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Fluorogenic Copper(I)-catalyzed Azide–Alkyne Cycloaddition Reactions and their Applications in Bioconjugation

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14.1 Click Reaction for Bioconjugation Applications

Bioconjugation has recently emerged as a fast growing technology that affects almost every discipline of life sciences. It aims at the ligation of two or more molecules (or supramolecules) to form a new complex with the combined properties of its individual components.[1] Taking advantage of the outstanding reaction profile, 'click chemistry', in particular the Cu(I)-catalyzed or copper-free alkyne-azide cycloaddition reactions,[2–4] was embraced by bioconjugation chemistry in its early developmental stages.

The first demonstration in bioconjugation application of the Cu(I)-catalyzed alkyne–azide cycloaddition (CuAAC) reaction was reported by Finn, Sharpless, Fokin and coworkers.[5] Cowpea mosaic virus (CPMV), a nonenveloped icosahedral plant virus, was chosen as a protein prototype and successfully labeled at all 60 identical protein asymmetric units of the capsid. Tris(carboxylethyl)phosphine (TCEP), a water-soluble reducing agent, was used to reduce Cu(II) to Cu(I) at 4 °C. Addition of tris[(1-benzyl-1*H*-1,2,3-triazol-4-yl)methyl]amine ligand (TBTA, **1**)[6] drastically enhanced the reaction rate and an almost quantitative amount of modified proteins was able to be recovered after the reaction (Figure 14.1). The azide and alkyne moieties could be attached to lysine, cysteine or tyrosine residues, and the reaction is quite inert to the structures to be conjugated.[5,7–13]

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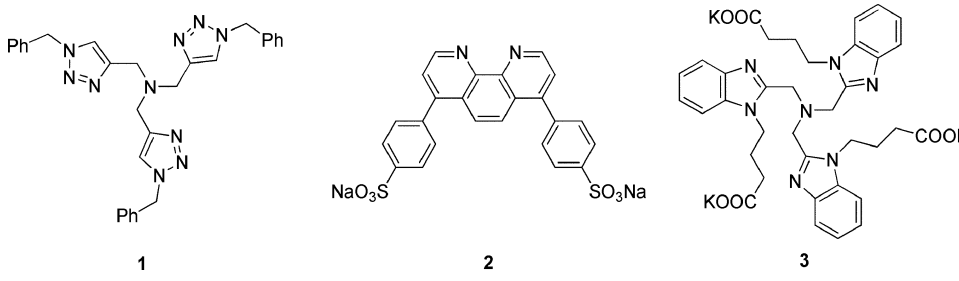
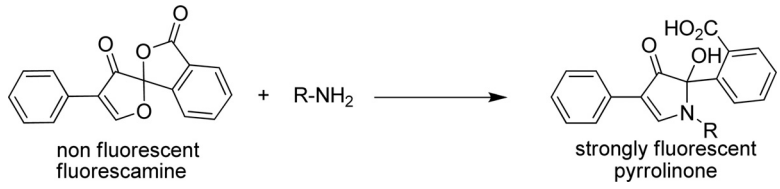


Figure 14.1 *Structures of the most well-known ligands used for CuAAC-based bioconjugation reactions.*

Many ligands and catalytic systems have been developed to make CuAAC reactions suitable for a bioconjugation process, where mild reaction conditions and high reaction efficiency are necessary. Besides ligand **1**, pyridine-containing compounds and benzimidazole-related ligands were found to be effective accelerating ligands to the CuAAC reaction.[14,15] The water-soluble bathophenanthrolinedisulfonic acid **2** and benzimidazole tricarboxylate **3** were confirmed to be remarkably reliable to catalyze a rapid and high-yielding synthesis of functionalized triazoles with an extremely low quantity of copper ion at room temperature or 4 °C (Figure 14.1). Being suitable for most of bio-platforms, CuAAC reactions have been followed by other groups for different bioconjugation applications. For example, Cravatt *et al*. used CuAAC reaction for activity-based protein profiling, where the proteomes (enzymes) of human breast cancer cells were labeled *in vivo*.[16,17] In this study, enzymes were functionalized with azides, homogenized and reacted with tetramethylrhodamine-alkynes. The labeled enzymes were detected and quantified, giving similar results with standard activity-based protein labeling. Tirrell *et al*. incorporated nonnatural azido-amino acids into the *E*. *col*i cell membrane protein OmpC, which were successfully modified with biotin–alkyne via a CuAAC reaction.[18,19] Schultz and coworkers introduced azido-amino acids or alkyne-amino acids into proteins in yeast[20,21] and the pIII protein of M13 filamentous phage.[22] These were sequentially reacted with fluorescent dyes or polyethylene glycols via click reaction. Ju and coworkers have applied the click reaction to fluorescently label DNA.[23,24] In addition, the CuAAC reaction has been applied to immobilize oligonucleotides on glass substrates in well-defined micropatterns.[25,26]

14.2 Significance of Fluorogenic Reactions in Bioconjugation

One important application of bioconjugation is to modify cellular components selectively with signaling probes for the research of *in vivo* imaging, proteomics, cell biology and functional genomics.[27–29] A multistep procedure is commonly employed: the cellular entity is first attached with a detectable tag, such as fluorescent dyes and biotin followed with purification of the ligated product and then detection of the conjugated tag with the target protein. However, excess prelabeled reagents (i.e. fluorescent dyes and biotin) are generally difficult to be removed from the intracellular environment or from tissues of living organisms, which prohibits the application of a multistep labeling procedure in many biological



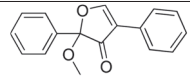
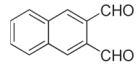
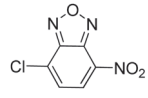
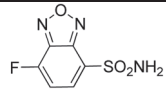
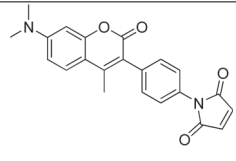
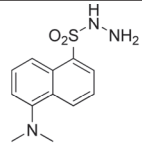
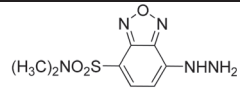
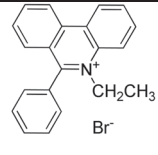
Scheme 14.1 *Fluorogenic reaction between fluorescamine and a primary amine.*

applications. An ideal alternative is a chemoselective process that is orthogonal to biological components, and the ligated product will afford strong detectable signal while the unbound reagent does not contribute to any background. Therefore, the fluorogenic reaction, a process where non- or weakly fluorescent reagents meet each other to give rise to visible fluorescence, would be invaluable for many bioimaging applications.

To date a wide array of fluorescent sensors and switches have been synthesized to recognize important events of chemistry, biology and materials. For example, selective ligands and ionophores for cations and ions are well established in optical sensing and *in vivo* probing.[30] Additionally, many fluorogenic dyes have been developed to detect neutral analytes based on noncovalent interactions.[31–33] In comparison, fewer reagents are available for covalent modification of biomolecules with high specificity and fluorogenic properties. Fluorescamine is one of the best-known reagents; it is intrinsically nonfluorescent but reacts rapidly with primary aliphatic amines to yield a blue-green-fluorescent pyrrolinone (Scheme 14.1). It has been broadly used in protein labeling, protein sequencing, determination of protein concentration and detection of low-molecule-weight amines in chromatography.[34–38]

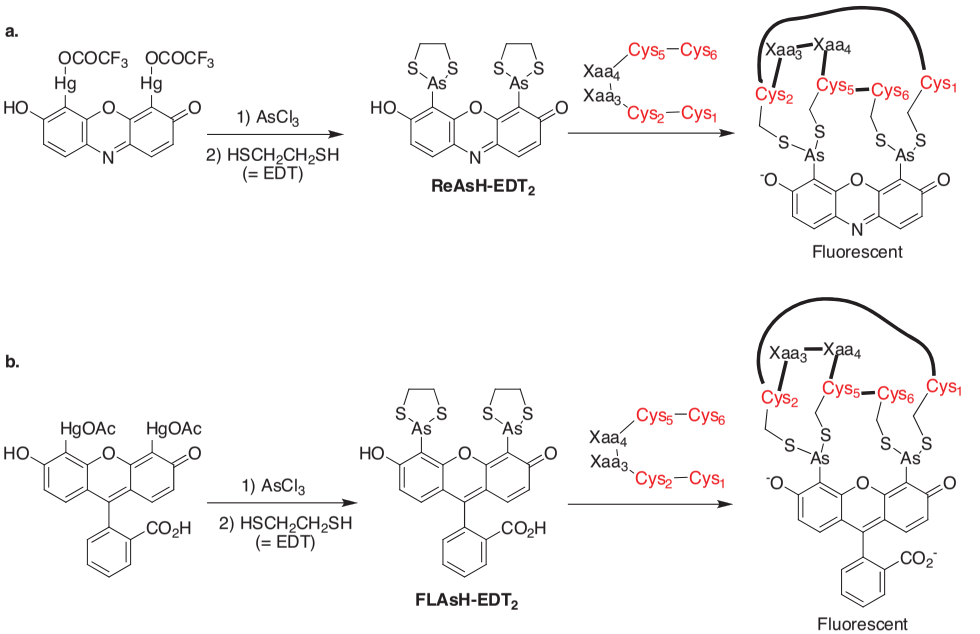
Table 14.1 lists a few other commercially available fluorogenic probes which are able to tag biomolecules containing functional groups like primary amines, thiols or carbonyls, as well as DNA or RNA.[30] However, since amine, thiol and carbonyl groups are the most abundant functional units in biosystems, all these reagents can hardly afford selective modification of a targeted biospecies under the complicated intracellular conditions. In order to distinguish the target protein among the surrounding components, genetically encoded tags such as green fluorescent protein (GFP) and its variants are routinely applied.[39] Although the development of the GFP technology in the past two decades has enabled the use of GFP (or its variants) to signal physiological activation and indicate its chemical environment, GFP is still potentially perturbative because its size (238 amino acids) is often larger than the protein of interest. Therefore, Tsien and coworkers designed a short peptide domain composed of six to 20 amino acids containing the sequence Cys–Cys–Xaa–Xaa–Cys–Cys (where Xaa is a non-cysteine amino acid), and this domain can be genetically incorporated into the protein of interest.[40,41] Two fluorogenic dyes, the bisarsenical derivatives of fluorescein (**FLAsH-EDT$_2$**) and resorufin (**ReAsH-EDT$_2$**), were synthesized (Scheme 14.2). The membrane-permeating **FLAsH-EDT$_2$** and **ReAsH-EDT$_2$** are nonfluorescent but become brightly fluorescent upon binding to the tetracysteine motif.[41] This fluorogenic reaction, designed by Tsien *et al*., is a powerful strategy to label proteins; however there is still a growing interest in visualizing biomolecules that are not amenable to such genetic modification. Furthermore, it is still not applicable for certain applications due to the potential cytotoxicity of bisarsenical compounds at high concentration.

Table 14.1 *Structure of representative commercially available fluorogenic reagents*

Compound[a]	Structure	Reaction partner
MDPF		Primary amine
NPA		
NBD-Cl		Primary amine, thiol
ABD-F		Thiol
D-346[b]		
D-100[b]		Carbonyl
DBD-H		
Ethidium bromide		DNA

[a] MDPF, 2-methoxy-2,4-diphenyl-3(2H)-furanone; NPA, naphthalene-2,3-carboxaldehyde; NBD-Cl, 4-chloro-7-nitro-2,1,3-benzoxadiazole; ABD-F, 4-fluoro-7-aminosulfonylbenzofuran; D-346, 7-diethylamino-3(4′-maleimidylphenyl)-4-methylcoumarin; D-100, 5-dimethylamino-naphthalene-1-sulfonyl hydrazine; DBD-H, 4-(*N*,*N*-dimethylam- inosulfonyl)-7-hydrazino-1,3-benzoxadiazole.

[b] Compound references in Molecular Probes catalog.



Scheme 14.2 *Non-fluorescent bisarsenical dyes* ***ReAsH-EDT₂*** *(a) and* ***FLAsH-EDT₂*** *(b) and proposed structures of complexes with an a-helical tetracysteine-containing peptide or protein domain. Reprinted with permission from B. A. Griffin* et al., *(1998),* Science, ***281****, 269–271. Copyright 1998 AAAS.*

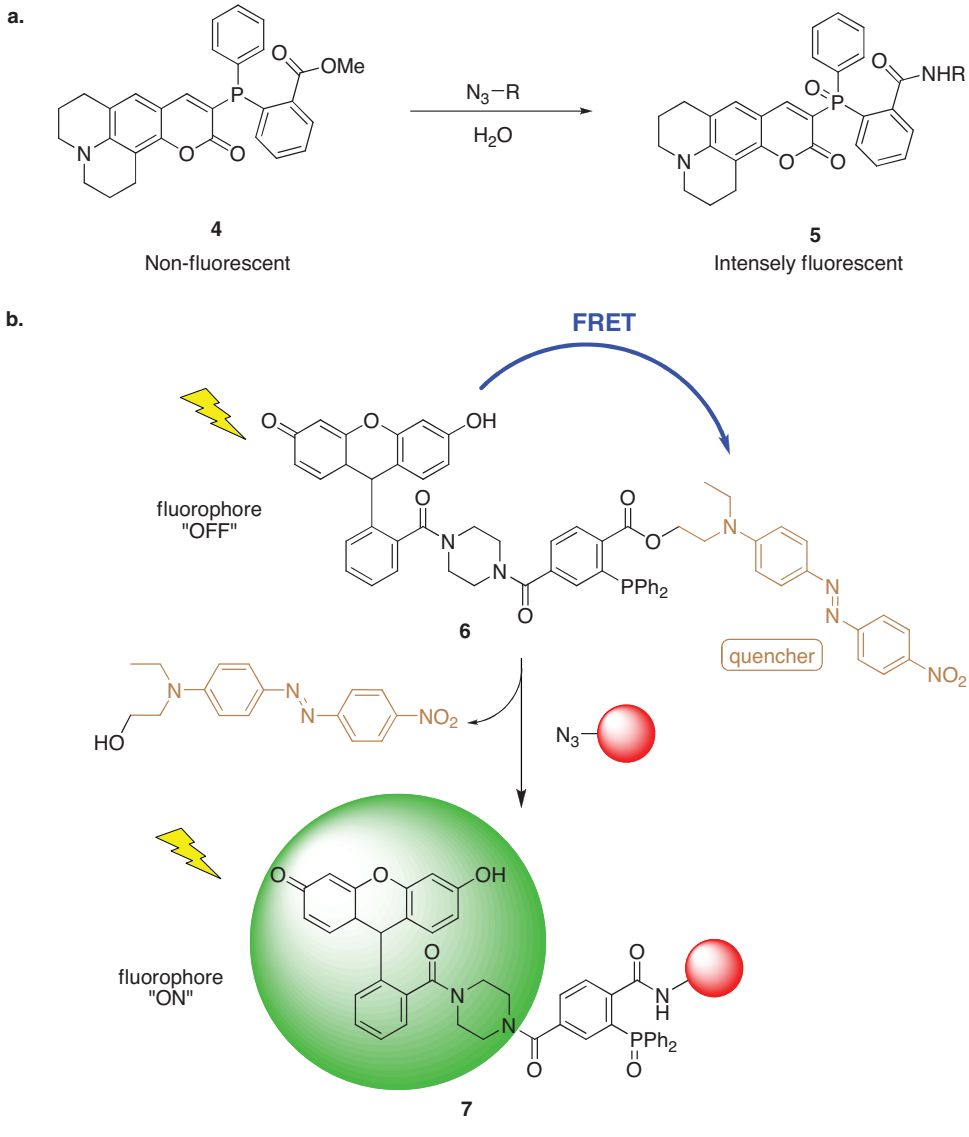
Being a highly energetic functional group, the organic azide is stable and unreactive with most biomolecules under physiological conditions as demonstrated by Bertozzi and coworkers with a modified version of the Staudinger reaction (see Chapter 3 for further details).[42] In brief, the product of the classical Staudinger reaction between a phosphine and an azide is an aza-ylide. Hydrolysis of the aza-ylide produces an amine and a phosphine oxide. The Bertozzi group elegantly placed an electrophilic trap adjacent to phosphine, which could react with the aza-ylide to form a stable amide adduct via an intramolecular electrophilic addition reaction. This reaction is highly efficient and specific even in the presence of water and a variety of other functional groups. They further designed a fluorogenic Staudinger reaction using a coumarin core.[43] The 3-position of the coumarin core is known to strongly influence its fluorescent properties. In compound **4**, the lone pair on phosphine quenches the fluorescence of coumarin [Scheme 14.3(a)]. After the formation of phosphorous oxide through the modified Staudinger reaction, the electron-donating phosphorous is switched into an electron-withdrawing functionality, and the fluorescence was activated.

Another fluorogenic Staudinger reaction was also reported for live-cell imaging.[44] In this study, phosphine compound **6** was synthesized with fluorescein-based fluorophore that is quenched intramolecularly by an ester-linked fluorescence resonance energy transfer (FRET) quencher. The reaction performed between **6** and benzyl azide in aqueous solution gave a compound whose fluorescence was enhanced 170-fold due to the free of the quencher [Scheme 14.3(b)]. With that in mind, HeLa cells were incubated with peracetylated *N*-azidoacetylmannosamine for 40 h in order to introduce *N*-azidoacetyl sialic acid into their surface cells, and the cells incubated with **6** for 8 h at 37 °C. After the incubation, fluorescence microscopy images showed highly localized fluorescence on the cell surfaces with little to no background with the cells that were not labeled with azidoacetylmannosamine.[44] The work by Bertozzi and coworkers highlighted that potential of using an azido group as the anchorage for bioconjugation and the merits of the fluorogenic reactions in real-time imaging of cellular components.

14.3 CuAAC-based Fluorogenic Reaction

As a prototype of 'click chemistry',[45–48] the recent advance of CuAAC reaction affords superior regioselectivity and almost quantitative transformation under extremely mild conditions.[2,49] Alkyne and azide groups are very small in size, are highly energetic, and have a particularly narrow distribution of reactivity. They can be conveniently introduced to organic compounds, and are quite insensitive to solvent and pH. Therefore, the CuAAC reaction becomes an ideal candidate to develop new fluorogenic reactions for the bioconjugation purpose. Figure 14.2 shows a schematic illustration of fluorogenic 1,3-cycloadditions between azides and alkynes, which have been reported to covalently link two biomolecules or supramolecular complexes for imaging or as reporters to monitor the ligation efficiency. In most situations, some prefluorophores are designed as the starting materials and the fluorescent signals can be triggered by the formation of triazole rings.

The coumarin was simultaneously chosen to develop fluorogenic CuAAC reactions by Fahrni *et al*. and Wang *et al*.[50,51] Coumarins are easy to synthesize and biocompatible and their photophysical properties are well known: substitution by electron withdrawing group at the 3-position and substitution by electron donating group at the 7-position strongly



Scheme 14.3 *Fluorogenic Staudinger reactions.[43,44] (b) Reprinted with permission from M. J. Hangauer, C. R. Bertozzi, (2008), A FRET-based fluorogenic phosphine for live-cell imaging with the Staudinger ligation,* Angew. Chem. Int. Ed., ***47****(1). Copyright 2008 Wiley-VCH.*

enhance their fluorescence intensities while addition of electron donating groups at the 4-, 6- or 7-positions or electron-withdrawing groups at the 3-position shift the fluorescence band to longer wavelengths (Figure 14.3).[52–54]

The challenge was the design and synthesis of coumarin derivatives whose fluorescence could be quenched with an azido or alkyne moiety. Zhou and Fahrni synthesized a coumarin-based fluorogenic probe **8** (Scheme 14.4) bearing an alkyne at the 7-position.[51]

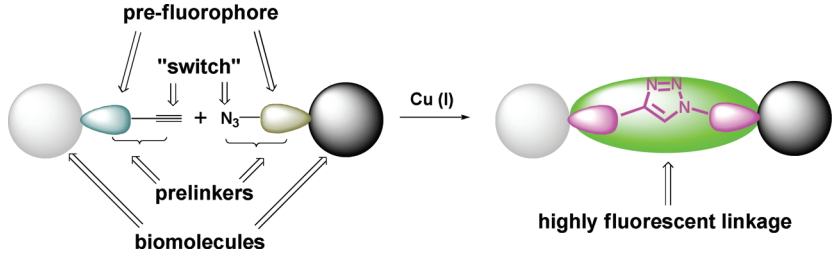


Figure 14.2 *Schematic representation of fluorogenic CuAAC reaction. Reprinted with permission from K. Sivakumar* et al.*, (2004), A fluorogenic 1,3-dipolar cycloaddition reaction of 3-azidocoumarins and acetylenes,* Org. Lett.*, **6**, 4603–4606. Copyright 2004 ACS.*

The formation of the triazole ring after CuAAC with an azide increases the electron-donating strength at the 7-position and consequently strongly enhances the fluorescence signal of the cycloaddition product. This fluorescence triggering was also confirmed by a semiempirical quantum calculation study on the electronic frontier orbital of starting material **8** and the final triazolo-compound **9**.

Wang and coworkers designed a series of fluorogenic 3-azidocoumarins **10** as shown in Scheme 14.5.[50] The fluorescence of these compounds is quenched due to the electron-rich α-nitrogen of the azido group. After the formation of the triazolo compound via the CuAAC reaction, the electronic density at the 3-position is reduced because the lone pair electrons contribute to the aromatic ring, which strongly enhances fluorescence. Moreover, they have applied these fluorogenic CuAAC to a wide range of alkynes, allowing the synthesis of triazolocoumarin dyes combinatorially. The formation of the triazolocoumarins **11** was directly detected by fluorescence screening of a 96-well plate upon irradiation at 365 nm (Figure 14.4). These triazolocoumarins can be prepared in large quantity by a simple filtration. Because of the high reactivity of aromatic azides used in the synthesis, the cycloaddition can be completed even at 0 °C, which benefits a real application of ligation between biomolecules, for which elevated temperature is usually destructive and physiological conditions are requested.

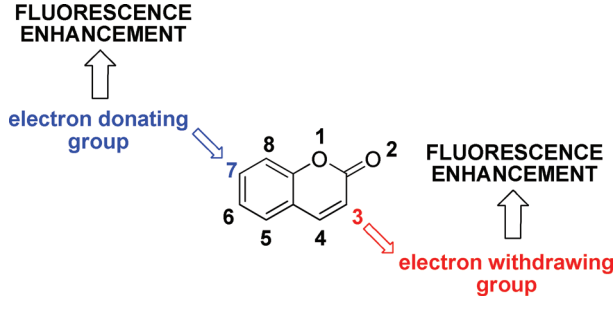
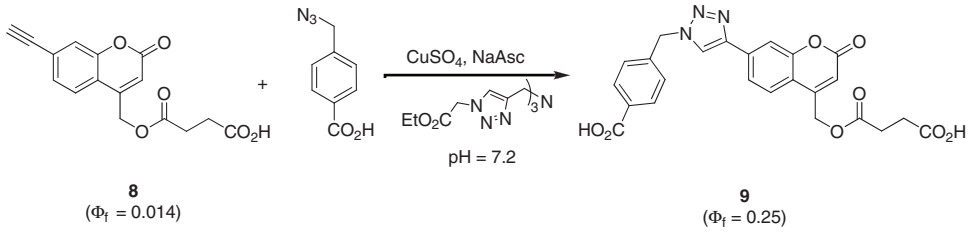


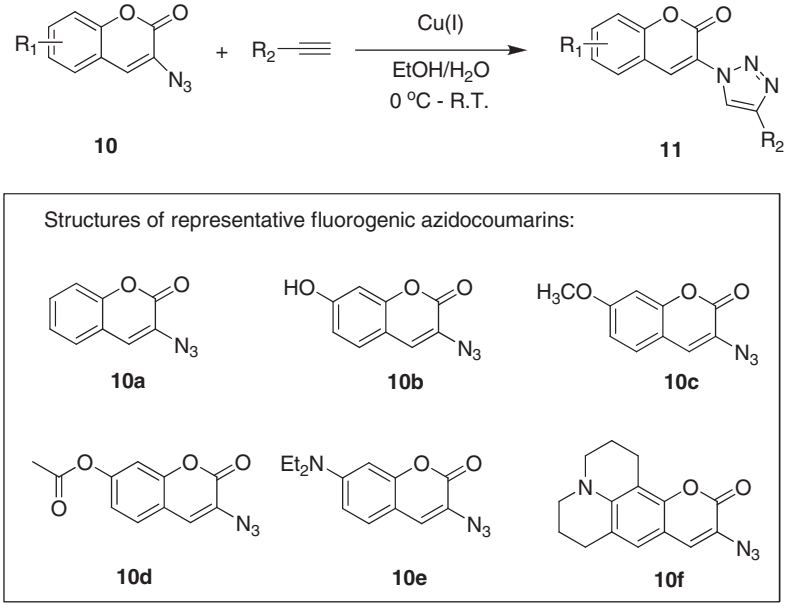
Figure 14.3 *Electron donating group at the 7-position and electron-withdrawing groups at the 3-position of the coumarin scaffold enhance its fluorescence.*



Scheme 14.4 *Fluorogenic CuAAC reaction based on the nonfluorescent 7-alkynylcoumarin* ***8****.*

Two CuAAC reaction-activated fluorescent probes based on 1,8-naphthalimide were reported by Wong and coworkers (Scheme 14.6).[55] The substitution of 1,8-naphthalimide at the 4-position by an electron donating group is known to strongly affect the fluorescence.[56] The 1,8-naphthalimide derivatives **12a** and **12b**, bearing at the 4-position either an alkyne or an azide, respectively, showed no fluorescence. Upon conjugation with complementary azido- and acetylene-modified L-fucose, **13a** and **13b**, respectively, they afforded strongly fluorescent triazolo-compounds.

Recently, Wang and coworkers proposed a new type of fluorogenic reaction based on the PET (photoinduced electron transfer) process of anthracenes.[57] In their work, an azido group



Scheme 14.5 *Fluorogenic 3-azidocoumarins and their CuAAC reaction. Reprinted with permission from K. Sivakumar* et al.*, (2004), A fluorogenic 1,3-dipolar cycloaddition reaction of 3-azidocoumarins and acetylenes,* Org. Lett.*,* ***6****, 4603–4606. Copyright 2004 ACS.*

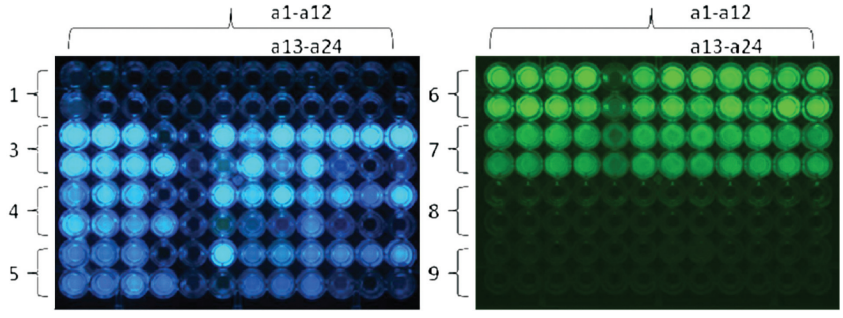
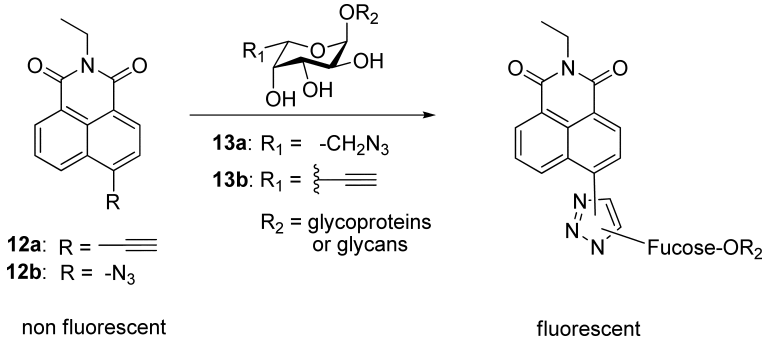


Figure 14.4 *Combinatorial synthesis and screening of triazolocoumarins (11) library in microtiter plates. The colors shown here do not represent the true fluorescent wavelengths due to the use of UV filters. Reprinted with permission from K. Sivakumar* et al.*, (2004), A fluorogenic 1,3-dipolar cycloaddition reaction of 3-azidocoumarins and acetylenes,* Org. Lett.*, **6**, 4603–4606. Copyright 2004 ACS.*

was introduced close to the anthryl core via a nonconjugated linker to allow a favorable electron transfer from the azido donor to the excited anthryl core inducing quenching of fluorescence. After the CuAAC reaction, the lone pair of electrons of the nitrogen is a part of the aromatic system, thus the nitrogen is a weaker electron donor, which does not permit the PET process and induces fluorescence activation (Scheme 14.7). In pure DMSO, the fluorescent emission intensity of product **15a** (R_2 is a phenyl group) was 75-fold stronger than that of **14a**, while **15a** shows almost the same absorption intensity as **14a** (Figure 14.5). The quantum yield of **15a** was 0.96, much higher than that of **14a** (~0.02). Moreover, there was no shift in emission and excitation wavelength accompanying the change of fluorescence intensity. All these results were consistent with a PET process between the azido group and the anthryl core. This fluorogenic CuAAC was tested between a series of azido-anthracene derivatives and a wide range of alkynes. The mild reaction



Scheme 14.6 *Fluorogenic ligation between 6-modified fucose analogs and 1,8-naphthalimide. Reprinted with permission from M. Sawa* et al.*, (2006), Glycoproteomic probes for fluorescent imaging of fucosylated glycans* in vivo, Proc. Natl Acad. Sci. USA, ***103***, *12371–12376. Copyright 2006 National Academy of Sciences, USA.*

14a: R_1 = H
14b: R_1 = -CH_2OH

15a: R_1 = H
15b: R_1 = -CH_2OH

Scheme 14.7 *Fluorogenic reaction of azido-anthracene 14. Reprinted with permission from F. Xie* et al., *(2008), A fluorogenic "click" reaction of azidoanthracene derivatives,* Tetrahedron, ***64*** *(13), 2906–2914. Copyright 2008 Elsevier.*

conditions and high fidelity of the Cu(I)-catalyzed process allowed them to screen the fluorogenic properties of the cycloaddition reactions combinatorially.

A fluorogenic click reaction using borondipyrromethene (BODIPY) as pre-fluorophore has also been developed.[58] Since the fluorescence properties of BODIPY dyes can be changed by modifying the 3- (or 5-) position of the pyrrole ring, an azido group was introduced to the 3-position of the pyrrole ring to quench the fluorescence of BODIPY **16**. The formation of triazole rings via CuAAC with alkynes reduces the electron-donating effect and increases strongly the fluorescence of derivatives **17** (Scheme 14.8). It was also noticed that the fluorescence enhancement is higher when the alkynes bear strong electron-withdrawing than when they bear strong electron-donating groups.[58]

14.4 Applications of CuAAC in Bioconjugation

The very mild conditions of the fluorogenic CuAAC reaction as well as the biocompatibility of the functional groups make it an ideal reaction for a wide range of *in vitro* and *in vivo* bioconjugation applications (Figure 14.6).

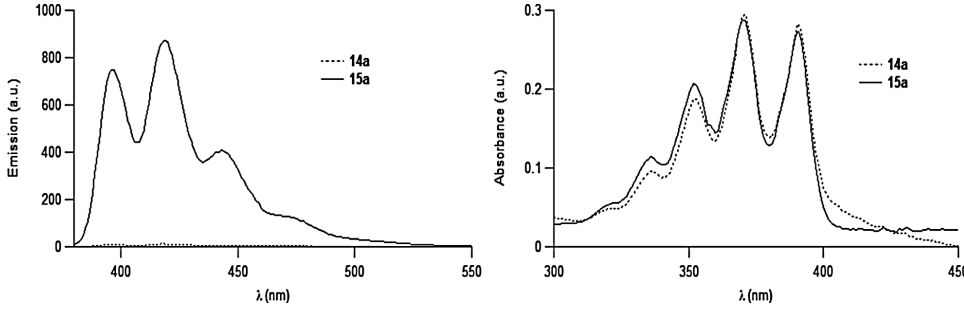
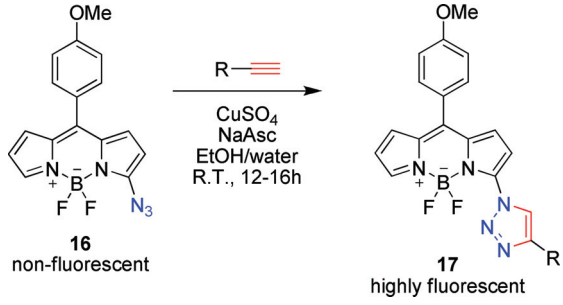


Figure 14.5 *Comparison of fluorescent emission (left) and absorption spectra (right) of* ***14a*** *and* ***15a*** *(R_2 = phenyl) in DMSO (10 μM for emission spectra and 50 μM for absorption spectra). Reprinted with permission from F. Xie* et al., *(2008), A fluorogenic "click" reaction of azidoanthracene derivatives,* Tetrahedron, ***64*** *(13), 2906–2914. Copyright 2008 Elsevier.*



Scheme 14.8 *Fluorogenic CuAAC reaction of a 3-azido-BODIPY compound.*

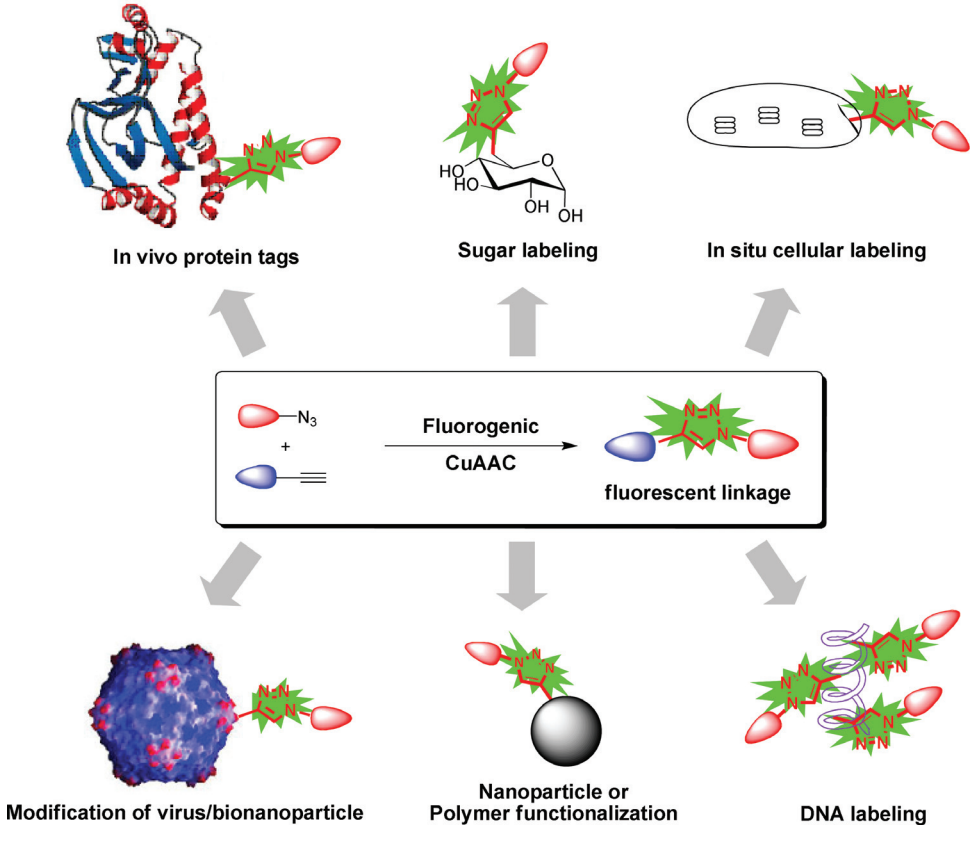


Figure 14.6 *Schematic illustration of the bioconjugate applications of the fluorogenic CuAAC reaction.*

14.4.1 Fluorogenic Probing of Cellular Components

In situ labeling proteins is of particular interest in biology because it allows the localization of the cell–cell interaction and the newly synthesized proteins. Fluorogenic CuAAC can be a useful tool in the imaging of proteins because it allows the use of profluorophore combining advantages, including small size, membrane permeability, intense fluorescence after activation and bioorthogonal reactivities.

Tirrell and coworkers have incorporated noncanonical amino-acids homopropargylglycine (Hpg) or ethylnyl-phenylamine (Eth) into recombinant barstarproteins by cotranslation [Figure 14.7(a)].[59] These two alkynyl amino-acids substituted methionine (Met) and phenylalanine (Phe) residues in the protein, respectively, and provided a triple bond for a possible ligation with an azido-profluorophore. An overnight treatment of cell cultures containing the recombinant barstar protein with the membrane-permeant 3-azido-7-hydroxycoumarin **10b**, Cu(I) and **1** at 4 °C was performed for *in situ* imaging. Excitation at 395 nm of cells gave a very strong fluorescent signal at 470 nm with a fluorescent enhancement up to 14-fold higher. The obtained fluorescence suggests that the protein is localized in inclusion bodies and a study by gel electrophoresis confirms that dye-labeling occurs mainly on the barstar [Figure 14.7(b)].

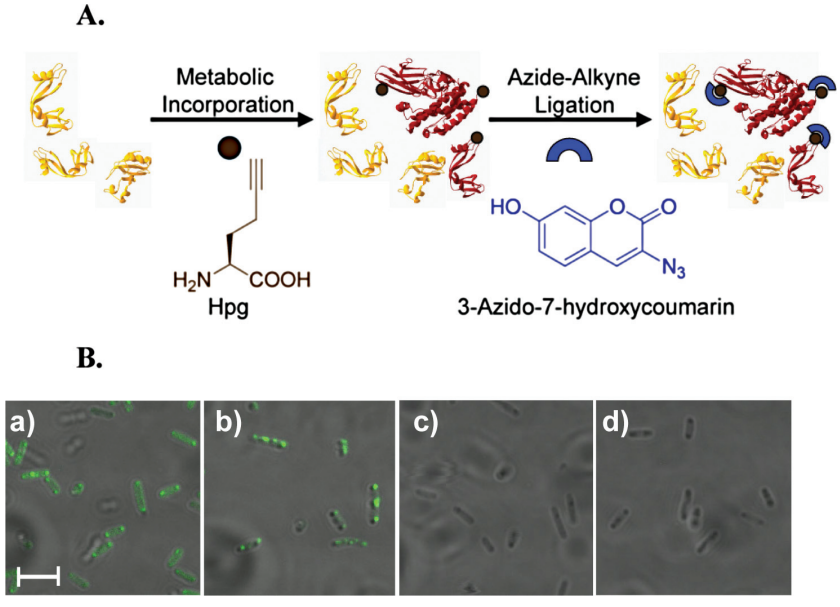


Figure 14.7 *(a) Bioorthogonal labeling of newly synthesized proteins. Reprinted with permission from K. E. Beatty* et al.*, (2006), Fluorescence visualization of newly synthesized proteins in mammalian cells,* Angew. Chem., Int. Edn, ***45****, 7364–7367. Copyright 2006 Wiley-VCH. (b) Fluorogenic labeling of barstar in* E. coli *cells after CuAAC reaction with* ***10b****. Cells were induced in media supplemented with 19 amino acids and one of the following amino acids: Hpg (a); Eth (b); Met (c); Phe (d). Scale bar is 5 μm. Reprinted with permission from K. E. Beatty* et al.*, (2005), Selective dye-labeling of newly synthesized proteins in bacterial cells,* J. Am. Chem. Soc.*,* ***127****, 14150–14151. Copyright 2005 American Chemical Society.*

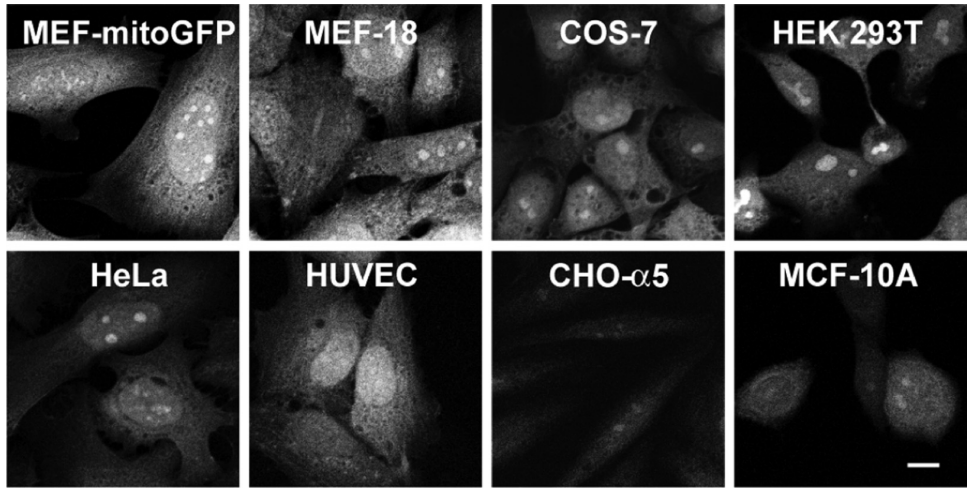


Figure 14.8 *Fluorogenic labeling of proteins in different type of cells. Scale bar is 10 μm. Reprinted with permission from K. E. Beatty* et al.*, (2006), Fluorescence visualization of newly synthesized proteins in mammalian cells,* Angew. Chem., Int. Edn, ***45****, 7364–7367. Copyright 2006 Wiley-VCH.*

Using the same method, Tirrell and coworkers also attempted to label newly synthesized proteins in a wide variety of mammalian cells.[60] First, mouse embryonic fibroblasts that express a mitochondrially targeted GFP (MEF-mitoGFP) were pulse-labeled with Hpg for 4 h, then reacted overnight in the dark at room temperature with coumarin **10b**, $CuSO_4$, TCEP and ligand **1** and then washed before visualization. The cell viability does not seem to be affected by incorporation of Hpg. A variety of different parameters have been optimized to find the conditions to visualize coumarin fluorescence by confocal microscopy. This imaging strategy was extended to different kind of mammalian cells (fibroblasts, endothelial and epithelial cells) and to different species (human, mouse, monkey and hamster). Most of the labeled cells show intense fluorescence in nuclear structures where the ribosome biogenesis takes place (Figure 14.8). Evidently, the fluorogenic CuAAC reaction enables a very efficient labeling and *in vivo* imaging of newly synthesized proteins in a wide range of mammalian cells.[60]

Wong *et al*. also reported the application of a fluorogenic CuAAC reaction to label fucosylated glycans *in vivo*.[55,61] Glycosylation is a co- or posttranslational phenomenon which takes place in more than half of eukaryotic proteins. Because L-fucose is the final sugar on glycans and participates in cell–cell interactions and cell migration processes in connection with biological processes such as embryogenesis, lymphocyte trafficking and cancer metastasis, fucosylation is a very important glycosylation process.[62–65] However, due to its high structural complexity of carbohydrates and the diversity of glycans, many functions of fucosylated glycoconjugates remain to be elucidated and a simple strategy for tagging glycans is of particular interest. In this study, acylated 6-azidofucose was incorporated in human hepatoma cell line (Hep3B) through the salvage biosynthetic pathway by incubation for 3 days.[55] Then cells were fixed, washed with PBS buffer and then reacted

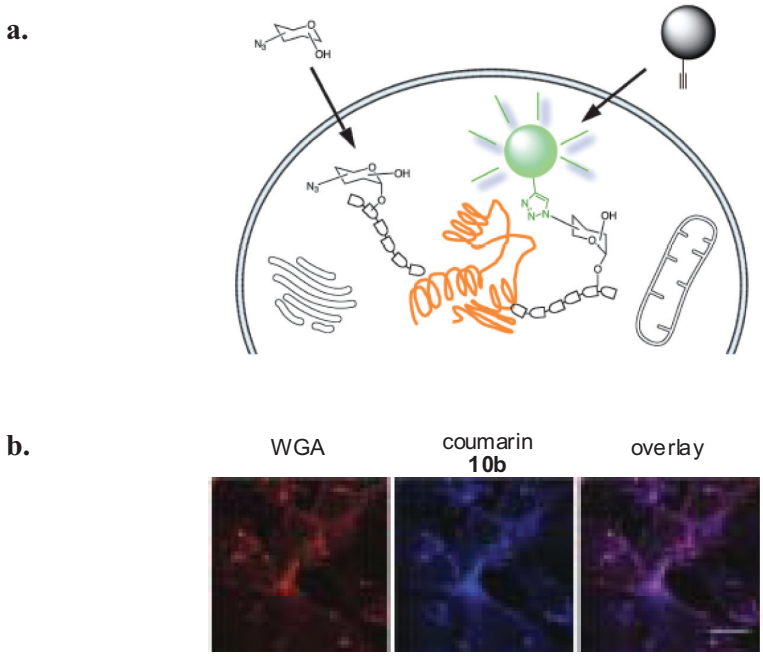


Figure 14.9 *(a) Schematic illustration of specific fluorescent labeling of fucosylated glycans in cells. Reprinted with permission from M. Sawa* et al.*, (2006), Glycoproteomic probes for fluorescent imaging of fucosylated glycans* in vivo, Proc. Natl Acad. Sci. USA, ***103***, *12371–12376. Copyright 2006 National Academy of Sciences, USA. (b) Fluorescence imaging of sialyl glycoconjugates in Hep3B cells using CuAAC activated probe* ***10b****. Cells were treated with 25 mm alkynyl ManNAc for 3 days, clicked with* ***10b*** *and stained with WGA lectin (Alexa Fluor 594). Scale bar is 20 μm. Reprinted with permission from T.-L. Hsu* et al.*, (2007), Alkynyl sugar analogs for the labeling and visualization of glycoconjugates in cells,* Proc. Natl Acad. Sci. USA, ***104***, *2614–2619. Copyright 2007 National Academy of Sciences, USA.*

with naphthalimide derivative **12a** in the presence of CuBr [Figure 14.9(a)]. After CuAAC, the engendered fluorescence in the cell by the formation of the triazolo-compound was visualized by fluorescence microscopy.[55]

However, acylated 6-azidofucose was found to be quite toxic for the cells, then alkynyl ManNAc was used instead because of its low toxicity.[61] Several human cancer cell lines were treated with it and then CuAAC reaction with **12b** was achieved: fluorescent-labeling of cell surface glycoconjugates was measured by flow cytometry and intracellular glycan labeling was controlled by fluorescence microscopy [Figure 14.9(b)], showing localization in the Golgi.

14.4.2 Fluorogenic Conjugation of DNA

Incorporation of labeled nucleosides into DNA is of particular interest for DNA diagnosis of genetic disorders and for exploration of structure, dynamics and interactions of nucleic acids.[66] Unfortunately incorporation of such tags is very difficult: enzymatic replacement of

natural oligonucleotides by labeled ones relies on highly modified protocols and chemical modification of bases by solid-phase synthesis gives poor yields. Postsynthetic introduction of labels has also been tried but coupling yields are very low. Thus click reaction has been recently used as an easy and successful alternative to incorporate tags via introduction of small functional groups on DNA.

Carell *et al*. developed a postsynthetic method for high density labeling of DNA.[67] Alkyne-modified uridine nucleosides **18** and **19** have been prepared, transformed into their corresponding phosphoramidites and incorporated into a series of oligodeoxyribonucleotides (ODNs) via solid-phase synthesis (Figure 14.10). Once triple bonds were introduced, click reactions were performed with azides **10b**, **20** and **21** using standard conditions and Cu(I)-complexing ligand **1** in order to avoid strand breaks in DNA. The high-density reliable modification of all alkyne sites was achieved by using flexible alkynes **19**, whereas rigid alkynes **18** led to partially labeled DNA, showing that linker length plays an important role in the efficiency of the reaction. Bioconjugation by means of CuAAC reaction is very important because it allows efficient introduction of labeled nucleosides into DNA and permits DNA imaging either by fluorescence (fluorescence is triggered off via reaction with **10b** or by introduction of fluorescein via **21**) or by Ag staining (**20** is a sugar enabling Ag staining).

They also managed to functionalize DNA with up to three different labels by successive click reactions.[68] Using the same method as in their previous work they incorporated the cytidine building block **22** and the thymidine building blocks **23a** and **23b** into ODNs (Figure 14.11). The first click reaction was performed directly on the resin by shaking it with a solution of CuBr, ligand **1**, sodium ascorbate and azido-profluorophore. Once modified, the oligonucleotide was cleaved from the support by using aqueous solution of ammonia which also removed the trimethylsilyl (TMS) protecting group [but did not deprotect the triisopropylsilyl (TIPS) group] and then purified by HPLC. The second click reaction was performed in solution followed by its precipitation from ethanol and deprotection of the TIPS group by tetrabutylammonium fluoride. The last click reaction and precipitation afforded the triple-modified oligonucleotide with an overall good yield (50%). Therefore, click chemistry can be used to incorporate very sensitive labels into DNA with good efficiency and simple work-up.

Seela *et al*. thoroughly investigated the conjugation of functionalized nucleoside with nonfluorescent 3-azido-7-hydroxycoumarin **10b**.[69–71] Alkynyl chains were introduced into oligonucleotides and incorporated into ODNs for further tagging. The DNA duplexes obtained with these modified oligonucleotides shows an enhancement of the stability compared with natural oligonucleotides. Functionalization of modified nucleosides or ODNs was easily achieved by reaction with **10b** via CuAAC using *t*-BuOH–H_2O–DMSO–THF mixture in the presence of 1:1 complex of $CuSO_4$-ligand **1** and TCEP to afford strongly fluorescent 1,2,3-triazolyl oligonucleotide conjugates. This bioorthogonal fluorogenic CuAAC reaction which allows incorporation of tags into DNA without destabilizing DNA duplexes is useful for DNA detection in solution or in DNA–protein complexes and can be used for the *in vivo* labeling of DNA. Moreover they demonstrated that enzymatic hydrolysis of 1,2,3-triazolyl oligonucleotide conjugates shows a strong fluorescence quenching for 7-deazapurines compared with pyrimidines. This nucleobase specific quenching, which is probably due to an electron transfer between the nucleobase and the coumarin, can be used to monitor conformational dynamics of nucleotides in solution.

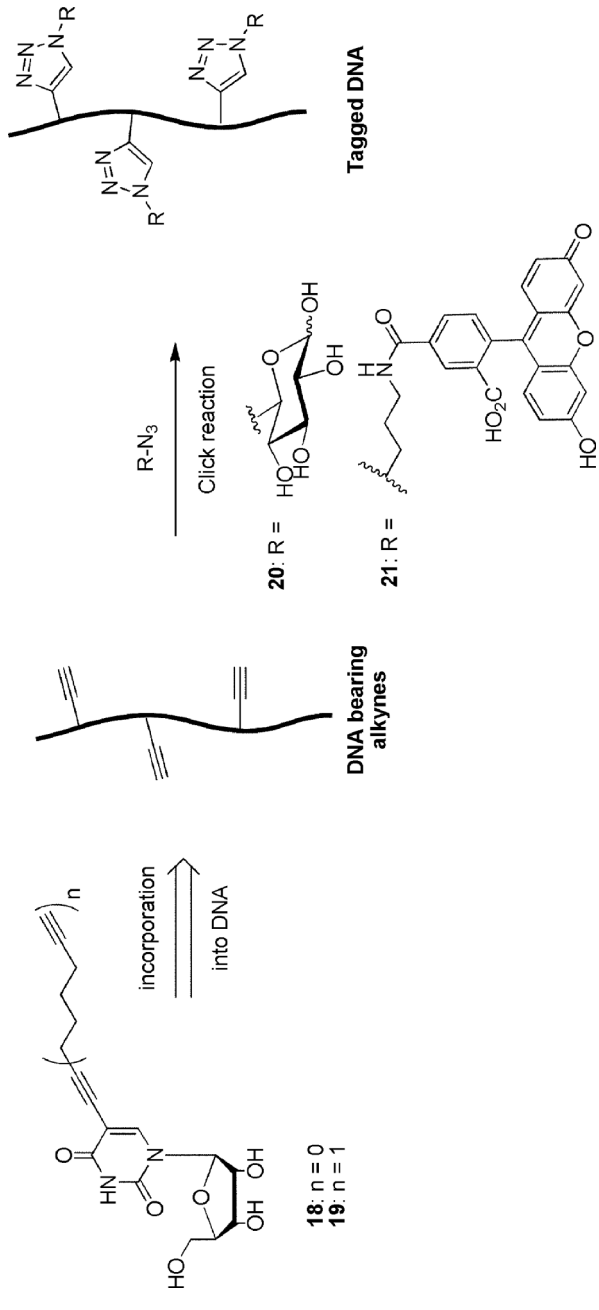


Figure 14.10 *Modified DNA by click reaction and structures of alkyne modified uridine nucleosides (**18** and **19**) and azide labels (**20** and **21**).*

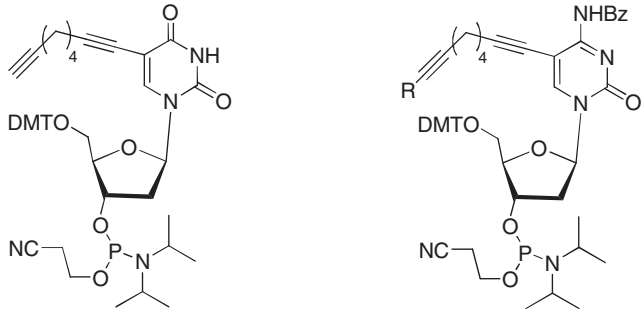
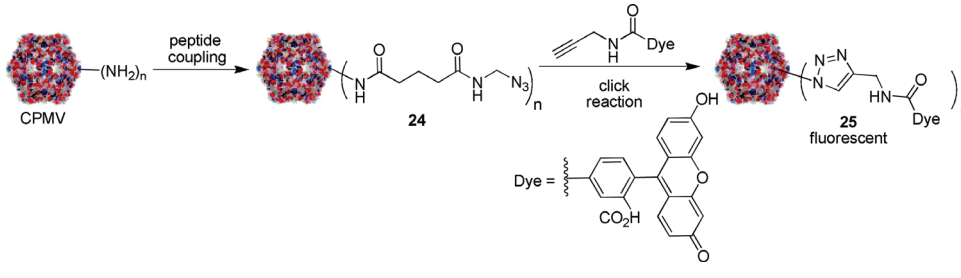


Figure 14.11 *Phosphoramidites **22** and **23**, DMT = 4,4′-dimethoxytriphenylmethyl. TMS = trimethylsilyl; TIPS = triisopropylsilyl; Bz = benzoyl.*

14.4.3 Fluorogenic Conjugation of Viruses

Finn *et al*. succeeded in labeling the cowpea mosaic virus (CPMV) with fluorescein.[5] CPMV is a stable and structurally well-characterized particle available in large quantities. Its capsid is composed by 60 identical copies of a two-protein asymmetric unit which enveloped the single-stranded RNA genome in the core. The outside of the capsid was decorated with azide or alkyne using either amide coupling or thio-ether formation at lysine or cysteine residues. The fluorescein derivatives were then conjugated to these three different virus-azides or virus-alkynes by click reaction under different conditions (Scheme 14.9). Addition of tris(triazolyl)amine **1** in CuAAC reaction showed acceleration of the reaction rate and a quasi-quantitative yield of the modified virus **25** was then obtained.[13]

They also replaced tris(triazolyl)amine **1** by the water-soluble sulfonated bathophenanthroline **2** as a ligand to modify the CPMV surface.[72] Tris(triazolyl)amine **1** is not very water-soluble, which can lead to some damage to the protein if the amount of available ligand is not enough in heterogenous solution. Use of sulfonated bathophenanthroline **2** under the same conditions permitted a decrease in concentration of labeled substrate and



Scheme 14.9 *Bioconjugation of CPMV particle via CuAAC reaction.*

also permitted, with modified procedures, modification of a wide range of molecules such as complex carbohydrates, peptides, proteins or polymers in high yield.[73,74]

Wang *et al*. revisited the surface modification of tobacco mosaic virus (TMV) using CuAAC reaction.[7] TMV is a rod-shaped virus of 300 nm length and 18 nm diameter that can be obtained in large quantity. It is made from 2130 identical protein subunits arranged helically around genomic single-strand RNA that also stabilizes the coat protein assembly. TMV is a very attractive to be used in different fields such as nanoelectronics and energy harvesting devices[75–77] or as a template to grow metal or metal oxide nanowires.[78–80] Tyrosine residues were transformed into alkynes by means of an electrophilic substitution reaction at the *ortho* position of the phenol ring by using a diazonium salt generated *in situ* from the 3-ethynylaniline. CuAAC reactions between alkyne-derivatized TMV and a series of azides using $CuSO_4$ and sodium ascorbate were performed to modify the TMV surface with molecules such as peptides or polymers.[7] A double surface modification was also achieved using a mixture of azides under the same reaction conditions.

In particular, Wang and coworkers demonstrated that fluorogenic CuAAC reaction can be employed to titrate the reactivity of alkyne groups in a polyvalent system. For example, trispropynyloxybenzene **26**, alkyne-derivatized CPMV **28** and alkyne-derivatized TMV **30** containing 3, 60 and 2130 terminal alkyne moiety, respectively, were reacted with nonfluorescent azido-anthracene derivatives **14a** and **14b** [Figure 14.12(a)]. Conversion of **28** into intensely fluorescent triazolo-anthracene **29** was confirmed by gel electrophoresis [Figure 14.12(b)] and its integrity by TEM and size-exclusion chromatography [Figure 14.12(c, d)]. Based on the intensity of the fluorescent emission of the final conjugate product, they can quantitatively determine the reaction efficiency of the polyvalently displayed alkyne groups towards CuAAC reactions.

14.4.4 Fluorogenic Conjugation of Nanoparticles/Polymers

Self-assembly of amphiphilic block copolymers into polymeric micelles with a core–shell type structure is interesting as a drug delivery vehicle, as the hydrophobic core of the micelles can encapsulate a lipophilic molecule, whereas the hydrophilic shell stabilizes the micelles in aqueous media and protect the contents of hydrophobic core from possible degradation. The cross-linking of micelles in the core or in the shell plays an important role for the robustness of nanoparticles as well as relative ratio of block length, composition and molecular weight of amphiphilic block copolymers. Thus with the introduction of appropriate functionalities into specific parts of the copolymer it is possible to enhance their performance as drug delivery systems. The difficulty lies in the control of the presence of the functional group into the polymer.

As a classical example, Wooley, Hawker and coauthors synthesized a new class of block copolymers with acetylene group in the hydrophobic block using reversible addition fragmentation chain transfer techniques.[81] These acetylene-functionalized block copolymers were then self-assembled and cross-linked to give shell cross-linked knedel-like (SCK) nanoparticles **32** with acetylene groups in the core domain. As the presence and, more importantly, the reactivity, of the alkyne within the nanoparticle core cannot be detected by standard analysis such as NMR and MS, fluorogenic CuAAC reaction becomes a unique tool. Therefore, CuAAC with nonfluorescent coumarin derivative **10b** was performed at room temperature for 2 days using an organic copper(I) catalyst [$CuBr(PPh)_3$] and triamine

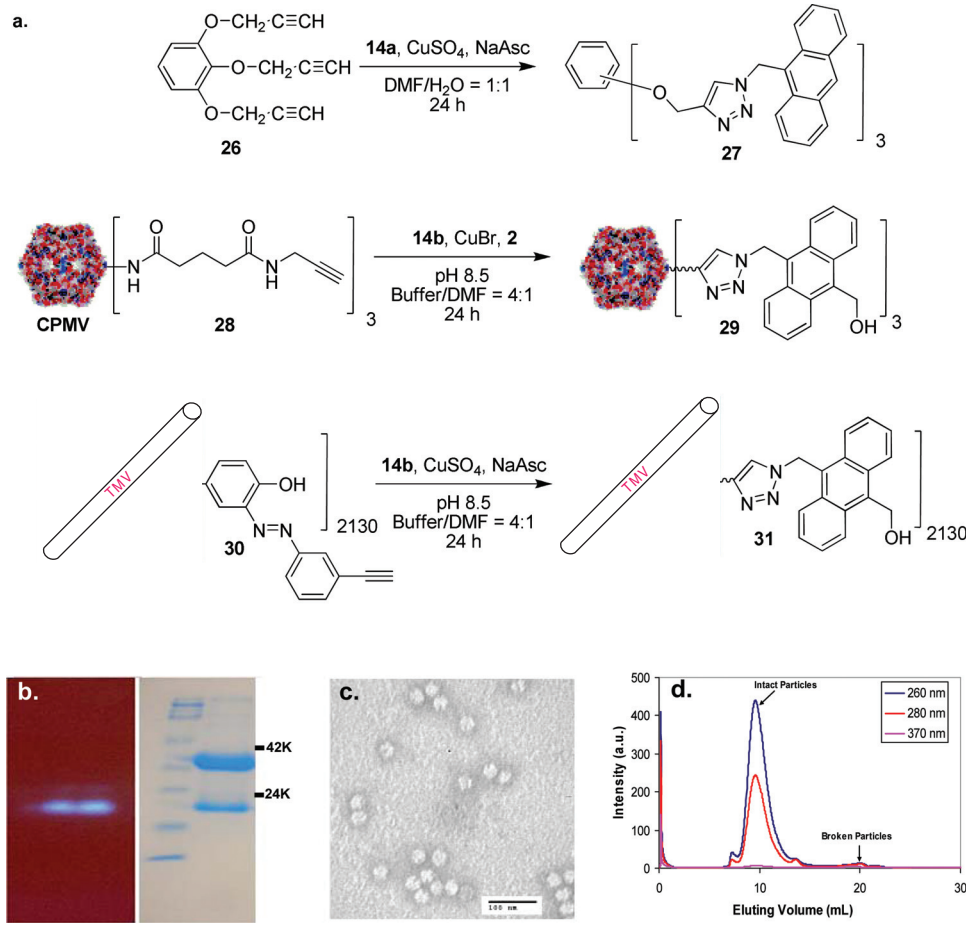
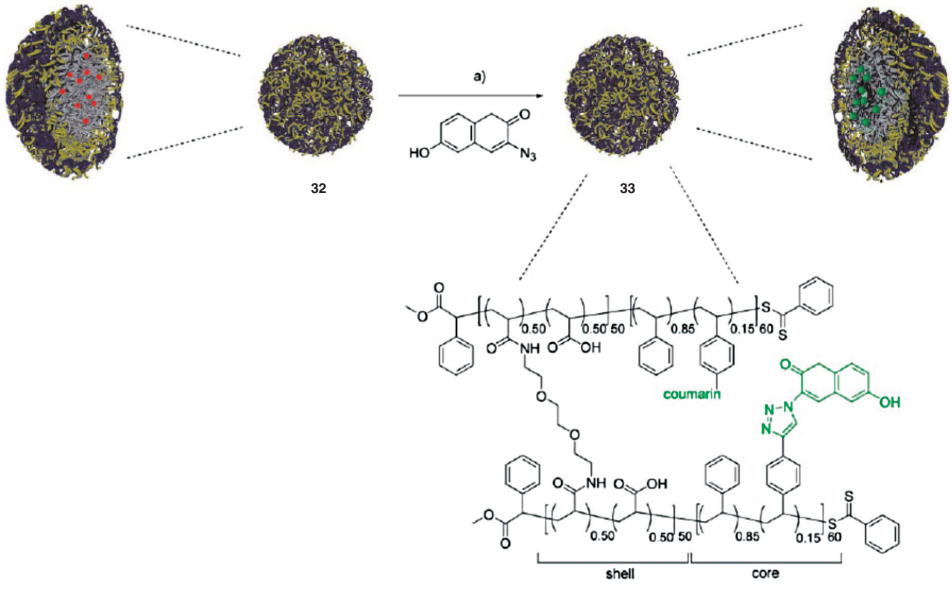


Figure 14.12 *(a) Virus modifications by CuAAC reactions. (b) SDS-PAGE of* ***29*** *visualized under UV irradiation (left) and upon staining with Coomassie blue (right). (c) TEM image of* ***27****. The scale bar is 100 nm. (D) Size exclusion FPLC analysis of* ***29****. Reprinted with permission from F. Xie* et al.*, (2008), A fluorogenic "click" reaction of azidoanthracene derivatives,* Tetrahedron*,* ***64****, 2906–2914. Copyright 2008 Elsevier.*

to afford the fluorescent particle **33** (Scheme 14.10). The formation of triazolo-compound was easily confirmed by fluorescence measurements and analytical ultracentrifugation proved that acetylene moieties were available within the core domain. In this case, click reaction allows the validation of the presence of triple bonds into the core and to image the nanoparticle.

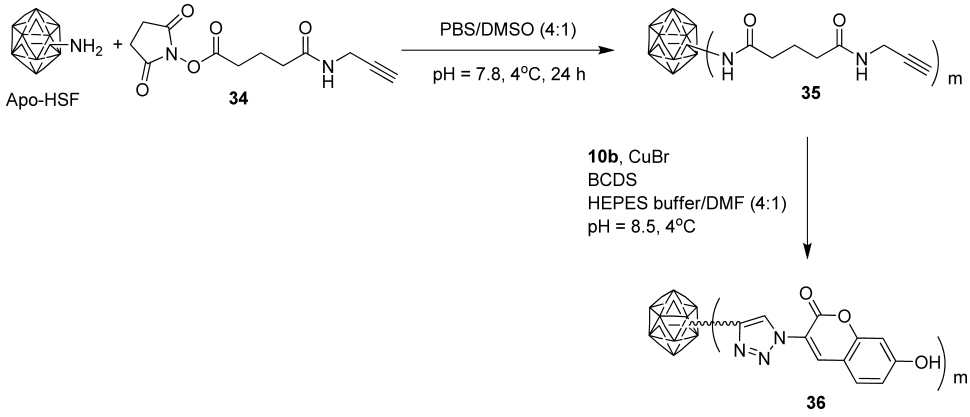
In another example, O'Reilly *et al*. reported the copolymerization of a terpyridine functionalized styrene monomer with styrene using nitroxide-mediated polymerization.[82] The terpyridine moiety of the nanostructure was selectively located within their hydrophobic core domain and was functionalized by metal complexation to afford novel



Scheme 14.10 *Fluorogenic click reaction on shell cross-linked knedel-like (SCK) nanoparticles. (a) Dialysis of* ***32*** *into THF/H_2O 4:1 for 3 days, then addition of [CuBr(PPh)$_3$] (0.1 equiv), and DIPEA (1.0 equiv),* ***10b*** *(1.11 equiv to acetylene functionality), RT, 2 days, followed by dialysis against THF–buffered H_2O 1:4 for 10 days, and then dialysis against pH 7.3 phosphate buffered saline, 4 days. Reprinted with permission from R. K. O'Reilly* et al., *(2006), Fluorogenic 1,3-dipolar cycloaddition within the hydrophobic core of a shell cross-linked nanoparticle,* Chem. Eur. J., ***12****, 6776–6786. Copyright 2006 Wiley-VCH.*

metal-functionalized polymer nanostructures. The terpyridine was utilized to complex metal centers (Fe, Cu and Ru) in the core domain. To test if the Cu-tethered metal complex within the nanostructures was an active catalyst, they used the fluorogenic CuAAC reaction by adding phenylethynyl and nonfluorescent 3-azidocoumarin to a solution of the nanoparticles. After 4 h reaction time, a high-fluorescence enhancement was noticed at 550 nm, confirming that Cu–terpyridin complex is active in click catalysis.

Wang and coworkers reported the chemoselective modification of horse spleen apoferritin (apo-HSF) by means of a CuAAC reaction.[83] Apo-HSF is derived from ferritin and its cage contains 24 identical subunits arranged into a hollow and spherical shell with an inner diameter of 8 nm and an outer diameter of 12.5 nm. The modification of lysine residues was achieved by acylation with NHS ester reagent to afford an alkyne on the biomolecule. In order to know that this chemoselective ligation has been achieved, a fluorogenic click reaction with the nonfluorescent coumarin **10b** in the presence of CuBr and ligand **2** (combination of $CuSO_4$–NaAsc or $CuSO_4$–phosphine did not give the triazolo-compound since aggregation and denaturation of apoferritin were observed) was realized to afford a triazolo-derivative, which shows very strong fluorescence at 474 nm upon excitation at 340 nm (Scheme 14.11). The triggering of fluorescence via the CuAAC reaction confirmed the modification of apo-HSF with an alkyne group. Twenty triazolo-coumarins by



Scheme 14.11 *Bioconjugation of apoferritin by CuAAC reaction.*

apoferritin particle (i.e. about one coumarin by subunit) were found in the protein by measuring the fluorescence intensity of final conjugates.

14.5 Conclusions

The CuAAC reaction has become a major ligation tool in bioconjugation in the past few years. The reaction between alkyne and azide presents several advantages – easy preparation of alkynes and azides, bioorthogonality of starting materials, high yielded reaction and very mild reaction conditions – which make this reaction very suitable to label cellular elements. The fluorogenic CuAAC, particularly, have shown very interesting results for tagging diverse biomolecules (viruses, proteins, sugars, etc.), both *in vitro* and *in vivo*, which enable the localization of biological processes in an intracellular environment. Moreover, obtention of fluorescent triazolo derivatives from nonfluorescent polymers and nanoparticles allows detection of the presence of functionalities such as acetylene or azido groups other than by classical techniques. Finally, fluorogenic CuAAC is an easy way to introduce labels into DNA in order to detect genetic diseases or investigate nucleic acid interactions.

New perspectives have recently emerged for fluorogenic reaction including monitoring the progress of specific reactions by increase in fluorescence. For example, Rozhlov *et al.*[84] have reported a new fluorogenic transformation based on formation of C–C bonds catalyzed by palladium, allowing the screening of reaction variables such as base, ligand, temperature, etc. However the fluorescence of the final product can be quenched by catalysts if used in high quantities, which can lead to detection problems. Tanaka *et al.* have synthesized new fluorogenic imines to detect Mannich-type reactions of phenol in water, which could also be very interesting for the screening of catalysts and conditions to tag reactions of phenol-bearing molecules.[85] Additionally, Marchand *et al.* have implemented this reaction in ionic liquid micro-reactors to evaluate the efficiency of alternative mixing methods on the reaction kinetics, opening up a wide subject.[86] Finally, fluorogenic copper-free Huisgen cycloaddition reactions have recently been developed for intracellular labeling to avoid the potential cytotoxicity of copper,[43,44] which is introduced in Chapter 3.

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