Copper-Catalyzed Azide–Alkyne Click Chemistry for Bioconjugation

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ABSTRACT

The copper-catalyzed azide-alkyne cycloaddition reaction is widely used for the connection of molecular entities of all sizes. A protocol is provided here for the process with biomolecules. Ascorbate is used as reducing agent to maintain the required cuprous oxidation state. Since these convenient conditions produce reactive oxygen species, five equivalents of a copper-binding ligand are used with respect to metal. The ligand both accelerates the reaction and serves as a sacrificial reductant, protecting the biomolecules from oxidation. A procedure is also described for testing the efficiency of the reaction under desired conditions for purposes of optimization, before expensive biological reagents are used. *Curr. Protoc. Chem. Biol.* 3:153-162 © 2011 by John Wiley & Sons, Inc.

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INTRODUCTION

A reliable set of procedures for the copper-catalyzed azide-alkyne cycloaddition (CuAAC) reaction (Fig. 1) is described here, based on investigations of the reaction mechanism and optimization using accelerating ligands for bioconjugative applications (Chan et al., 2004; Lewis et al., 2004; Himo et al., 2005; Rodionov et al., 2005; Sen Gupta et al., 2005; Chan and Fokin, 2007; Rodionov et al., 2007a,b; Hong et al., 2008, 2009; Buckley et al., 2010; Presolski et al., 2010). In practical terms, "bioconjugation" is usually used to signify making covalent bonds to biological molecules (principally oligopeptides, proteins, oligonucleotides, nucleic acids, and fatty acids), assemblies of biological molecules (such as lipid bilayers and vesicles, virus particles, and protein aggregates of many kinds), and living systems (usually cells in culture). However, these procedures are also applicable to polymers, having been particularly important for the attachment of polyethylene glycol to many compounds, or to any molecules that are handled in aqueous solvents at low concentrations. High concentrations and nonaqueous reaction conditions require a different catalyst formulation for demanding situations, as has been discussed elsewhere (Presolski et al., 2010).

The basic CuAAC process requires only copper ions in the +1 oxidation state. These may be supplied by a discrete Cu^{I} complex (Rostovtsev et al., 2002; Tornøe et al., 2002), by metallic copper (Rostovtsev et al., 2002), or by copper-impregnated materials (Lipshutz et al., 2006) that expose cuprous ions to the reaction solution, or, most conveniently, by a mixture of a Cu^{II} salt and a reducing agent, sodium ascorbate being by far the most popular (Rostovtsev et al., 2002). The development of accelerating ligands for the reaction is primarily driven by the need to maintain a sufficient concentration of Cu^{I} in solution, since copper ions can undergo fast and debilitating redox and disproportionation reactions if not properly bound by chelating ligands.

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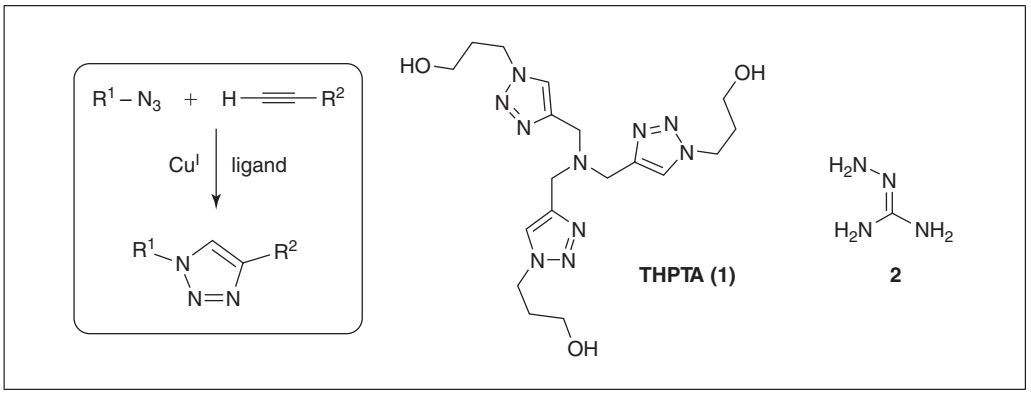

Figure 1 The general CuAAC reaction, and structures of accelerating ligand THPTA (**1**) and aminoguanidine additive (**2**).

BASIC PROTOCOL

COPPER-CATALYZED AZIDE-ALKYNE CYCLOADDITION FOR COUPLING OF CARGO-AZIDE TO BIOMOLECULE-ALKYNE

The basic procedure below describes the ligation of a functional ("cargo") azide to a biomolecule-alkyne. It can be used equally well in the reversed sense (biomolecule-azide + cargo-alkyne), and the cargo can also be a biomolecule. Aminoguanidine is recommended when side reactions between dehydroascorbate and protein side chains (principally arginine) are to be suppressed, but is not otherwise helpful and should be omitted, if possible. Tris(3-hydroxypropyltriazolyl-methyl)amine] (THPTA, structure 1, Fig. 1) serves the dual purpose of protecting biomolecules from hydrolysis by Cu(II) byproducts, and sacrificially intercepting the radicals and/or peroxides derived from O_2/Cu/ascorbate that oxidize histidine and other residues. An excess of this ligand does not dramatically slow the reaction, so more than five equivalents can be used, if necessary. Note that other water-soluble versions of the tris(triazolylmethyl)amine motif have been reported (Hong et al., 2008; Soriano del Amo et al., 2010), and are also suitable.

NOTE: All of the materials are commercially available from standard suppliers, except for the ligand (THPTA). THPTA can be prepared by the published procedure (Hong et al., 2009) or is available from the authors in small quantities until it becomes commercially available.

Materials

Biomolecule-alkyne of interest
100 mM potassium phosphate buffer, pH 7 (see Critical Parameters regarding buffers)
5 mM cargo-azide
20 mM $CuSO_4$ in water
50 mM ligand THPTA (structure 1 in Fig. 1; available in small quantities from the authors, *mgfinn@scripps.edu*), in water
100 mM sodium ascorbate: prepare fresh just before use by adding 1 ml of water to 20 mg ascorbate
100 mM aminoguanidine hydrochloride (structure 2 in Fig. 1): add 1 ml of water to 11 mg aminoguanidine)

2-ml microcentrifuge tubes
End-over-end rotator

1. In a 2-ml microcentrifuge tube, combine the following reagents in the order indicated:

   a. Biomolecule-alkyne + buffer to make 432.5 μl of solution that is 57.8 μM in alkyne.

      The procedure has been successfully done with 2 μM and higher concentrations of biomolecule-alkyne.

   b. 10 μl of 5 mM cargo-azide.

      The amount of cargo-azide added should be in ∼2-fold excess with respect to alkyne groups on the biomolecule, down to 20 μM (in other words, if the alkyne concentration is very low, more than two equivalents of azide are needed for fast reaction).

   c. A premixed solution of 2.5 μl of 20 mM $CuSO_4$ (2.5 μl) and 5.0 μl of 50 mM ligand THPTA (structure 1).

      *Final concentrations in the microcentrifuge tube: $CuSO_4$, 0.10 mM (note that this can be adjusted as desired between 0.05 and 0.25 mM); ligand **1**, 0.50 mM (ligand to copper ratio, 5:1).*

   d. 25 μl of 100 mM aminoguanidine.

      Final concentration of aminoguanidine in the tube, 5 mM.

   e. 25 μl of 100 mM sodium ascorbate.

      Final concentration of sodium ascorbate in the tube, 5 mM.

2. Close the tube (to slow the introduction of oxygen), mix by inverting the tube several times, or attach to a slow end-over-end rotator (∼30 rotations per min). Allow the reaction to proceed for 1 hr.

   Workup will depend on the particular application. Copper ions can be removed by washing or performing dialysis with solutions of buffered ethylenediamine tetraacetic acid (EDTA). The addition of an excess of EDTA relative to Cu also serves to stop the reaction when it is not desirable to simply expose the mixture to air and allow any remaining reducing agent to be used up (thus generating reactive oxygen species in the process, as described above). Copper-adsorbing resins such as Cuprisorb (http://www.seachem.com) are useful in cases of preparative organic synthesis, but tend to bind biomolecules and thus be of lesser value for bioconjugation. In such cases the conjugates are purified directly after the reaction in such a way as to leave small molecules behind.

DETERMINING THE EFFICIENCY OF BIOCONJUGATION CuAAC WITH A FLUOROGENIC PROBE

SUPPORT PROTOCOL

It is often helpful to test the reactivity of a biomolecule-alkyne in a way that allows an easy readout. For this purpose, the fluorogenic coumarin azide of Wang and coworkers is usually employed (structure **3** in Fig. 2; Sivakumar et al., 2004). A convenient assessment can be made of CuAAC efficiency under a particular set of conditions by first reacting 3 with an excess of a small-molecule "model" alkyne such as propargyl alcohol or phenylacetylene to ensure completion of the click reaction. The resulting solution is then diluted to the same concentration at which the biomolecule alkyne will be used. For a better control, also include the biomolecule without its alkyne appendage to provide a good mimic of the bioconjugation environment. The fluorescence of that solution can be used to define 100% reaction. The CuAAC reaction on the desired biomolecule-alkyne can then be performed, and the fluorescence intensity used directly to estimate the progress of the reaction. The assumption that the fluorescence wavelength and intensity of triazole **4** (Fig. 2) will not be much changed when attached to the biomolecule is usually a reasonable one, as has been demonstrated previously (Hong et al., 2009).

The example here describes a representative procedure for optimizing CuAAC conditions for a protein ("X") that has been decorated with an aliphatic terminal alkyne group,

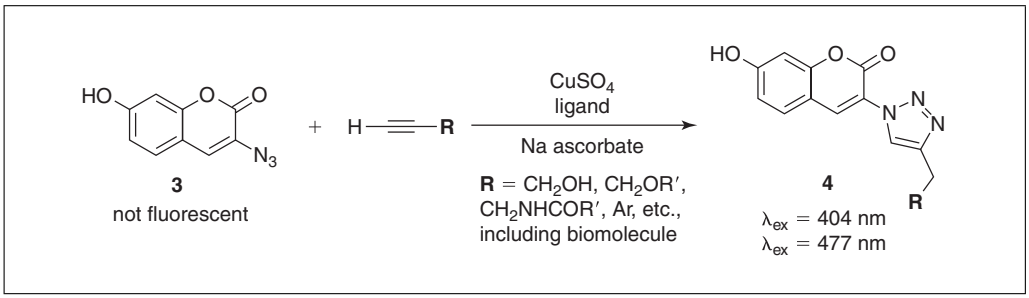

Figure 2 CuAAC reaction with Wang's fluorogenic azide (**3**), used to investigate reaction conditions before using the expensive biomolecule or cargo reagent(s).

designated "X-alkyne," to be used at a concentration of 25 μM (1 mg/ml of a 40-kDa protein bearing one alkyne). Compound 3 serves as a surrogate for the cargo-azide, to be used at a concentration of 50 μM (two equivalents with respect to alkyne). The buffer used here should be the same buffer that will be used in the desired conjugation process.

***Additional Materials** (also see Basic Protocol)*

The materials used here are identical to those used in Basic Protocol 2, except for the "cargo-azide," which is compound **3** in Figure 2; this molecule is available from Glen Research (*http://www.glenresearch.com*, cat. no. 50-2004-92), or it can also be prepared by following the published procedure (Sivakumar et al., 2004); the solid or DMSO stock solution of this compound should be stored in a refrigerator and protected from light

Protein X (see above) or surrogate protein, e.g., bovine serum albumin (BSA)

Fluorometer

Perform the CuAAC reaction

1. In a 2-ml microcentrifuge tube, combine the following reagents in the order indicated:

   a. Propargyl alcohol + buffer to make 446.2 μl of solution that is 560 μM in alkyne.

      Propargyl alcohol is used as the model alkyne.

   b. 10 μl of 5 mM azide 3 stock solution

      *The final concentration of azide **3** in the tube will be 100 μM.*

   c. A premixed solution of 6.3 μl of 20 mM $CuSO_4$ and 12.5 μl of 50 mM ligand THPTA (structure 1).

      *The final concentration of copper in the tube will be 0.25 mM and the final concentration of ligand **1** in the tube will be 1.25 mM (ligand to copper ratio, 5:1).*

   d. 25 μl of 100 mM sodium ascorbate.

      The final concentration of sodium ascorbate in the tube will be 5 mM.

2. Close the tube (to prevent more oxygen from diffusing in), mix by inverting the tube several times, or attach to an slow end-over-end rotator (~30 rotations per min). Allow the reaction to proceed for 1 hr.

3. Dilute the reaction mixture by a factor of 4 with buffer to obtain a solution approximately 25 μM in triazole **4**. In this dilution step, include protein X, if possible (or a surrogate protein such as bovine serum albumin), so that the final concentration of protein in the diluted solution is also 25 μM. Read the fluorescence intensity at 477 nm (excitation wavelength 404 nm), which should be substantially greater than that of a 25 μM solution of **3**.

   *Routine users of the CuAAC bioconjugation reaction may wish to prepare a supply of triazole **4** using propargyl alcohol and phenylacetylene to act as standards for*

reactions involving aliphatic and aromatic biomolecule-alkynes, respectively. These pure compounds can then be diluted to the appropriate concentration in the buffer/protein mixtures of interest to establish an approximate expected fluorescence intensity readout of a complete click reaction under desired conditions.

Determine CuAAC efficiency

4. In a 2-ml microcentrifuge tube, combine the following reagents in the order indicated:

   a. Combine biomolecule-alkyne + buffer to make 437.5 μl of solution that is 28.6 μM in alkyne.

   b. 5 μl of 5 mM azide 3.

      *The final concentration of azide **3** in the tube will be 50 μM.*

   c. A premixed solution of 2.5 μl of 20 mM $CuSO_4$ and 5.0 μl of 50 mM ligand THPTA (structure 1).

      *Final concentrations in the microcentrifuge tube: $CuSO_4$, 0.10 mM (note that this can be adjusted as desired between 50 and 250 μM); ligand **1**, 0.50 mM (ligand to copper ratio, 5:1).*

   d. 25 μl of 100 mM aminoguanidine.

      The final concentration of aminoguanidine in the tube will be 5 mM.

   e. 25 μl of 100 mM sodium ascorbate.

      The final concentration of sodium ascorbate in the tube will be 5 mM.

5. Close the tube (to prevent more oxygen from diffusing in), mix by inverting the tube several times, or attach to a slow end-over-end rotator (~30 rotations per min). Allow the reaction to proceed for 1 hr.

6. Read the fluorescence intensity of the mixture at 477 nm (excitation wavelength 404 nm) and compare to the intensity observed for the model reaction as described above.

   The observation of substantially lower fluorescence suggests the need for adjustment of the reaction conditions.

COMMENTARY

Background Information

The marriage of azides and alkynes in biological molecules has emerged from an appreciation of the insights made possible by the application of earlier bioorthogonal reactions in chemical biology (Lemieux and Bertozzi, 1998; Prescher and Bertozzi, 2005). A bioorthogonal reaction is performed in the presence of, or with the participation of, biological molecules, in which the molecular components of the native structures do not participate. For example, Bertozzi's use of the Staudinger ligation to attach carbohydrate molecules to proteins helped popularize the use of azides in this approach, the application being notable for the fact that efficient connections were achieved without engaging the many hydroxyl, carboxylic acid, amine, indole, imidazole, thioether, and even thiol groups present on one or both of the participating partners (Hang and Bertozzi, 2001).

Azides and alkynes are particularly useful because they are small and chemically unobtrusive. Lacking the ability to engage in strong hydrogen bonding, acid-base, hydrophobic, coulombic, dipolar, or π-stacking interactions, they are unlikely to perturb the biological molecules to which they are attached as long as their density of presentation on such scaffolds is not overwhelmingly great. An outstanding example of their invisible nature is provided by the growing number of examples in which azide- or alkyne-derivatized nutrients or cofactors are taken up and incorporated into biological molecules by living cells (Kiick et al., 2002; Prescher et al., 2004; Laughlin et al., 2006; Baskin and Bertozzi, 2007; Gierlich et al., 2007; Hsu et al., 2007; Stabler et al., 2007; Wirges et al., 2007; Ning et al., 2008; Chang et al., 2009; P.S. Banerjee et al., 2010; Breidenbach et al., 2010; Rangan et al., 2010). While exquisitely

selective, the uncatalyzed 1,3-dipolar cycloaddition reaction of standard azides and alkynes is quite slow unless the alkyne is rendered sufficiently electron-deficient to open up conjugate addition pathways and thus compromise its bioorthogonality (Huisgen, 1962, 1989). Copper catalysis of the reaction between azides and terminal alkynes represents one solution to this problem, first described independently in 2002 by the groups of Meldal in Denmark (in the context of solid-phase peptide modification; Tornøe et al., 2002) and Sharpless/Fokin in the U.S. (in the context of solution-phase reactivity; Rostovtsev et al., 2002). This reaction has come to represent "click chemistry" for many, although other reactions are also included in this concept (Kolb et al., 2001). Another general solution to the azide-alkyne rate problem is to make the alkyne highly strained in a ring structure, and is sometimes referred to as "copper-free click chemistry." This ligation method is not discussed here, but the reader should be aware that recent developments from the Bertozzi laboratory (Jewett et al., 2010) have made this process fast enough to be applicable to most bioconjugation conditions. In such situations, the major practical differences between the two triazole-forming reactions are the size of the participating alkyne group, favoring the CuAAC process which uses only a small and easily installed terminal alkyne group, versus the need for a catalyst, which of course only the CuAAC reaction requires.

Critical Parameters

The authors have found, in reviewing the literature and discussing click chemistry with others, that several practices of questionable value are sometimes employed in the execution of the CuAAC reaction. Several are listed below in approximate order of frequency. Note that these practices are not necessarily fatal; the reaction usually still works, just not at optimal rates.

1. *The use of strong base in nonaqueous conditions*. Hünig's base ($i\underline{P}r_2NEt$) is often used and yet is unnecessary and likely to diminish cycloaddition rates. The CuAAC reaction involves both the deprotonation of alkyne and reprotonation of Cu-triazolyl intermediate. While alkynes are not very acidic, there is almost never a need to accelerate Cu-acetylide formation with base, since this is already a very fast process.

2. *Failure to use an appropriate accelerating ligand*. One of the reasons for the popularity of the CuAAC reaction is its permissive nature: a wide variety of functional groups in substrates and solvates are tolerated, and ligands are often not required. However, when higher temperatures cannot be used, or rate accelerations beyond those that can be achieved by heating are required, accelerating ligands can solve may problems, since the CuAAC reaction is strongly aided by the correct coordination environment.

3. *Failure to use rudimentary methods to protect reactions from oxygen*. Even when excess sodium ascorbate is present to maintain a sufficient concentration of cuprous ions, it is useful to at least cap reactions to minimize oxygen exposure. Otherwise, copper will catalyze the oxidation of ascorbate, eventually depleting the reducing agent, killing the CuAAC catalyst, promoting Cu^{II}-mediated alkyne-alkyne (Glaser) coupling, and generating larger amounts of reactive oxygen species than is necessary. While many CuAAC reactions are fast enough to withstand these challenges, it is recommended that exposure to oxygen be limited by all convenient means, especially for reactions involving small amounts or low concentrations of azide and alkyne reagents, as are present in most bioconjugation situations.

4. *Unrealistic expectations*. Even a reaction as fast and reliable as CuAAC cannot connect two reactants to each other in a few hours if both are present in low nanomolar concentrations. In such situations, either the concentration of one of the reaction partners must be increased, or the two partners must be engineered to interact with each other in a preorganized fashion, such as via the binding of two complementary oligonucleotide chains. Also note that in this protocol, and in most CuAAC bioconjugation reactions, the copper complex is not used in catalytic amounts, but rather is present in stoichiometric or excess amounts relative to azide and alkyne. This is because the rate is dependent on copper concentration in a non-obvious way: for most catalysts of the type described here, a threshold behavior has been observed, such that little reactivity occurs below 50 μM in Cu, and maximal activity is reached at approximately 250 μM Cu (Rodionov et al., 2005; Presolski et al., 2010).

5. *The use of cuprous iodide as a copper source*. Iodide ions are good ligands for Cu^{I} and can therefore interfere with the CuAAC reaction under most bioconjugation conditions. In other cases, iodotriazoles can be formed via formation of intermediate iodoalkynes (Hein et al., 2009).

6. *The use of TCEP as reducing agent*. While the use of TCEP was reported early on (Wang et al., 2003), it was later found that the Cu-binding and azide-reducing properties of phosphines can interfere with the CuAAC reaction. When ascorbate cannot be used, hydroxylamine can function as a reducing agent for Cu^{II}, or Cu wire can be used to protect a preformed Cu^{I} complex if the reaction mixture is not exposed to excessive amounts of oxygen.

7. *Use of nonoptimal buffers or solvent mixtures.* With the catalyst described here, the reaction has been shown to proceed well over a broad pH range (Presolski et al., 2010); however, a pH around 7 is recommended for most cases. Buffers that contain very high concentrations (greater than approximately 0.2 M) of chloride ion are to be avoided because chloride can compete for Cu at these concentrations. Tris buffers can also slow CuAAC reactions because of Cu binding by the Tris molecule. Cu-phosphate complexes are often insoluble, but if the Cu source is premixed with the ligand, such complexes do not form (or do not precipitate) even in phosphate-based buffers, and reaction rates are high. Phosphate, acetate, HEPES, or MOPS buffers are commonly employed, but others are almost certainly suitable as long as they do not contain Cu-binding species.

Similarly, the role of DMSO and other coordinating co-solvents is an important one to appreciate. See Presolski et al. (2010) for a complete account, the overall lesson of which is that when high concentrations of such co-solvents are required (greater than approximately 30% to 50% of the solvent volume), a different ligand is suggested.

8. *Failure to turn up the heat.* The CuAAC process benefits greatly from higher temperatures; even modest increases that are tolerated by some biological molecules can produce good results. We speculate that many cases in which CuAAC bioconjugation does not work well suffer from the sequestering of the metal by competing coordinating species in solution, such as donor solvent molecules or donor groups in protein or other species present (Presolski et al., 2010). In such situations, the great kinetic lability of Cu^{I} centers may be compromised, and a little heat may be all that is needed to free the Cu ions.

9. *Safety notes.* Hazards associated with azide decomposition do not exist in bioconjugation situations for which the amounts of azides used tend to be small and the molecules to which they are attached are large. Still, hazardous practices in the synthesis of organic azides should be mentioned here. Of greatest concern is the potential for the generation of hydroazidoic acid (HN_3) in preparative-scale reactions using an electrophile and sodium azide. When working up such reactions that contain excess inorganic azide, exposure to acid will generate HN_3, which is volatile, highly toxic, and explosive. In general, therefore, such workups should avoid acid; in large amounts, azide ion should be quenched by nitration (Clusius and Effenberger, 1955; Stedman, 1960) as follows: In a fume hood, treat a stirred aqueous solution containing no more than 5% azide ion with sodium nitrite ($\sim$7 ml of 20% aqueous $NaNO_2$ per gram NaN_3, representing a 40% excess). Slowly add aqueous sulfuric acid (20% solution) with stirring until the reaction mixture is acidic. Note that it is important for sodium nitrite to be introduced first. When the evolution of nitrogen oxides (toxic—keep this reaction in the hood) ceases, test the acidic solution with starch iodide paper. If it turns blue, excess nitrite is present, indicating complete azide decomposition.

Although not generally used with biomolecules, chlorinated solvents (particularly CH_2Cl_2 and $CHCl_3$) should be avoided with azide ion since it is possible to generate $CH_2(N_3)_2$ and $CH(N_3)_3$, which can be highly explosive when concentrated, such as in the trap of a vacuum line. Similarly, small molecule-azides should never be isolated away from solvent in significant quantities, such as by distillation, precipitation, or recrystallization.

Troubleshooting

Troubleshooting suggestions for common problems encountered with the CuAAC reaction are presented in Table 1, along with explanations of possible causes and recommended solutions for overcoming or avoiding these problems.

Anticipated Results

This CuAAC protocol has been used successfully for the attachment of a wide variety of ligands [small molecules (Astronomo et al., 2010; Hong et al., 2010; Pokorski et al., 2011) proteins (D. Banerjee et al., 2010) DNA (Cigler et al., 2010), and organic polymers (Manzenrieder et al., 2011)] to a wide variety of biological molecules, such as the surfaces of

Table 1 Troubleshooting Guide for Bioconjugation by the CuAAC Click Reaction

Problem	Cause	Solution
A dextran-alkyne at 25 mM would not click in water, even when accelerating ligands were used	Collapse of hydrophobic regions, burying the alkynes and making them inaccessible; similar challenges can occur with proteins and oligonucleotides	Perform the reaction in denaturing or solvating conditions such as the use of lots of DMSO, along with appropriate ligand (ligand **7** in Presolski et al., 2010)
Low yields in complex biological systems, such as with the product resulting from enzymatic incorporation of an alkyne into RNA	Potential failure of one or more steps preceding the CuAAC step	Perform a test reaction with coumarin azide **3**. If little or no reaction is observed, add propargyl alcohol. Continued failure of the reaction suggests that the biological substrate is sequestering the copper catalyst, in which case excess Cu can be added. It is also possible to release active Cu by the addition of Zn^{2+}.
Substrates are incompatible with ascorbate (such as showing excessive sensitivity to reactive oxygen species or dehydroascorbate byproduct formation, not solved by the recommended use of excess ligand and aminoguanidine)	Oxidation, binding, etc.?	(a) Use hydroxylamine as reducing agent for Cu^{II} (typically 10 mM). (b) Use a Cu^{I} complex such as CuBr, CuOAc, or CuOTf, and protect the reaction from air. (c) Generate Cu^{I} electrochemically.(Hong et al., 2008).
Invitrogen Click-It kit doesn't work	Various	Use the procedure outlined in this unit
Failure of this protocol involving protein, DNA, RNA, gold nanoparticles, or other species that can bind Cu ions	Sequestration of Cu away from azide and alkyne reactants	(a) Use excess Cu and ligand. (b) Add Zn^{II} as a sacrificial metal to whatever is removing Cu from the reaction. (c) Use alternative ligand as previously described (Presolski et al., 2010) in the proper ligand:Cu ratio of 1:1 or 2:1.
CuAAC doesn't work in fresh cell lysate or under other conditions that may contain free thiols	Strong Cu-thiolate binding ties up the metal (CuAAC with THPTA usually tolerates glutathione up to 1 mM, but not more than that)	Using an accelerating ligand will help, and excess Cu, Zn^{II} or Ni^{II} can often occupy the thiols and leave some Cu^{I} free to mediate the CuAAC reaction
Failure of His_6-tagged proteins to engage in CuAAC ligation	The His-tag binds copper	Use excess copper or sacrificial metals, such as Zn^{II} or Ni^{II}; may also switch to a FLAG or other peptide tag
DNA damage observed, sometimes more seriously when the reaction mix is vortexed	Oxidation from excess reactive oxygen species (and, in one case, the use of cupric nitrate)	Use $CuSO_4$ + ascorbate, but minimize agitation of the solution and keep it capped as much as possible. In extremely sensitive cases, CuOAc with Cu wire provides the mildest conditions.
Reactions are slow with CuBr, CuI, TCEP	Low solubility and bad reducing agent	Use $CuSO_4$, sodium ascorbate, and DMSO, DMF, or NMP as co-solvent (up to 10%). These solvents appear to be biocompatible and help to dissolve most small molecules of interest.

virus-like particles. In many cases, the attachments result in polyvalent displays of triazoles on the biomolecular scaffold, and so yield can be difficult to quantify. However, when yields can be measured, CuAAC reactions usually give quantitative or near-quantitative yields, with excellent recovery of the desired conjugates, showing that the reaction conditions do not induce substantial cleavage of biological molecules.

Time Considerations

In general, the CuAAC reaction is easy to set up and perform. When the reactants are present in sufficient concentration (greater than 10 μM each), the reaction is performed properly, and no unexpected sequestration of copper ions takes place, the CuAAC reaction can be expected to provide quantitative yields of triazoles within an hour or two at room temperature. The time required for workup and purification varies from minutes for simple precipitation or molecular weight cutoff filtration to separate biomolecules from the catalyst and small-molecule reagents, to hours for chromatographic techniques.

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