REVIEW ARTICLE

RECORDS OF PHARMACEUTICAL AND BIOMEDICAL SCIENCES

Click Chemistry as a Promising Protocol for Fluorogenic Reactions

Heba A. Aref [a]*, Mohamed A. Helal [b,c], Safaa M. kishk [c], Shaimaa Mohamed Aboukhatwa [d], Ismail Salama [c]

[a]Medicinal Chemistry Department, Faculty of Pharmacy, El Mounufia University, El Mounufia ,32511, Egypt; [b]Biomedical Sciences Program, University of Science and Technology, Zewail City of Science and Technology, Giza, 12587, Egypt; [c]Medicinal Chemistry Department, Faculty of Pharmacy, Suez Canal University, Ismailia, 41522, Egypt; [d]Medicinal Chemistry Department, Faculty of Pharmacy, Tanta University, Tanta ,31511, Egypt.

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* Correspondence Author:

Tel: +201140230606

E-mail address:

heba.abdelfattah@phrm.menofia.edu.eg

hebaaref@ymail.com

1. Introduction

1.1. The click reaction concept

The need for innovative conjugation reactions that non-organic chemists (such as analysts and biochemists) can utilize efficiently increased as organic compounds started to take their place as readily tunable and functional materials. Such a reaction ought to be easier to carry out with good selectivity and high yield. Click chemistry is a collection of such reactions that has evolved as an efficient tool for ligation. Click chemistry refers to joining molecular fragments as simply as clicking a seatbelt buckle's two halves together (Zimam, 2015). The concept of click chemistry was initially presented by the Nobel Prize winner K. Barry Sharpless and coworkers in 2001 at the Scripps Research Institute describing a new strategy for organic synthesis through a group of reactions that must meet specific requirements. This set of reactions was identified as a direct result of Mother Nature's strategy which using a very limited number of monomers for achieving astonishing biological diversity (i.e. proteins from amino acids, nucleic acids from nucleotides, etc.). Dr. Sharpless put up a novel strategy that would make it simple to create enormous combinatorial libraries by connecting readily available building blocks with click reactions. Like in nature, the starting compounds must be small in size, react quickly and completely convert to the desired product in a selective manner. From this concept, the "click chemistry" philosophy was born and described by K.B Sharpless et al. Although Sharpless presented the idea of click chemistry to offer an efficient conjugation strategy in drug discovery, this concept and methodology has quickly acquired acceptance in nearly all areas of science and technology that use organic compounds. It opened a myriad of applications in combinatorial chemistry, medicinal chemistry, biomedical research, material and polymer science. In addition, click chemistry became a newly-emerging technique in biosensing systems for biorthogonal ligation of DNA and proteins. Besides it has labeling applications for selective detection of biomolecules, small molecules and ions as well (Moses et al., 2007, Meghani et al., 2017, Varazo et al., 2009).

1.2. Criteria of click reactions

Any reaction that can efficiently and selectively create conjugate molecules from smaller units under simple reaction conditions has potential to find a place in the "click chemistry" world. In defining click chemistry, Sharpless *et al.* compiled a list of requirements that must be fulfilled for a given reaction to fall under the umbrella term "click" (Meldal et al., 2020). For a process to be labeled "click chemistry" the reaction should be:

- Modular in approach with wide scope and depends on readily starting materials and reagents.
- Versatile in nature.
- Selective, giving a single product in high yield, with no or only inoffensive byproducts.
- Performed in benign and easily removable solvents; reaction can proceed in water, as well as in organic solvents.
- Efficient under simple reaction conditions (ideally insensitive to water and oxygen and can be performed rapidly at room temperature).
- Regio- and stereospecific, stereoselective and not necessarily enantioselective.
- Reaction work-up and product isolation should be as simple as the synthesis process, and if purification is necessary, it must be feasible using nonchromatographic techniques like distillation or crystallization.
- Have high thermodynamic driving force, often larger than 20 kcal/mol. A large ΔG of reaction (50 kJ/mol) confirms Quasiclassical trajectories (QCT) at low concentrations.
- The click-functional groups should be inert to all other reaction conditions.
- All other functional groups should be inert to the click reaction conditions.
- Products must be reasonably stable under physiological conditions.

The limitations of click chemistry may appear demanding, but there are various reactions with different mechanisms which fit into this category and can be regarded as click reactions as long as they follow a simple common reaction trajectory. The five most prevalent click reactions include (Knight et al., 2019):

- Copper-Catalyzed Azide–Alkyne 1,3-Dipolar Cycloaddition (CuAAC) Reaction.
- Ruthenium-Catalyzed Azide–Alkyne Cycloaddition (RuAAC) Reactions.
- Strain-Promoted Azide–Alkyne Cycloaddition (SPAAC) Reactions.
- Inverse Electron Demand Diels-Alder Reaction (IEDDA).
- Azide-Phosphine Coupling (Staudinger Ligation).

Among the different "click" reactions meeting this concept's requirements, one has considered as the centerpiece of "click chemistry" and sometimes being mistakenly defined as click reaction in itself. It fulfills all of the criteria of click chemistry perfectly and is usually referred to the Copper-catalyzed Azide–Alkyne Huisgen 1,3-Dipolar Cycloaddition reaction.

2. Copper-catalyzed Azide–Alkyne Huisgen 1,3-Dipolar Cycloaddition Reaction

The copper-catalyzed Azide–Alkyne Huisgen 1,3-Dipolar Cycloaddition (entitled as CuAAC reactions) is the jewel in the crown and it is considered as "the premier example of a click reaction". It is worth noting that since 1893 the non-catalyzed azide alkyne cycloaddition reaction has been known when A. Michael proposed that regioisomeric triazoles were produced in a reaction between dimethyl but-2-ynedioate and phenyl azide at 100 °C in a sealed tube (Sultana et al., 2020). Nevertheless, it was only in the 1960s that Huisgen identified this kind of reaction for its mechanism and generality and coined the term Huisgen 1,3-dipolar cycloaddition reaction (Scheme1) (Santos et al., 2020). Undoubtedly, the Huisgen reaction is of a great importance to the synthesis of numerous five membered heterocycles. However, it was limited by the remarkable disadvantages of this

type of uncatalyzed addition reaction such as the necessity of high temperatures and pressure, long reaction times, low yield and poor regioselectivity (Breugst et al., 2020). These problems made this reaction ignored for decades as an effective conjugation technique until solved at the dawn of last decade by Sharpless and Meldal via the addition of Cu(I) catalysis termed as 'Click Chemistry'. This revolutionary modification led to a great enhancement in both rate and regioselectivity; in the so-called copper(I)-catalyzed azide- alkyne cycloaddition reaction (Scheme 1) (Kolb et al., 2001). The wonderful advantages of this Cu(I) catalysis can be summarized as follow: First, the cycloaddition reaction becomes both kinetically and thermodynamically favorable (26 and 50 kcal/mol, respectively) and 10^7 times faster than the uncatalyzed reaction. This makes it possible for the reaction to occur within a reasonable amount of time, such as minutes or hours as opposed to days. (Phillips et al., 2021). Second, it is regioselective, as only the 1,4- regioisomers of 1, 2, 3-triazoles are formed in very high yields with shortest workup and purification steps. Whereas the non-catalyzed Huisgen reaction lacks regioselectivity, producing both the 1,4- and 1,5-disubstituted isomers (Yu et al., 2013). Third, it proceeds at room temperature whereas the non-catalyzed reaction occurs only at elevated temperatures. Fourth, neither the sterics nor the electronics of the azide or the alkyne component have any impact on the catalyzed variant. Primary, secondary, tertiary, and aromatic azides with different substitutions can easily take part in this transformation. Excellent tolerance for variations in the acetylene component is also observed (Liang et al., 2011). All the previously mentioned advantages increase the popularity of this cycloaddition among the other click reactions.

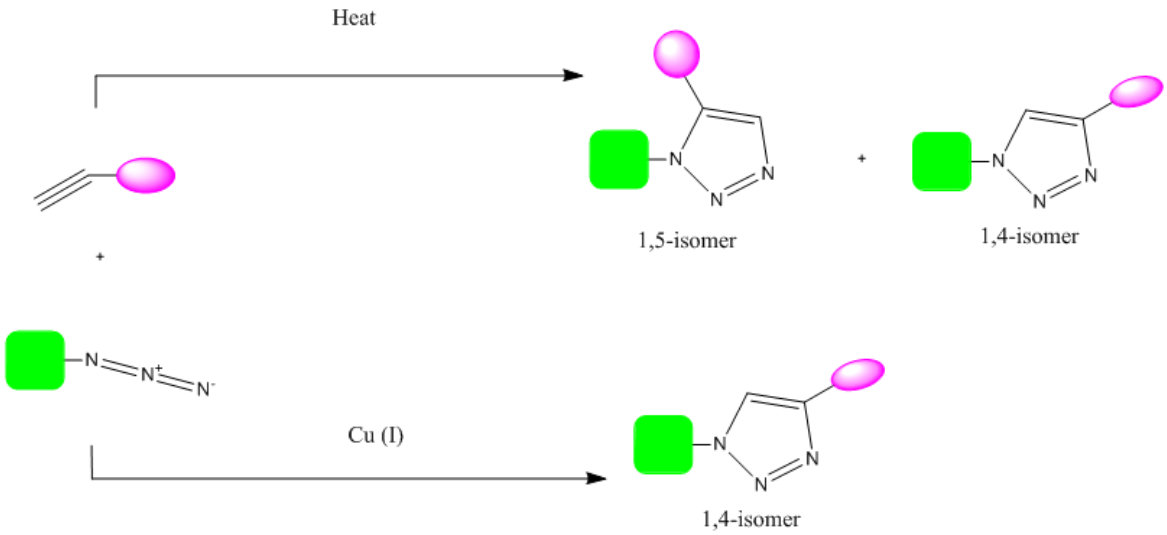
2.1. Mechanism of CuAAC Click Reactions

Wang et al., presented a thorough mechanistic study on CuAAC (scheme 2)(Wang et al., 2016a). According to studies, the activation energy of the Cu-catalyzed cycloaddition reaction is 11 kcal/mol lower than that of the uncatalyzed reaction, which has an activation energy of 26 kcal/mol. The CuAAC reaction proceeds through a stepwise mechanism starting with the formation of a Cu–alkyne π complex following by deprotonation of the alkyne proton to form a copper acetylide. The acetylenic proton becomes more acidic when copper is coordinated with the alkyne, increasing its acidity by up to 9.7 pH units. This permits the deprotonation to take place in aqueous environments even in the absence of a base. The copper acetylide is in equilibrium between a monomer and a dimer. The azide nitrogen is activated by coordination with one of the Cu ions in the dimer. This complex undergoes cyclization to form a metallacycle through a nucleophilic attack of the terminal nitrogen of the azide group on the internal carbon of the alkyne. The energy calculation revealed a notably low energy barrier for this step, which accounts for the higher rate of the catalyzed reaction comparing to the uncatalyzed one. After that a ring contraction of the metallacycle occurs via a transannular interaction between the C=Cu bond and the lone pair of electrons on the substituted nitrogen of the azide. A Cu triazolide is produced in this relatively faster step which then protonated to liberate the 1,4-disubstituted triazole and regenerate the Cu(I) catalyst (Liang and Astruc, 2011).

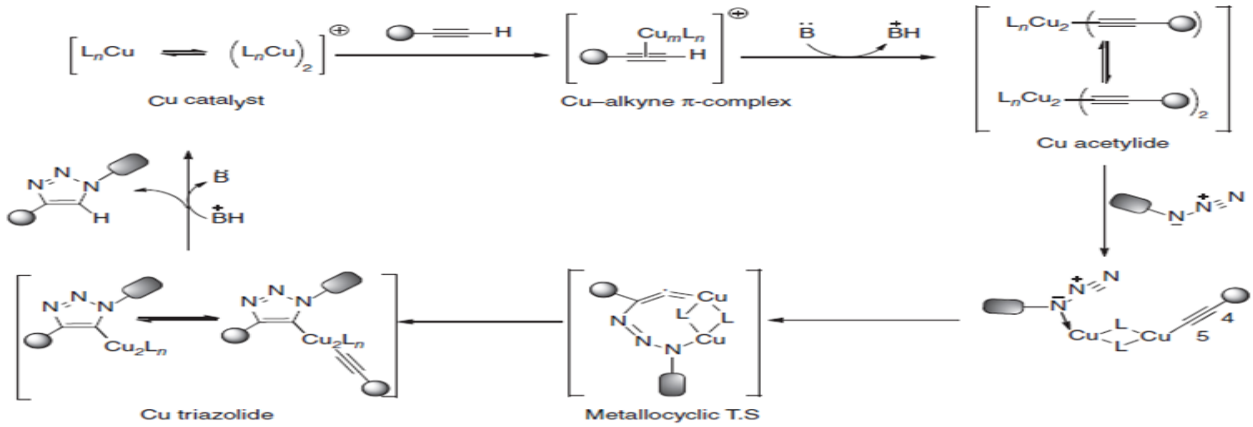
2.2. Catalysts, solvents, additives, azide and alkyne substrate in the CuAAC reaction.

2.2.1. Catalysts

As was already indicated, the Huisgen reaction was rejuvenated after using copper as a catalyst by lowering the activation barrier by 11 kcal/mol, which is enough to propel the reaction forward quickly with great selectivity. The promising success of CuAAC click reactions encouraged researchers to search for better and more stable catalysts for this reaction. Several efforts have been exerted for this purpose, various copper salts such as $CuSO_4$, $CuCl_2$ and Cu $(OAc)_2$ without any reducing agent, noncopper catalysts like $ZnCl_2$, $InCl_3$, AgCl, AgI, and silver metal were investigated but no one of them could provide the desired triazoles and Cu(I) is found to be the best catalyst (Becer et al., 2009). The distinctive activity of Cu(I) over other metal ions is a result from its capability of involvement in the terminal alkynes in both σ and π interactions and the possibility of immediate replacement of the ligands in its coordination sphere (generally in aqueous medium). This interaction between terminal alkynes and Cu(I) improves the 1,3-dipolarophilic properties of the terminal alkynes and consequentially facilitate their reaction with azides. According to some reports, Cu(I) salts can speed up this reaction by up to 10 million times. Additionally, the copper catalyst only promotes the synthesis of one of these regioisomers, the 1, 4-disubstituted-1,2,3 triazole at room or at moderate temperatures (Hein et al., 2010).



Scheme 1: 1,3-dipolar cycloaddition between alkynes and azides.



Scheme 2: Mechanism of the CuAAC reaction (Wang et al., 2016).

An essential point is that Cu(I) is thermodynamically unstable and, under aerobic conditions, it oxidizes to Cu(II) or disproportionately to a mixture of Cu and Cu(II) (C.f.D.E.a.R.C. U.S. Department of Health and Human Services Food and Drug Administration). The generation of Cu (II) halts the reaction since it is catalytically inactive. Therefore, the introduction of Cu(I) to a reaction mixture needs special consideration due to its thermodynamic instability (Singh et al., 2016). Meldal has compiled and summarized all Cu(I) sources in an extensive list (Meldal et al., 2008). The three main sources of Cu(I) are from a Cu(I) salt, from a Cu (II) salt and a reducing agent, or through oxidation of Cu found in copper wire, turnings, or powders (C.f.D.E.a.R.C. U.S. Department of Health and Human Services Food and Drug Administration). The most popular method is to *insitu* reduce Cu (II) salts, such $CuSO_4 5H_2O$ to create Cu(I) salts. Usually, sodium ascorbate is utilized in excess by 2 to 10 times as the reducing agent, but other reducing agents, including hydrazine and tris(2-carboxyethyl) phosphine (TCEP), have been applied with acceptable results (Hein et al., 2008). The benefits of this approach are the following: it is inexpensive and affordable; it functions well in aqueous solutions and does not require an inert environment or a deoxygenated atmosphere to prevent atmospheric oxygen from oxidizing copper (I) into copper (II). Additionally, this strategy prevents the production of undesirable byproducts associated with the use of

Cu(I). The Cu (II) catalyst ($CuSO_4$) removal can be simply attained by washing the product with water or by using metal resin scavengers, such as Chelex resin. The only drawback of this method is probability of reducing Cu (II) down to Cu (C.f.D.E.a.R.C. U.S. Department of Health and Human Services Food and Drug Administration) by the reducing agent. However, this can typically be avoided by the use of an appropriate reducing agent to catalyst ratio and/or the addition of a copper stabilizing agent, such as tris-(hydroxypropyltriazolylmethyl)-amine (THPTA) (Hein et al., 2008). The second method for creation the catalyst is the direct addition of Cu(I) salts. Over the past few years, many of these compounds, including CuBr, CuI, etc. have been used. While this method doesn't need a reducing agent, it does require a deoxygenated atmosphere and organic solvent (or a mixed solvent. The use of water is excluded owing to the inherent thermodynamic instability of Cu(I) which facilitate its oxidation to Cu (II). In the absence of water (which normally deprotonate the terminal alkyne to form the acetylide), Cu(I) salts need an equivalent of a base containing nitrogen to facilitate the reaction. Additionally, it has been found that amines and certain solvents such as acetonitrile participate in stabilization of the Cu(I) species through coordination, thus they prevent its degradation through oxidation or disproportionation. However, the use of Cu(I) salts as catalyst is accompanied with a higher possibility of side product formation. The use of CuI under certain conditions has been shown to produce 1-iodoalkynes, which then undergo cyclization with azides to give 5-iodotriazole products (Liang and Astruc, 2011). The third method is the use of elemental copper. This method comes with a considerable number of drawbacks. Since this approach produces quite less Cu(I) ions in solution, longer reaction durations and larger amounts of copper are required. This approach is more expensive and necessitates a somewhat acidic environment to dissolve the metal, which may be harmful to any reactants with acid-sensitive functional groups. The isolation of products with minimal copper contamination is a benefit of this approach. Various other forms of copper such as Cu(I)-modified zeolites, copper oxide nanoparticles, or copper nanoparticles adsorbed on charcoal have all been used for CuAAC reactions successfully (Ramapanicker et al., 2016). Among the various available sources of Cu(I), the *in-situ* reduction of the cheap and air stable $CuSO_4.5H_2O$ by ascorbic acid or sodium ascorbate in aqueous/alcohol solvent systems remains the most popular method of choice and known as the Sharpless-Fokin conditions (Wang et al., 2016b).

2.2.2. Additives

A potential drawback of this methodology is the cytotoxicity of copper. Therefore, it is necessary to find alternatives to the high copper concentrations needed for the acceleration of the CuAAC. Using ligands is one potential strategy to speed up the reaction without using high copper concentrations. Although the reaction proceeds on its own smoothly, several ligand systems promote the rate enhancement of the CuAAC. Most notably, the Tris((1-benzyl-4-triazolyl)methyl)amine **(**TBTA) has been shown to significantly speed up the reaction, allowing for lower catalyst loadings. Although TBTA is still the most used ligand, other relevant examples have recently arisen, including phosphites and histidine derivatives, water soluble sulfonated bathophenanthroline, pybox ligands and benzimidazoles. The addition of a ligand in the CuAAC has two functions. In one case, it prevents Cu(I) from being oxidized by various external species by stabilizing the oxidation state of Cu(I). This provides a high level of the catalytically active complexes in solution. Additionally, it is hypothesized that the reaction medium contains a variety of Cu(I) complexes and that the presence of ligands can change the equilibrium between the multi-nuclear copper complexes, causing stable clusters to dissociate and forming active complexes. (Campbell-Verduyn et al.).

2.2.3. Solvents

One of the most notable characteristics of the CuAAC reaction is the variety of solvents that can be used from organic to aqueous. The reaction is significantly exothermic and robust in all solvent polarities with very large ΔG due to triazole's aromaticity (Meldal and Diness, 2020). The comprehensive list of various solvent conditions provided by Meldal in his review (Meldal and Tornøe, 2008) which categorized into:

a) polar solvents such as alcohols, acetone, acetonitrile, dimethyl sulfoxide and dimethylformamide.

b) aqueous solvents including mixtures of water with an alcohol, acetone, tetrahydrofuran, acetonitrile, dimethyl sulfoxide and dioxane.

c) non-coordinating solvents such as toluene,

dichloromethane and chloroform.

d) weakly coordinating solvents such as pyridine, dioxane and tetrahydrofuran.

As mentioned previously, Cu (II) with ascorbate catalyst is preferred to be used in aqueous solvents, while CuI catalysts is preferred to be used in non-aqueous solvent. Traditionally, the reactions have been successful when water and an alcohol have been utilized as the solvent because this solvent mixture enabled the metal salt as well as the organic components needed to be conjugated to be effectively dissolved. Furthermore, the capability of water to act as a base permits these reactions to be carried out without the need of an external base such as N,N′-diisopropylethylamine (DIPEA). Moreover, it eliminates the requirement of protecting groups (O–H and N–H functional groups basically are "invisible" in aqueous solvents) and finally water is safe for the environment.

The click reaction conditions that are commonly used are those described by the Fokin and Sharpless group in 2002 in which the catalyst was $CuSO_4$ and 10 equivalents of sodium ascorbate in an aqueous solvent such as water with an alcohol (t-BuOH, EtOH or MeOH) to solubilize the substrate and retain the favorable aqueous medium as well (Binder et al., 2002).

2.2.4. Azide and alkyne substrate

Acetylenes and azides are highly stable across a wide range of organic reaction conditions as well as in biological environments, yet they are highly energetic functional groups. Their combination to form triazoles is irreversible and highly exothermic. In the azide-alkyne cycloaddition, alkyne serves as the dipolarophile and azide as the dipole. At atmospheric pressure and room temperature, azide and alkyne show little reactivity towards one another. This stability, which is purely kinetic in origin, explains the slow behavior of the cycloaddition reaction and is responsible for the inertness of these functional groups to biological molecules and towards the reaction conditions inside living systems (i.e. aqueous, and mild reducing environments). Additionally, the non-polar nature of azides and alkynes allows them for being hidden from and unreactive with most other functional groups therefore this permits their installation early on in a synthetic scheme (Rodionov et al., 2007). The CuAAC reaction is fairly successful with a wide range of azides and alkynes. The impact of the electronic and steric effects on the rate of the CuAAC reaction has been investigated by Matyjaszewski's team. They have found that azides with electron-withdrawing groups and less steric hindrance exhibited the fastest reactions. Moreover, they have found that alkynes with carbonyl groups in this reaction are more reactive than alkyl alkynes (Liang and Astruc, 2011). Typically, terminal alkynes are synthesized via dehydrohalogenation of vicinal dihalides in the presence of 3 equivalents of the alkoxide bases at high temperatures (Shaw et al., 2020). Organic azides can be simply synthesized either from primary amines with trifluoro methane sulfonyl azide or from halogenated alkyl compounds with sodium azide (Bräse et al., 2005). It is significant to highlight that low molecular weight azides, particularly those with several azide functionalities, can be hazardous and unsafe to handle and should never be separated from solvent. So it is advisable to generate these derivatives *in situ* just before the click reaction by SN2 reactions with organic halides or aryl sulfonates and sodium azide (Barral et al., 2007).

3. Pharmaceutical applications of CuAAC

The resulting 1,2,3-triazoles have been proven to own a number of Attractive characteristics in the field of medicinal chemistry as well as analytical chemistry. From medicinal chemistry point of view, triazoles are rigid, relatively inert heteroaromatics. They exhibit high aromatic stability because they are resistant to both acid and basic hydrolysis as well as reductive and oxidative conditions. In addition, this moiety is not hydrolyzed under physiological conditions and regarding metabolic degradation it is relatively resistant. More importantly, in terms of atom arrangement and electrical characteristics, the 1,2,3-triazole ring can roughly resemble an amide bond. At the same time, thanks to their intermediate polarity and high dipolar moment (of ∼5 D) they are capable of participating actively in hydrogen bond formation, dipole–dipole and π-stacking interactions as well. Depending on these interesting features, 1,2,3-triazoles are frequently regarded as active pharmacophore moieties in medicinal chemistry rather than a neutral linkage. They easily bind to biological targets by dipole interactions and hydrogen bonding interactions. They interestingly, displayed various biological activities, including antituberculosis, anticancer activities, anti-HIV and also antimicrobial activity (Kolb et al., 2003, Nwe et al., 2009, Tron et al., 2008). From analytical chemistry point of view, fluorescent triazoles,

generated using azide or alkyne based chemical reporters are currently successfully used in bioimaging, biolabeling, biosensing and also as chemosensors. This will be discussed in details in the next section (Finn et al., 2010, Ji et al., 2015, Le Droumaguet et al., 2010).

3.1. CuAAC based fluorogenic reactions

One of the most promising applications of the CuAAC reaction is its use as a fluorogenic reaction in which azide or alkyne moiety can be specifically derivatized using non- or weakly fluorescent alkyne or azide, respectively (Scheme 3). The design of chemo selective fluorogenic probes can be effectively achieved based on the electron-donating characters of the triazole ring formed in the azide-alkyne ligation and these promising probes could find a range of applications in biology, analytical chemistry, or material sciences. The resulting highly fluorescent 1,2,3-triazoles can provide various significant roles in sensing through binding of the target analyte, acting as a linker between the reporter and the binding site, or contributing to the reporter, usually as part of a conjugated fluorophore. This approach offers a novel green and sustained protocol for the synthesis of hetero bioconjugates with high fluorescence properties and it becomes a growing prevalent motif in chemical sensors. The remarkable advantages of this fluorogenic CuAAC reaction make it a reaction of choice for analytical chemists and biologists since it would be a powerful method for *in vivo* and *in vitro* tracking of biomolecules as well as small molecules in biological fluids. The characteristics of CuAAC as a fluorogenic reagent can be summarized as follow (Le Droumaguet et al., 2010):

(a) Ease of preparation of alkynes and azides. Azide and alkyne are very small groups and highly energetic. They can be easily introduced to organic compounds and are quite insensitive to solvents and pH.

(b) High selectivity since only the azide and alkyne components are involved in this reaction without any interference from any other organic groups present in biological fluids, such as amino, hydroxy and carboxy groups. (c) Biorthogonality of starting materials because azides and alkynes are hardly ever found in naturally occurring biomolecules making this reaction can be carried out selectively under physiological conditions even in the presence of many other functionalities in biological systems.

(d) Friendly reaction condition. Usually, it doesn't need a rise in temperature. It proceeds at room temperature but can be carried out over a broad range of temperatures (0–160°C), in a variability of solvents (including water).

(e) Reaction is simple, quick and quantitative allowing preparation of nanomoles of conjugates in diluted solutions.

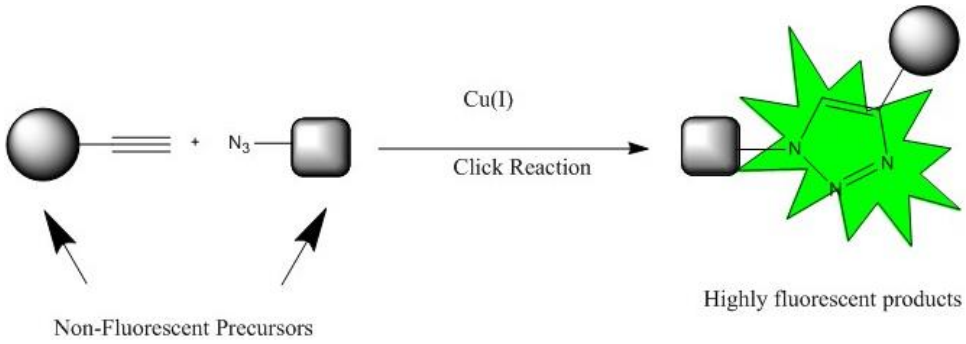
(f) The reaction is not sensitive to pH unlike some other conjugation chemistries. There is no need to control pH or add any special buffer, acid or base in reaction mixture. In fact, CuAAC shows a good performance in pH interval of 4-12.

(g) The resulting triazole is rigid, stable in a very high yield.

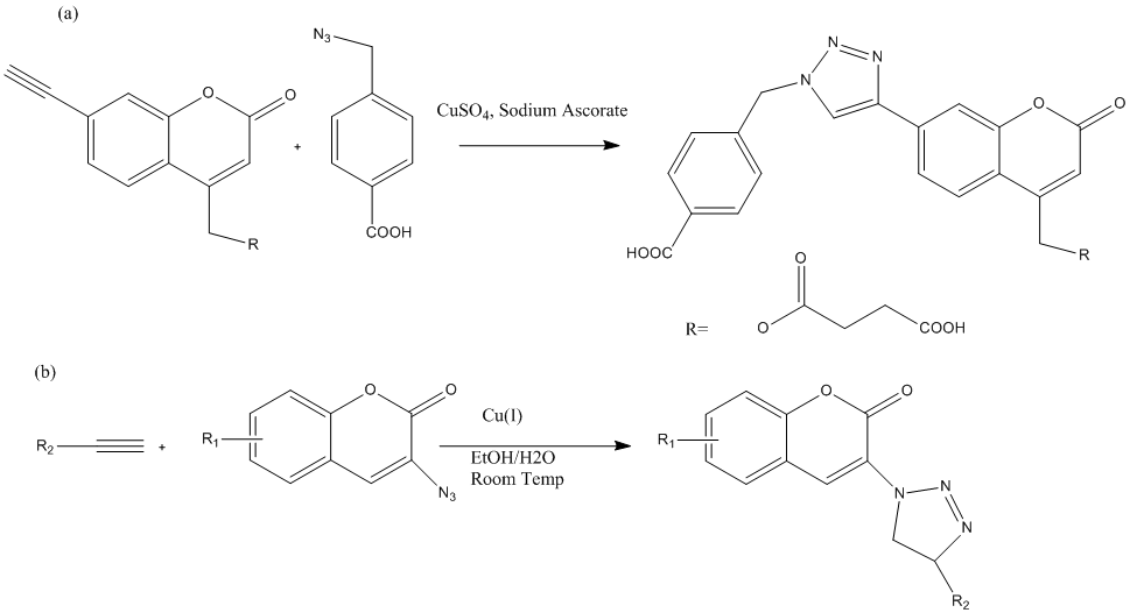
3.2. Applications of fluorogenic reactions based on CuAAC

In the light of the previously mentioned advantages several reports have been published about novel azide and alkyne bearing fluorescence probes that exploiting the advantageous changes in the electronic structure associated with the triazole ring formed in the Cu(I)-catalyzed azide-alkyne ligation reaction. The following are just a few examples; **Wang and coworkers** reported a series of fluorogenic 3-azidocoumarins (Scheme 4) which do not fluoresce due to the presence of three electron-rich nitrogen atoms at the third position. After clicking with terminal alkynes, the extension of conjugation through the five-membered triazole ring make them fluorescent (Sivakumar et al., 2004). Also based on this principle, **Zhou and Fahrni** developed the non-fluorescent alkyne-functionalized coumarin (Scheme 4) in which the alkyne group quenches the fluorescence of the coumarin core which could be restored after CuAAC reaction with the azide, by the formation of the triazole compound (Zhou et al., 2004) .

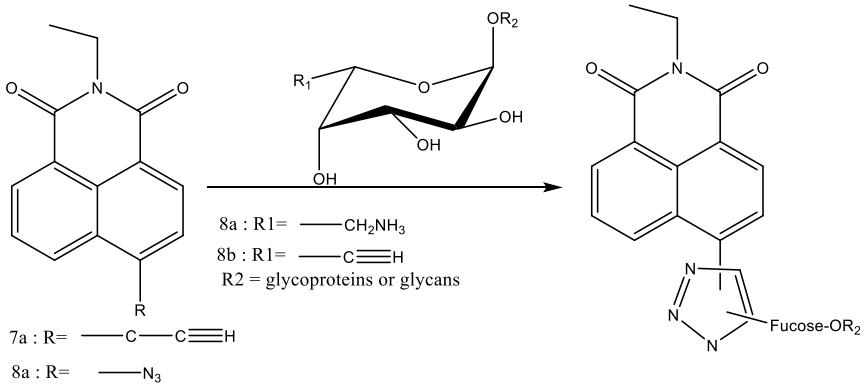
Next and coworkers presented novel clickable fluorescence probes using 1,8-naphthalimide dyes to label fucosylated glycans in vivo (Scheme 5). The electronic effect of the substituent at the 4-position of 1,8-naphthalimide has a significant influence on the fluorescence of molecule. Thus, non-fluorescent 1,8-naphthalimides 7a and 7b were synthesized with an alkyne or an azide at the 4-position. The CuAAC reactions with azido or acetylene counterparts 8a or 8b could activate the naphthalimide fluorescence signal (Sawa et al., 2006). **Wang and coworkers**



Scheme 3: Schematic representation of a typical fluorogenic click reaction (Le Droumaguet et al., 2010).

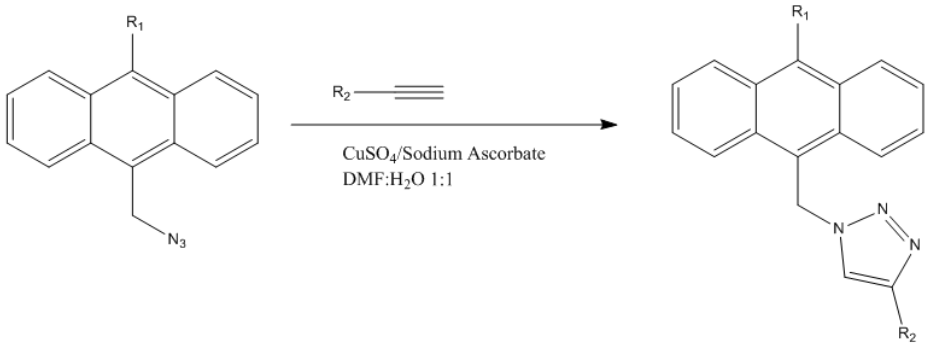


Scheme 4: Fluorogenic CuAAC reactions to afford fluorescent 3 and 6 based on non-fluorescent coumarin derivatives (Sivakumar et al., 2004, Zhou et al., 2004).

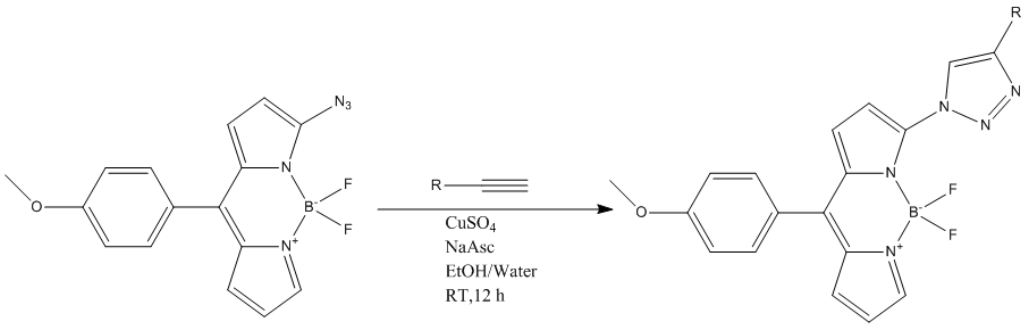


Scheme 5: Fluorogenic CuAAC reaction based on non-fluorescent1,8-naphthalimides (Sawa et al., 2006).

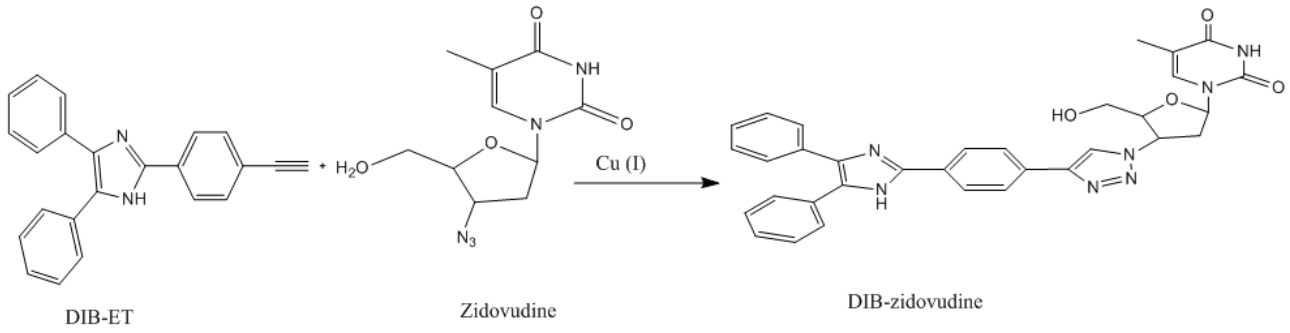
developed a new type of fluorogenic dye based on the photoinduced electron transfer process with anthracene as a fluorophore (Scheme 6). They reported the synthesis of five novel azido derivatized anthracenes and applied the CuAAC reaction to activate their fluorescence. The azido group allows an electron transfer to the excited anthracene, which quenches the fluorescence of the dye. The formation of triazole compounds via the CuAAC reaction reduces the electron donating effect of the nitrogen, blocks the PET process and restores the fluorescence (Xie et al., 2008). Boron-dipyrromethenes (BODIPY) are a class of fluorescent dyes whose fluorescence quantum yields are generally high. **Xie and Wang** prepared the non-fluorescent BODIPY with an azido-group at the 3-position of the pyrrole ring (Scheme 7), which quenches the fluorescence. After formation of triazole compounds via CuAAC reactions with alkynes, the triazole rings are weaker electron donors which enhances the fluorescence of products (Shie et al., 2014). Similarly, **Kruda and coworkers** reported a fluorescent alkyne, 2-(4-ethynylphenyl)-4,5-diphenyl-1H-imidazole (DIB-ET) as a specific fluorescence derivatization reagent for zidovudine as a model of azide compound (Scheme 8) (Maeda et al., 2014). Based on the same CuAAC reaction, **Sameh Ahmed and coworkers** reported Dansyl azide (DNS-AZ) as a novel fluorescence labeling reagent for the determination of Rasagiline mesylate (RSM) (Scheme 9) as a model example for drugs with terminal alkyne moiety (Ahmed et al., 2019). Later, **Jianjun Qi and Ching-Hsuan Tung** reported that non -fluorescent benzothiazole with an electron-deficient alkyne group at 2-position could react with azide containing molecules to form fluorescent adducts (Scheme 10) (Qi et al., 2011). More importantly, the rigidity of the 1,2,3-triazoles makes this reaction perfect choice for bioconjugations. Viruses, bacteria such as E. coli, proteins, polysaccharides, oligonucleotides have all been successfully conjugated to various substances using this approach (Le Droumaguet et al., 2010).



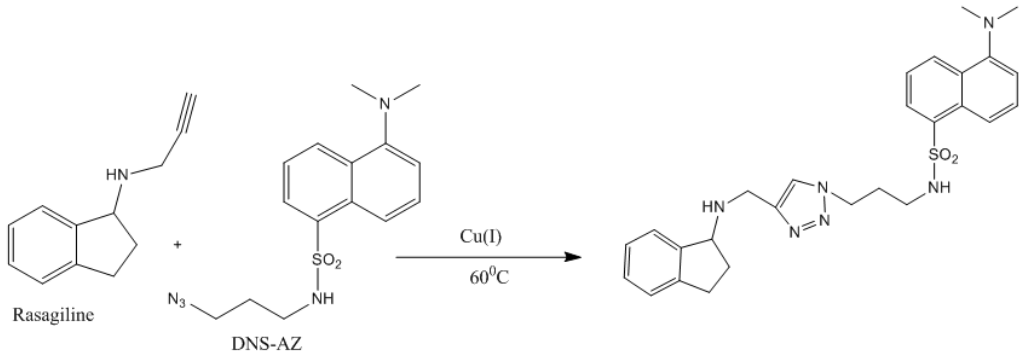
Scheme 6: The fluorogenic CuAAC reactions of azido-anthracenes and alkynes (Xie et al., 2008).



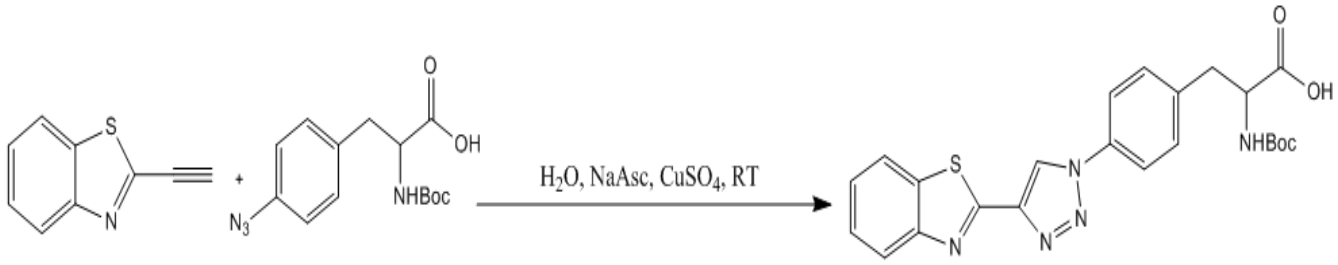
Scheme 7: Synthesis of BODIPY derivatives from azides (Shie et al., 2014)



Scheme 8: Fluorescence derivatization reaction of zidovudine with DIB-ET based on the Huisgen reaction (Maeda et al., 2014).



Scheme 9: Click chemistry derivatization reaction scheme for RSM with DNS-AZ (Ahmed et al., 2019).



Scheme10: Fluorogenic CuAAC reactions based on non-fluorescent benzothiazole alkyne 9 with azide (Qi et al., 2011).

4. The pitfalls of click chemistry

The novel philosophy of Cu(I)-catalyzed Huisgen 1,3-dipolar cycloaddition of terminal alkynes and azides is very tempting and may have given the idea that it is an "invincible" reaction that always gives high yields regardless of the circumstances. However, this reaction is imperfect and some important constraints must be taken into account. The most important challenge is the need of copper as catalyst but it is cytotoxic. The fluorogenic azide-alkyne cycloaddition reaction now has a copper-free variant, which is advantageous for intracellular labelling applications since it avoids the possible cytotoxicity of copper. Using ligands is another potential method to speed up the reaction without resorting to high copper concentrations. The second drawback is alkyne homocoupling in which alkynes react with another alkyne instead of the azide. This problem can be avoided when both the alkyne and the azide are at terminal positions on the reactants in order for the click reaction to function effectively.

Another restriction might be the instability of some azides. It is well known that an organic molecule should be regarded as explosive and extremely dangerous if the ratio of nitrogen to carbon atoms in the molecule exceeds, or is equal to, one. For pharmaceutical research, which often concentrates on bigger molecules with high carbon contents, this is fortunately not a significant problem in most cases. The last restriction covered in this paper is the fact that many "click-ready" building units and "click products" are currently not readily available in the marketplace. This implies that the pharmaceutical scientist is responsible for synthesizing their own chemicals or working with others who can. Despite the fact that click reactions are some of the simplest and highest yielding reactions known, this mission can seem overwhelming to some pharmaceutical scientist, specially whose original background is not in organic synthesis. Fortunately, the concept of click chemistry is still rather new. So, it is expected that as time goes and this approach receives wider acceptance more and more products would be commercially available (Hein et al., 2008). Despite of these considerable challenges, one may be certain that these needs will be satisfied in the near future.

5. Conclusions

Click chemistry has numerous applications in pharmaceutical sciences, polymer chemistry and materials research. Among various click reactions, the CuI-catalyzed Huisgen 1,3-dipolar cycloaddition has by far considered as the most popular one. This reaction has demonstrated itself to be an ideal candidate for fluorogenic reactions in account for its selectivity, specificity mild reaction conditions, simple work-up, and high yield. Consequently, it is anticipated that it will soon become a more common strategy for a variety of analytical chemistry applications. We can only hope that other new click reactions will be developed as time progresses, and expand our chemistry "tool box."

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Conflict of interest

There is no conflict of interest.

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