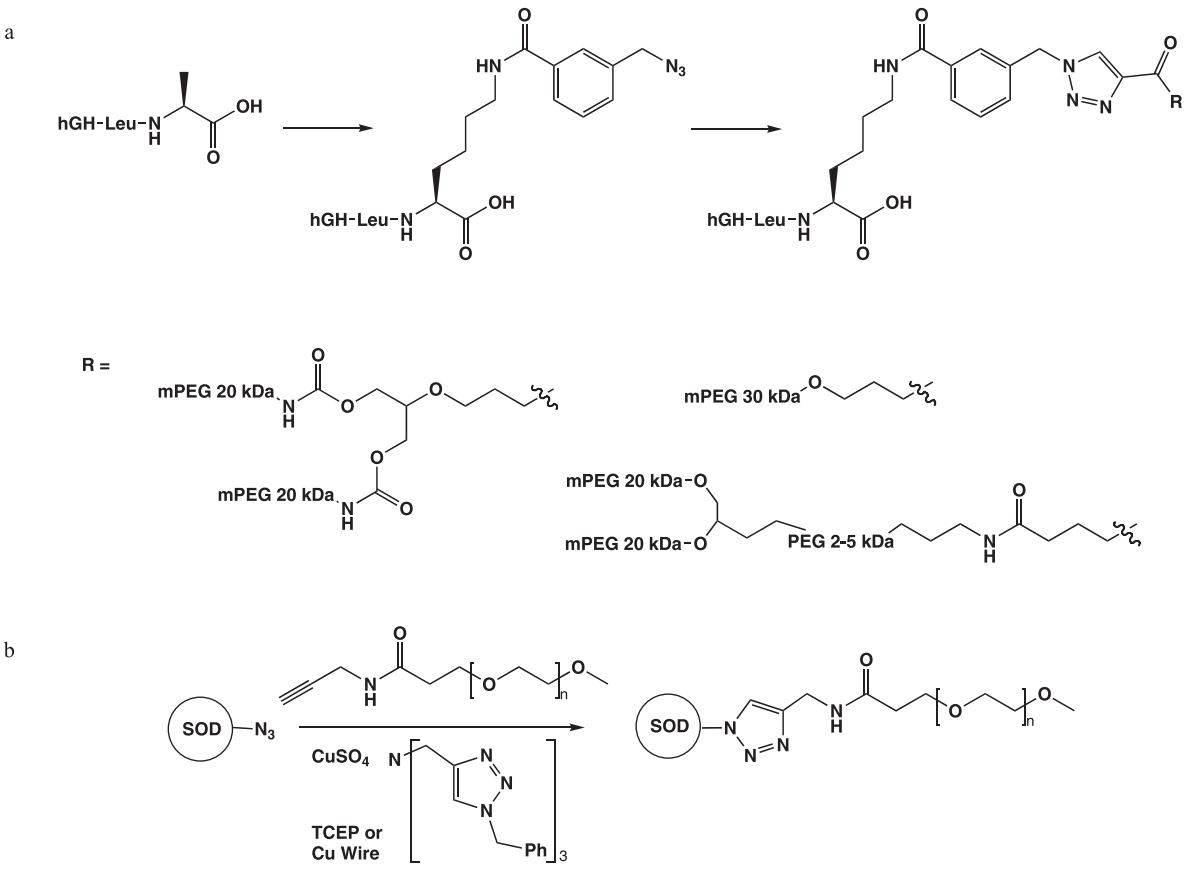


Figure 10.7 *PEGylation of (a) elongated hGH 64 (Reprinted with permission from ref.[64]. Copyright 2007 Elsevier) and (b) SOD with a site-specific incorporated azide functionality (Reprinted with permission from ref.[66]. Copyright 2004 Elsevier) using the CuAAC reaction.*

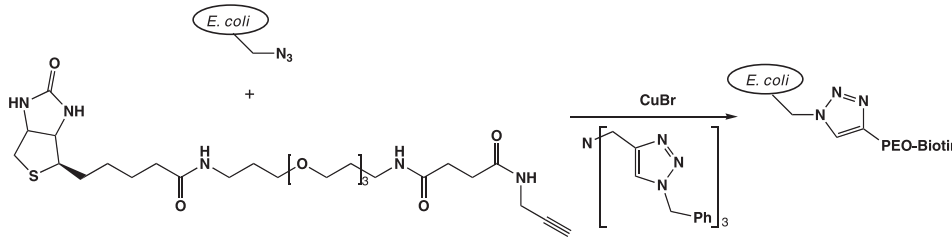


Figure 10.8 *Formation of an* E. coli*–biotin conjugate from a click chemistry reaction. Reprinted with permission from refs.*[71] *and* [72]*. Copyright 2003 and 2004 respectively, American Chemical Society.*

the unnatural amino acids.[71] More recent experiments were conducted with three different methionine surrogates, azidoalanine, azidonorvaline and azidonorleucine, but using highly pure copper bromide as the catalyst instead of $CuSO_4$/TCEP.[72] The highly active catalyst led to approximately 10-fold more extensive cell labeling than previously observed. This method has been used in practical applications in the discrimination of recent from old proteins in mammalian cells.[73] The incorporation of the azide-bearing amino acid azidohomoalanine is unbiased, nontoxic and was not found to increase protein degradation.

Viruses, self-assembled protein architectures, are often used as macromolecular scaffolds for bioconjugation as they provide robust architectures with multiple functional groups on the exterior.[16] These exterior groups can be used to conjugate biomolecules directly or can be modified by the attachment of an azide or an alkyne for CuAAC derivatization. The group of Finn has used lysine, cysteine and tyrosine residues of the Cowpea Mosaic Virus (CPMV) to introduce azide and alkyne moieties onto the structure.[74–77] These compounds were then successfully functionalized with fluorophores,[74,76,77] peptides,[74] proteins,[74] oligosaccharides[74] and glycopolymers[75] in yields and substrate loadings far superior to those possible with previously established procedures.[77–79] One example[74] involved carbohydrates being attached to the surface of the virus particle with the view of being useful for drug targeting, as well as for the elusive goal of antibody production against carbohydrate epitopes.[80] The azide functionalized tetrasaccharide, which binds the protein galectin-4, an early marker of breast cancer cells,[81,82] was subjected to a CuAAC reaction with a dialkyne fluorescein molecule [Figure 10.9(a)]. The resulting dye–alkyne derivatives were then successfully grafted onto the azide appended CPMV by a second CuAAC reaction [Figure 10.9(b)]. The retention of the activity of the tetrasaccharide was verified by the formation of a gel upon the addition of the conjugate to dimeric galectin-4. In order to determine the *in vivo* effects of CPMV, the virus was derivatized with an alkyne appended gadolinium complex of 1,4,7,10-tetraazacyclododecane-*N*,*N*′,*N*″,*N*‴-tetraacetic acid (DOTA) and the toxicity, biodistribution and pathology were determined in mice.[83] The virus was found to be a safe and nontoxic platform for biomedical applications.

Enzymes are also attractive molecules for the construction of biohybrid materials due to the array of chemical conversions they are capable of catalyzing. This functional activity means that they are of great interest as components in the preparation of biosensors and in the areas of catalytically active materials and surfaces. The catalytic activity of enzymes has been vastly studied in bulk, but only recently have their characteristics been studied at the

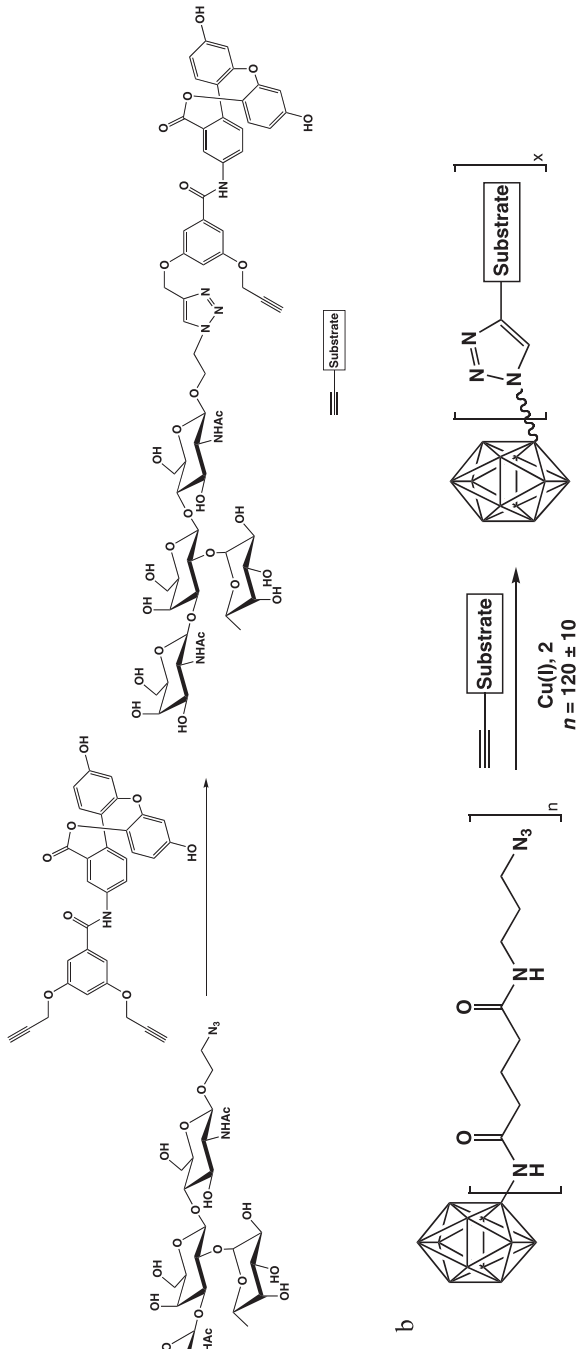


Figure 10.9 *The click reaction of a tetrasaccharide to a fluorescein molecule (a) and the subsequent reaction with CPMV (b) to form a protein conjugate. Reprinted with permission from ref.*[74]*. Copyright 2005 American Chemical Society.*

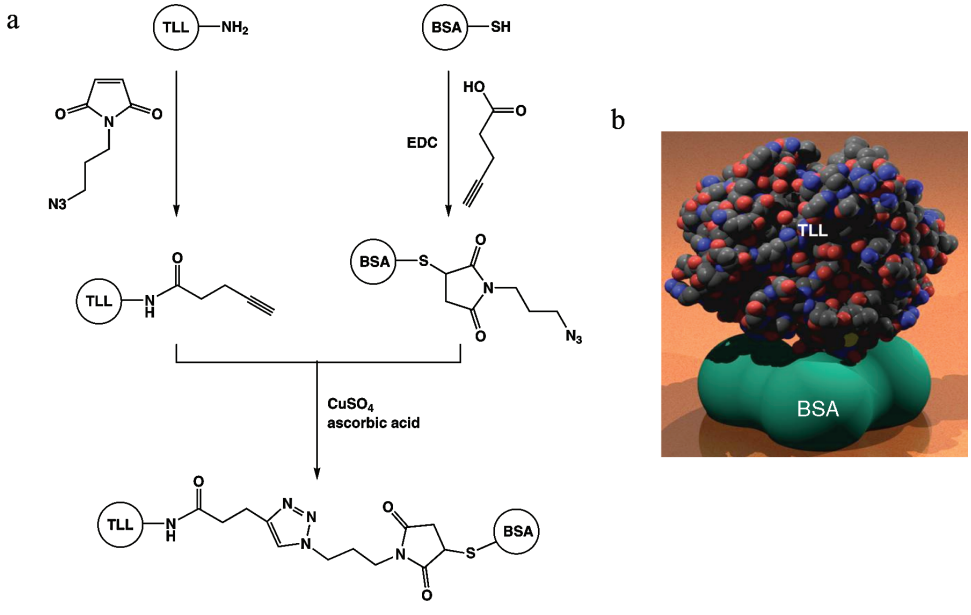


Figure 10.10 *Synthesis of a TLL–BSA heterodimer by means of a CuAAC reaction (a) and schematic representation (b). Reprinted with permission from ref.[88]. Copyright 2006 Royal Society of Chemistry.*

single molecule level.[84] In order to conduct the single molecule studies the group of Nolte employed the enzyme lipase B from *Candida Antarctica* (Cal B), which was adsorbed onto a surface and individually monitored by confocal fluorescence spectroscopy while converting the profluorescent BCECF-AM substrate into fluorescent BCECF acid.[85,86] While this method gave a wealth of information, the process of absorbing an enzyme onto the surface was uncontrolled and only a limited number of enzymes were found to remain active. To overcome this, a mutant of thermomyces lanuginosa lipase (TLL) for which the nonspecific adsorption characteristics were too inconsistent for successful single enzyme studies,[87] was used to construct a heterodimer with bovine serum albumin (BSA).[88] The role of the BSA is to act as a 'protein foot' to stick the enzyme onto the surface. The BSA was functionalized with an azide moiety and clicked to a monoalkyne-appended lipase, constructed by derivatization of the single accessible lysine residue (Figure 10.10). Deposition of the protein dimer resulted in all bound enzymes remaining active and exhibiting comparable behavior. The dimer also showed a two-fold increase in catalytic activity in the conversion of the profluorescent substrates 5-(and 6-)-carboxy fluorescein diacetate compared with that of the nonfunctionalized lipase, making this approach an ideal method for the construction of active enzyme surfaces.

10.4 Biohybrid Amphiphiles

The material and self-assembling properties of proteins have been shown to significantly improve on attachment to polymers,[89–92] making the synthesis of polymer–protein conjugates an important field of research for applications in areas such as nanotechnology

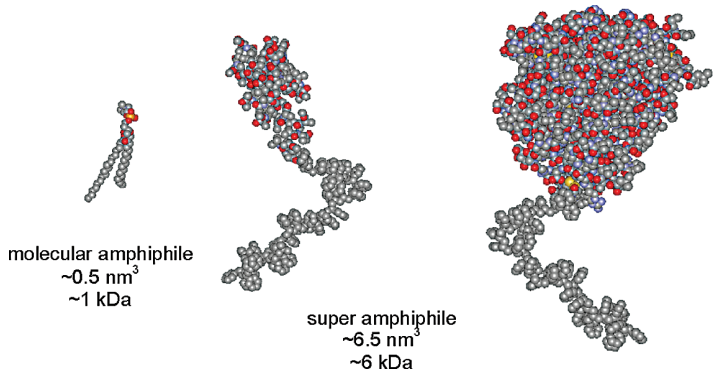


Figure 10.11 *Computer-generated models of molecular, super and giant amphiphiles. Reprinted with permission from ref.[96]. Copyright 2002 American Chemical Society.*

and medicine.[93] The polymers involved are usually water-soluble and in the majority of cases are PEG or PEG analogs. Less well studied is the attachment of a hydrophobic polymer to a biomolecule, a conjugation that results in a polymer biohybrid that is amphiphilic in character. It has been shown in studies of low-molecular weight and super amphiphiles (Figure 10.11) that the shape of the individual molecule determines the structure of the resulting aggregate,[94,95] therefore requiring a highly specific and selective strategy for their preparation. The requirement that, for example, only one tail is attached can be readily controlled using click chemistry making this strategy very attractive.

The Nolte group have formed protein–polystyrene conjugates, 'giant amphiphiles' (Figure 10.11), using the CuAAC reaction[32] and have demonstrated that these compounds exhibit self-assembling properties similar to those of the classical low molecular weight amphiphiles.[96–98] The peptide, H-GlyGlyArg-(7-amino-4-methylcoumarin) (H-GlyGlyArg-AMC) was used as the polar head group as it is easily accessible and contains the AMC fluorophore, a useful tool for characterization. The peptide was functionalized with an alkyne moiety through the reaction of the *N*-terminus with 3-butynyl chloroformate. The apolar PS tail was synthesized by ATRP and subsequent end-group modification. The CuAAC reaction of the two components generated the PS–GlyGlyArg–AMC biohybrid amphiphiles, which were found, by transmission electron microscopy (TEM) and scanning electron microscopy (SEM), to form vesicles in water upon injection from a THF solution [Figure 10.12(a)]. The same PS unit was then coupled to an alkyne-appended BSA molecule generating amphiphiles that self-organized into micellular structures [Figure 10.12(b)].[32] It was shown that the biological function of the protein head groups could be (partially) preserved on conjugation and self-assembly, demonstrating great promise for the construction of biologically active nano-sized assemblies.

Later work from the same authors involved the synthesis of ABC triblock architectures by a cofactor reconstitution approach, as outlined in the schematic in Figure 10.13(a), using well-defined PEG-*b*-PS diblock copolymers and hemeproteins.[99] The PEG-*b*-PS copolymer was chosen for these studies as it is known that this macromolecule, depending on the ratio of the two different blocks, can phase separate into various structures. The diblock copolymer was prepared by the functionalization of monomethoxy PEG with an ATRP initiator and the subsequent polymerization with styrene; the terminal bromine was then converted into

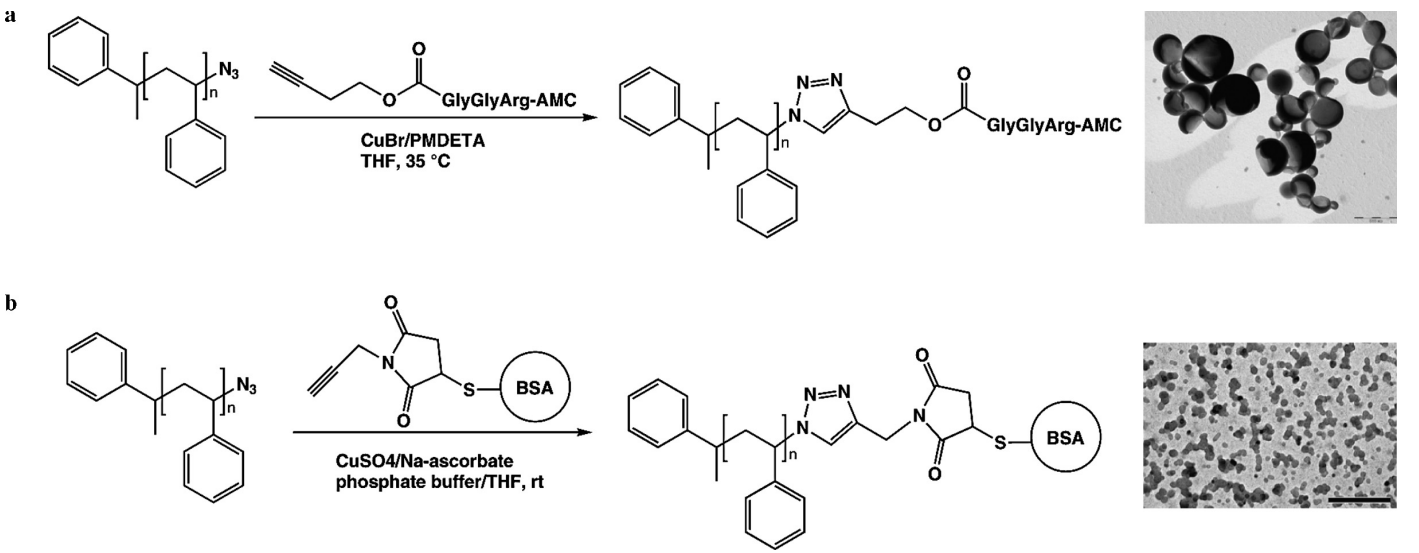


Figure 10.12 *Biohybrid amphiphiles from the click reaction of a PS azide with a tripeptide (a) and a BSA protein (b) and the corresponding TEM images. Reprinted with permission from ref.[32]. Copyright 2005 Royal Society of Chemistry.*

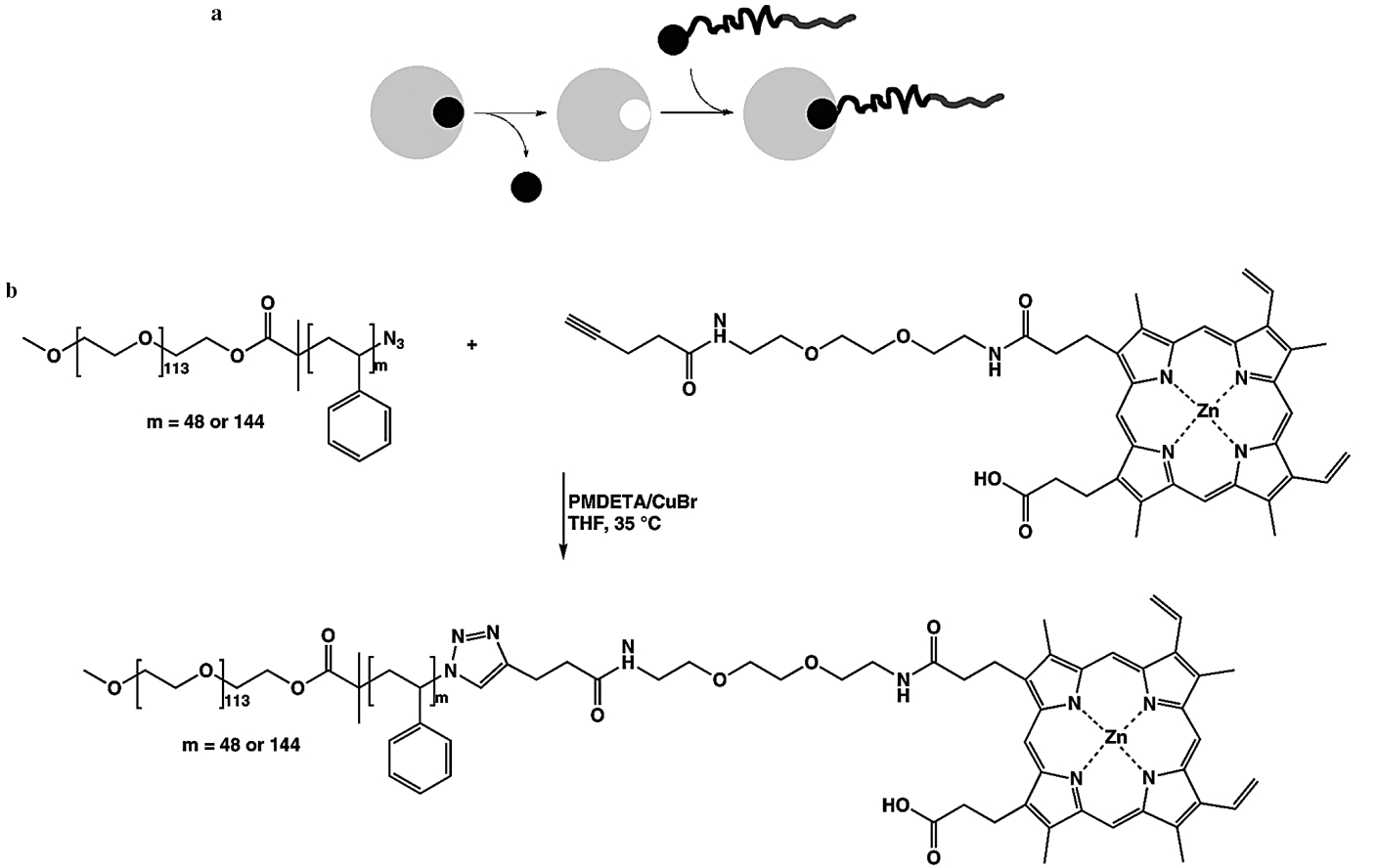


Figure 10.13 *(a) Schematic representation of the reconstitution method employed in the synthesis of ABC triblock polymers and (b) the click reaction of a PEG-b-PS diblock copolymer with heme factor ZnPPIX. Reprinted with permission from ref.[99]. Copyright 2007 American Chemical Society.*

an azide. An acetylene appended heme cofactor, Zinc protoporphyrinIX (ZnPPIX), was coupled to the diblock copolymer under CuAAC conditions [Figure 10.13(b)] and subsequently reconstituted with myoglobin or horseradish peroxidase (HRP). Reconstitution was achieved by the gentle shaking of a mixture of the polymer and the apoenzyme in a plastic tube leading to the gradual obtainment of stable aggregates.

Since the click reaction is performed prior to reconstitution, the latter proceeds elegantly in the absence of the copper catalyst, which has been known to interact with protein structures. Reconstitution with the proteins resulted in a range of more complex aggregate morphologies compared with those observed for PEG-*b*-PS, including micellular rods, vesicles, toroids, figure-eight structures, octopus structures and spheres with a lamellar surface.

The groups of Velonia and Haddleton introduced a post-functionalization approach to giant amphiphiles.[100] This differs from the above method in the sense that a protein is coupled to a hydrophilic polymer generating a water-soluble biohybrid, which can be easily isolated and purified – a difficult feat for some amphiphilic copolymers. The hydrophobicity responsible for the amphiphilic character of the final molecule can then be introduced by means of a click reaction. To demonstrate this, a hydrophilic α-maleimido poly-1-alkyne was prepared using ATRP. This polymer was then coupled to a BSA protein by the Michael addition of the terminal maleimide with a thiol group. The resulting hydrophilic multifunctional bioconjugate was isolated using protein purification and fully characterized before undergoing the CuAAC reaction with hydrophobic azides to generate the amphiphilic species (Figure 10.14). Confocal microscopy and TEM studies showed that these giant amphiphiles exhibit aggregation behavior similar to that reported for the direct coupling of a hydrophobic polymer to a protein. These types of compounds are particularly interesting for potential biomedical and biotechnological applications.

10.5 Glycoconjugates

Carbohydrates, or saccharides, are an essential part of life, whether as an energy source (starch), as structural materials (cellulose and chitin) or as the structural core of nucleic acids. Oligosaccharides, because of their involvement in intracellular and intermolecular communications in the majority of biological and physiological processes, have undergone significant investigation over the last decade in order to define and understand the complexity of multicellular life.[101–105] Because of the presence of polyvalent repeat units, carbohydrates can polymerize in a branched or a linear fashion at a number of linkage positions. This gives rise to many different geometries and a therefore a high degree of complexity. This is evident when it comes to their synthesis, which, in contrast to peptides and nucleic acids, is far from trivial as a result of the large variety of functional groups present and the need for control over chemo- and stereochemical factors. In addition, carbohydrates are often attached to other biomolecules, such as lipids, proteins and nucleic acids, highlighting the need for orthogonal coupling reactions that use mild conditions. Click glycochemistry has proven to be a valuable tool in the construction of glycosylated biohybrid materials and allows the construction of materials that are otherwise unobtainable[8].

10.5.1 Carbohydrate Clusters

A direct logical approach to carbohydrate containing biohybrid materials involves the conjugation of clickable sugar moieties onto functionalized macromolecular materials, such

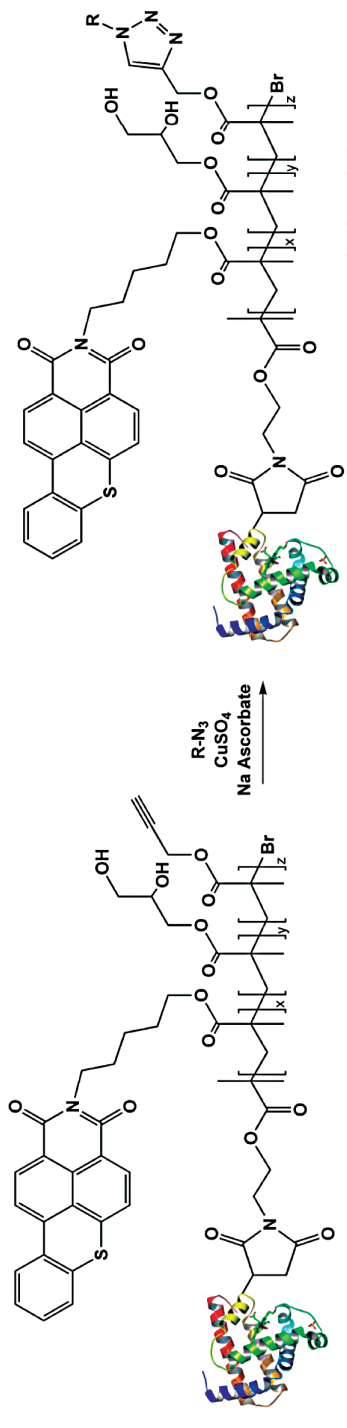


Figure 10.14 *Formation of giant amphiphiles by the post-functionalization approach. Reprinted with permission from ref.*[100] *. Copyright 2007 Royal Society of Chemistry.*

as polymers or dendrimers. Sharpless and coworkers prepared, using the CuAAC reaction, unsymmetrical dendrimers containing both mannose binding units and coumarin fluorescent units.[106] One such biohybrid, based on 2,2-bis(hydroxymethyl)propionic acid, involved the positioning of an alkyne group at the focal point for functionalization with a coumarin chromophore (Figure 10.15). Further alkyne groups were introduced onto the periphery of the dendrimer and coupled with unprotected 2-azido α-D-mannopyranoside molecules. This dendrimer was shown to be a highly efficient dual-purpose recognition/detection agent for the inhibition of pathological conditions, such as hemagglutination conditions, through multivalent interactions. The groups of Liskamp and Pieters also used dendrimers in conjunction with click chemistry. In this case, azidosugars were reacted with a series of alkyne-terminated dendrimers constructed from 3,5-di-(2-aminoethyoxy)-benzoic acid or 3,4,5-tris(3-aminopropoxy) benzoic acid repeat units.[107] Since, in this research, the base dendrimers are easily varied, contain significant rigidity and there is considerable distance between the clickable functional groups, this approach provides a basis for the preparation of a large array of multivalent biomolecular constructs.

In a different approach, Lee and coworkers prepared glycoclusters from an alkyne functionalized carbohydrate core unit.[108] Four individual alkyne groups were introduced onto a methyl β-D-galactopyranoside unit and functionalized, using CuAAC chemistry, with azido linked lactose or *N*-acetyl lactosamine derivatives (Figure 10.16). The resulting sugar-cored glycoclusters were found to be much stronger inhibitors of the RCA_{120} lectin compared with monovalent lactose.

10.5.2 Glycopeptides

The glycoproteins are a class of biomolecules involved in a large number of biological recognitions events.[109] They commonly consist of an oligosaccharide linked, through an *N*- or *O*-atom, to a protein.[80,110,111] This glycosyl–protein bond is intrinsically sensitive towards enzymatic hydrolysis, as is the case with most biological polymers, resulting in limited metabolic stability. In addition, the synthetic assembly of the *O*-glyco-peptides is hindered by the facile elimination of the carbohydrate portion due to β-elimination under basic conditions. The groups of Rutjes[112,113] and Dondoni[114] have independently investigated the incorporation of triazole linkages as stable isosteres for native glycosidic linkages. Although the 1,2,3-triazole functional group does not occur in nature, it is present in diverse biologically active substances exhibiting anti-HIV[115] and anti-bacterial[116] behavior, as well as selective β_3-adrenergic receptor agonism.[117] Interestingly, the triazole moiety has been postulated to act as an amide isosteres in terms of electronic properties and the placement of substituents.[7,118,119] There are, however, noticeable differences between these two functionalities, in particular an increase in the distance between R^1 and R^2 of 1.1 Å for a triazole compared with an amide [Figure 10.17(a)]. The triazole, in addition, possesses a stronger dipole moment (4.83 Debye compared with 3.92 Debye for the amide) – a feature that may enhance peptide bond mimicry by increasing the donor and acceptor properties of hydrogen bonding. The potential of a triazole moiety to act as an imide was demonstrated by Ghadrir and coworkers by the comparison of a triazole-containing octapeptide to a natural peptide.[119] The triazole isostere was found to have similar behavior to the natural peptide with both forming an extended network resulting in solvent-filled nanotubes [Figure 10.17(b)].

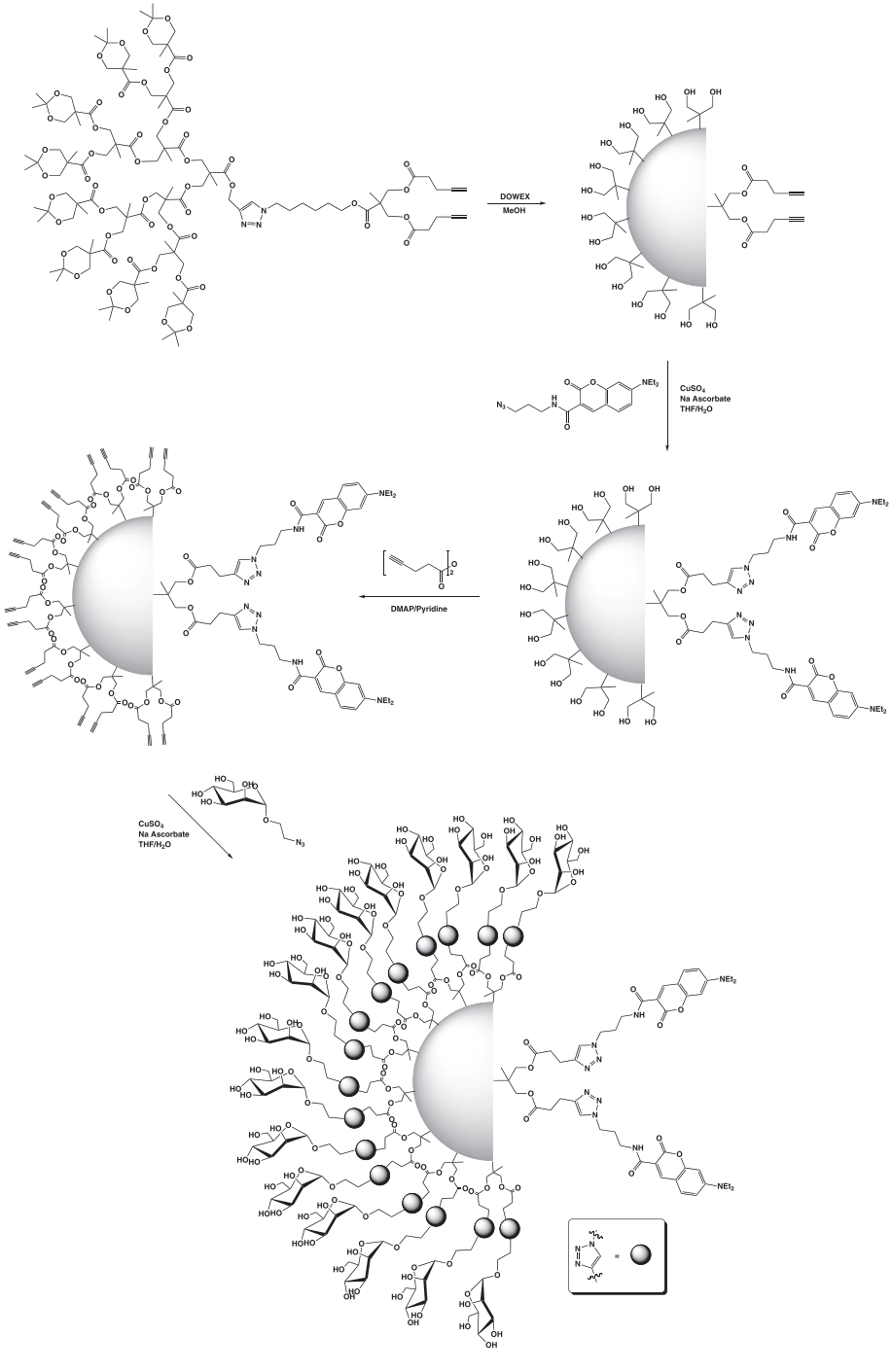


Figure 10.15 *Synthesis of a multivalent, asymmetrical glycodendrimer base on 2,2-bis(hydroxymethyl)propionic acid. Reprinted with permission from ref.*[106]*. Copyright 2005 Royal Society of Chemistry.*

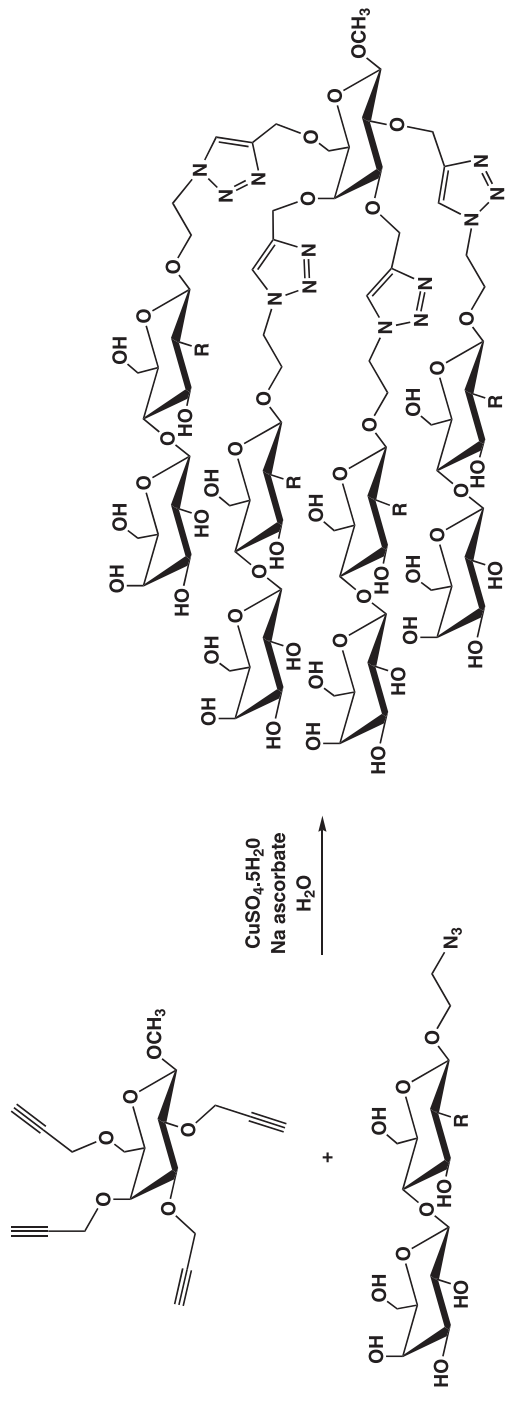


Figure 10.16 *Carbohydrate glycoclusters formed from the click reaction of sugar moieties to a tetraalkyne functionalized carbohydrate core. Reprinted with permission from ref.*[108]*. Copyright 2005 Elsevier.*

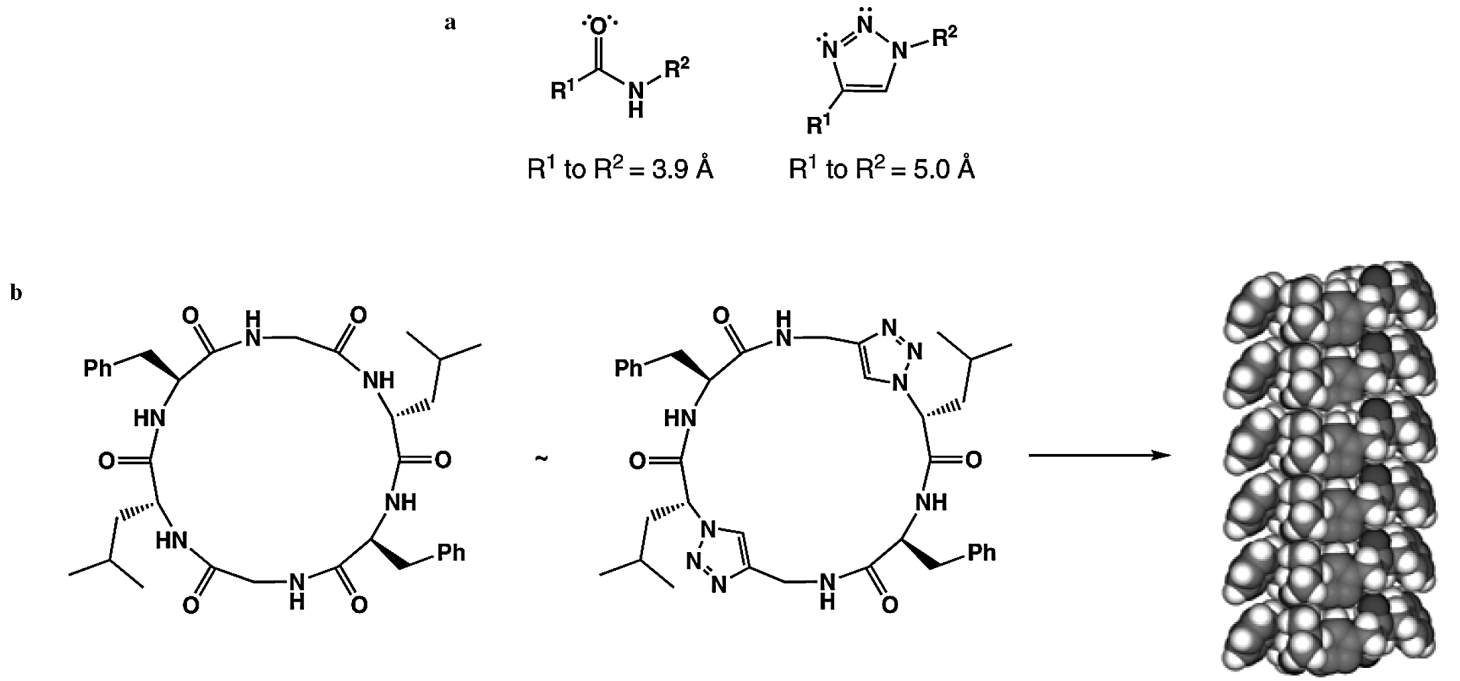


Figure 10.17 *(a) Physical properties of a triazole compared with an amide and (b) nanotube formation from the triazole containing octapeptide. Reprinted with permission from ref.[119]. Copyright 2003 American Chemical Society.*

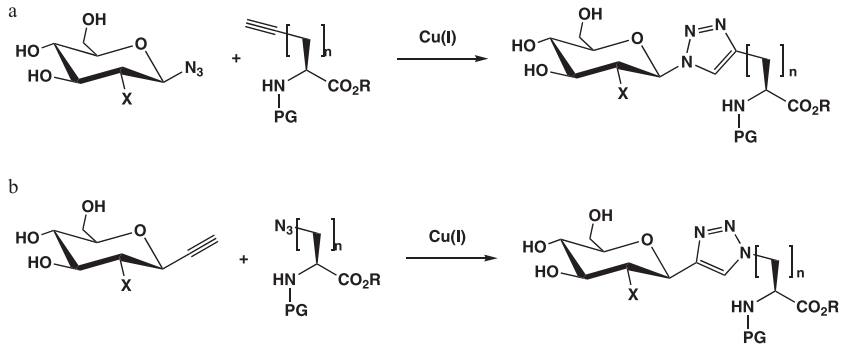


Figure 10.18 *Synthetic strategies for click glycosylation in which the sugar functions as the azide (a) or the alkyne (b). Reprinted with permission from ref.*[112]*. Copyright 2004 American Chemical Society.*

To determine the synthetic viability of triazolyl glycoamino acids, Rutjes *et al*. performed the CuAAC reaction with a range of anomeric azidoglycosides with *N*-Boc-propargylglycine methyl ester and isolated the triazole-linked products in good yields [Figure 10.18(a)].[112] Variation of the amino acids (either by differing the amino acid or by changing the protecting group of propargylglycine) was then investigated and found to generate the desired products in good yields. In addition, increasing the length of the side chain of the amino acid resulted in no significant change in formation of the triazole linkage. The scope of the click reaction was extended to dipeptides and disaccharides and in both cases the triazole-linked compounds were obtained in satisfactory yields. Finally, the inversion of the alkyne and azide moieties between the carbohydrate and amino acid groups [Figure 10.18(b)] also readily generated the conjugated products in good yields. The described methodology was later successfully combined with enzymatic C-terminal elongation of amino acids, performing click glycosylation either before or after an enzymatic peptide coupling step under the action of alcalase.[113]

To further evaluate the amido isosteric properties of the triazole moiety, Rutjes and coworkers synthesized side chain glycosylated cyclic arginine–glycine–aspartate (*c*RGD) derivatives for biological comparison studies.[120] The RGD peptides are found in proteins of the extracellular matrix, such as vitronectin, fibrinogen and laminin. This motif is specifically recognized by integrins (heterodimeric transmembrane proteins), which link the intracellular cytoskeleton with the extracellular matrix and therefore play an important role in cell signalling, cell–cell adhesion, apoptosis and cell–matrix interactions. To evaluate the binding affinities for $\alpha_v\beta_3$ integrin two glycosylated *c*RGD derivatives, one containing the triazole moiety, and two reference *c*RGD compounds (Figure 10.19) were synthesized by a combination of solid phase and solution phase techniques.

The affinities of *c*(RGDfV), *c*(RGDyV), *c*(RGDy-*N*-1-β-gluco-Asn]) and *c*(RGDy-^{N}TGA) for $\alpha_v\beta_3$ integrin were determined by competitive binding assays using dimeric^{111}In-DOTA-E-[*c*(RGDfK)]$_2$. It was found that the binding of the dimer to $\alpha_v\beta_3$ was inhibited by each compound in a concentration-dependent manner with only relatively small differences between the peptides observed [IC_{50}: 65 nM for *c*(RGDfV); 144 nM for *c*(RGDyV); 238 nM for *c*(RGDy-[*N*-1-β-gluco-Asn]); 144 nM for *c*(RGDy-^{N}TGA)],

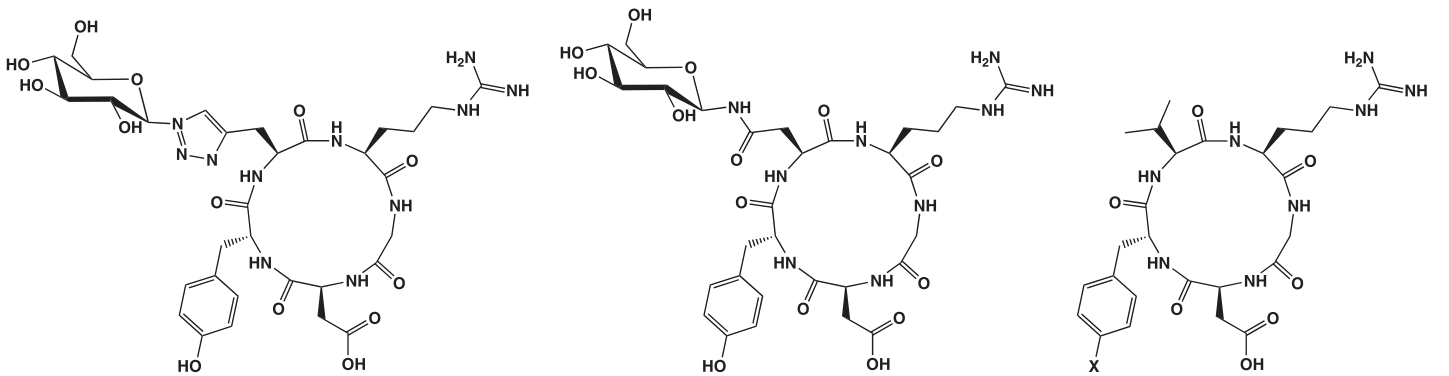


Figure 10.19 *Cyclic RGD peptides for integrin targeting. [aN]TGA = nitrogen-coordinated triazole-linked glycoamino acid. Reprinted with permission from ref.[120]. Copyright 2007 American Chemical Society.*

indicating that side-chain modification has only a limited effect on the $\alpha_v\beta_3$ binding affinity of compounds. Additionally, the fact that only small differences are observed between the *c*RGD peptides with carbohydrates attached indicates that the glycoamino acid binding characteristics are nearly unchanged upon substitution of the amide linkage with a triazole. Given that side chain glycosylation of peptides is known to improve the pharmacological properties of hydrophobic/lipophilic peptides,[121] the biodistribution of ^{125}I-*c*(RGDyV),^{125}I-*c*(RGDy-[*N*-1-β-gluco-Asn]) and ^{125}I-*c*(RGDy-^{N}TGA) in athymic mice with sc $\alpha_v\beta_3$-expressing tumors was determined. All peptides were found to rapidly clear from the blood and 2 h after injection the concentration of the glyco-containing *c*RGDs was higher in the tumor cells than in any of the other tissues examined. In order to determine the nonspecific uptake of the peptides, the biodistribution was also determined in the presence of excess unlabeled DOTA-E-[*c*(RGDfK)]. In this case it was found that, for each of the three compounds being tested, the major part of the uptake in the tumor was $\alpha_v\beta_3$-mediated; the triazole-linked glycopeptide revealed the highest tumor-to-blood ratio, although it showed a lower tumor uptake than that of the amide-linked analog. The carbohydrate-bearing *c*RGD peptides also showed $\alpha_v\beta_3$-mediated uptake in nontargeted organs such as the lung, spleen and intestine. These studies show that tumor uptake is not solely dependent on the binding affinity, but rely also on factors such as blood resident time, molecular weight, structure and charge.

10.5.3 Glycopolymers

The glycopolymers, synthetic macromolecules featuring pendant carbohydrate groups,[12] have been investigated in diverse applications including macromolecular drugs,[122–124] drug delivery systems,[125,126] surface modifiers[127,128] and as models of biological systems.[129] Many of these areas require polymers that have known molecular weight and glycosylation density, as well as the position of glycosylation. Glycopolymers have mostly been synthesized by either the polymerization of a sugar-containing monomer or by the post-functionalization of a pre-formed polymer with sugar moieties.[12] Using the click reaction for the post functionalization of polymers with sugars is an attractive method as libraries of glycopolymers with the same macromolecular features can be obtained by attaching different sugars to the polymer scaffold. This can be of great importance as the effect of the sugar moieties on biological behavior, for example carbohydrate–lectin recognition, can be strongly dependent on the sugar polymer chain length.[130] Haddleton and coworkers have used the post-functionalization of well-defined alkyne containing polymers with sugar azides in their studies of glycopolymers.[45,131,132] A trimethylsilyl methacrylate monomer was polymerized by living radical polymerization (LRP) to form the homopolymer.[45] Sugar azides were coupled to the polymers under CuAAC conditions, with particular focus on sugars able to bind lectins; concavalin A (Con A) was chosen as the model α-mannose-binding lectin as it is involved in a number of biological processes and there are many reports in the literature focusing on its chemical and biological behavior.[133–135] The alkyne functionalized homopolymer was used to form a library of polymers differing only in the amount of Con A-binding mannose ligand and was achieved by the co-clicking of mixtures of mannose- and galactose-based azides (Figure 10.20). The behavior was tested in the presence of Con A and it was observed that the clustering rate and the stoichiometry of the polymer–protein conjugates depended on the epitope density of the polymer,

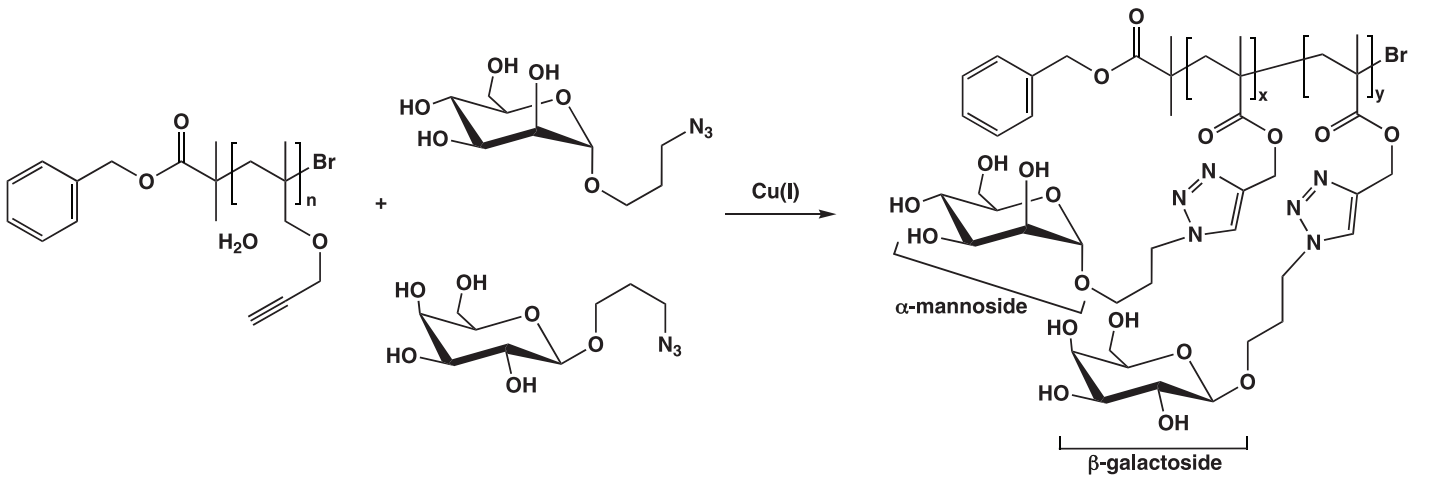


Figure 10.20 *Synthesis of random copolymers employed for Con A binding studies. Reprinted with permission from ref.*[45]*. Copyright 2006 American Chemical Society.*

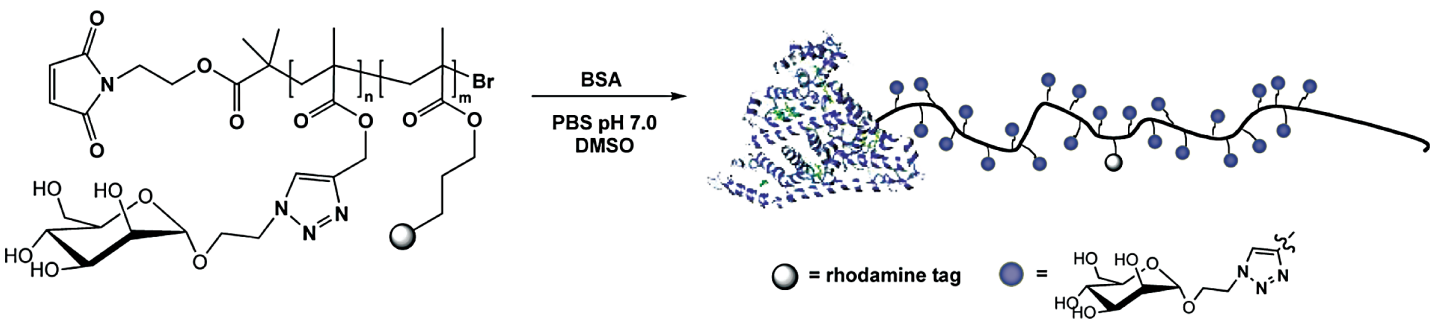


Figure 10.21 *Glycoprotein mimic prepared by the coupling of a clicked glycopolymer to BSA. Reprinted with permission from ref.[132]. Copyright 2007 American Chemical Society.*

that is, the number of Con A tetramers bound by each polymer chain was found to increase with an increase in the mannose units attached to that chain. This indicates that the glycopolymers studied here are able to function successfully as multivalent ligands. The interaction of lactose- and galactose-bearing glycopolymers with the lectin *Ricinus Communis* (RCA I) was also studied and the glycopolymers were again found to act as multivalent ligands.[131]

Further work by the same authors resulted in the site-directed conjugation of clicked glycopolymers to form glycoprotein mimics.[132] The maleimide-terminated glycopolymer was prepared by the click reaction of a mannose containing monomer with propargyl methacrylate. This then underwent living radical polymerization with a maleimide-protected initiator in the presence of a fluorescent rhodamine B comonomer (to facilitate characterization) to give the macromolecular intermediate, from which the furan protecting group was removed by a retro-Diels–Alder reaction. The glycopolymer was then conjugated, through the maleimide terminus, to the thiol group of BSA to give the glycoprotein mimic (Figure 10.21). Libraries of glycopolymers were prepared with the co-clicking approach by introducing appropriate mixtures of different sugar azides. The interactions of these glycopolymer–BSA conjugates with recombinant rat mannose-binding lectin (MBL) were then examined by surface plasmon resonance, which revealed clear and dose-dependent MBL binding to the conjugate. The immobilized glycopolymer–BSA conjugates showed a significantly enhanced capacity to activate the complement system (a biochemical cascade that helps clear pathogens from an organism) through the lectin pathway compared with unmodified BSA.

10.6 Conclusions

A variety of biohybrid materials from the copper-catalyzed reaction between azides and alkynes have been described in this chapter. The click reaction has found widespread use for the synthesis of these materials due to the ease with which it is employed and the ability to form products that are otherwise difficult to prepare. Several conjugates formed by the click reaction are already being used in biomedical areas with promising results, and given that this is a reasonably new field of research, there is an exciting future ahead.

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