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Fast, Cell-compatible Click Chemistry with Copper-chelating Azides for Biomolecular Labeling**

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Abstract

We report that azides capable of copper-chelation undergo much faster “Click chemistry” (copper-accelerated azide-alkyne cycloaddition, or CuAAC) than nonchelating azides under a variety of biocompatible conditions. This kinetic enhancement allowed us to perform site-specific protein labeling on the surface of living cells with only 10–40 μM $\text{Cu}^{\text{I/II}}$ and much higher signal than could be obtained using the best previously-reported live-cell compatible CuAAC labeling

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conditions. Detection sensitivity was also increased for CuAAC detection of alkyne-modified proteins and RNA labeled by metabolic feeding.

Keywords

Click chemistry; fluorescent probes; lipoic acid ligase; protein engineering; metabolic labelling

The copper-catalyzed azide-alkyne cycloaddition, or CuAAC, has been used extensively for the conjugation, immobilization, and purification of biomolecules.^[1] Despite excellent reaction kinetics, high specificity, and bioorthogonality, CuAAC has been used to a far lesser extent in cells and *in vivo*, because of toxicity caused by Cu^I-mediated generation of reactive oxygen species (ROS) from O₂.^[2] One approach that has been taken to address this problem is to remove the Cu^I requirement by using alkynes activated by ring strain^[3–5]. However, even the fastest of the strained cyclooctynes^[6] react with azides >10-fold more slowly than terminal alkynes in the presence of Cu^I ($k_{\text{obs}} \sim 1 \text{ M}^{-1}\text{s}^{-1}$ for (aza)dibenzocyclooctyne^[6] compared to k_{obs} of $10\text{--}100 \text{ M}^{-1}\text{s}^{-1}$ per $10\text{--}100 \mu\text{M}$ Cu^{I/II} for CuAAC^[7]). A second approach to improve cell compatibility has been to employ water-soluble ligands for Cu^I, such as THPTA^[8], BTAA^[9], and bis-L-histidine^[10]. These ligands both accelerate the cycloaddition reaction and act as sacrificial reductants, helping to protect cells and biomolecules from ROS^[8].

Here we explore a third approach to improve the cell-compatibility and performance of CuAAC. In general, reducing the copper concentration reduces the toxicity of CuAAC reaction conditions to cells, but this is accompanied by a large decrease in reaction speed^[9]. We reasoned that it might be possible to compensate for this reduction by using an azide reaction partner that contains an internal copper-chelating moiety (Figure 1A). This would raise the effective copper concentration at the reaction site. This concept has been explored previously for azide-alkyne reactions in organic solvent, with Cu^{II} rather than Cu^I, and at very high Cu (10 mM) and reactant (200–400 mM) concentrations^[11;12], but never before for conditions relevant to biomolecular labeling. The goal of our study was to examine the effect of substrate chelation assistance on CuAAC kinetics and bio-compatibility.

The rate-determining step of CuAAC is postulated to be the metallacycle formation between the Cu^I-acetylide and the organic azide^[15]. We wished to test whether an organic azide containing an internal Cu^I ligand could accelerate formation of the metallacycle and hence the overall rate of the CuAAC reaction. We prepared two azides with adjacent pyridine nitrogens to chelate Cu^I (picolyl azides **2** and **4**), in addition to their carbocyclic analogs, **1** and **3** (Figure 2).

CuAAC reaction timecourses were measured using 7-ethynyl coumarin, a fluorogenic alkyne whose quantum yield increases from 1% to 25% upon reaction with azides⁴ (Figure 2A). Assays were first performed with $10 \mu\text{M}$ CuSO₄ in the absence of Cu^I ligands. Reaction timecourses are shown in Figure S1 and values for percent conversion to product after 10 min and 30 min are given in Figure 2B. Whereas the conventional azides **1** and **3** give no detectable product after 30 min under these reaction conditions, the picolyl azides **2** and **4** give 81% and 38% product yields, respectively, after 30 min. We examined a few other picolyl azide derivatives as well. The methyl ester **5** was similar to the acid **4**. Substitution of the aromatic ring with an electron-donating methoxy group (azide **6**) further accelerated the CuAAC reaction, while an electron-withdrawing chloride substituent (azide **7**) dampened the accelerating effect. These observations are consistent with a mechanism in which rate acceleration is caused by pyridyl nitrogen coordination to Cu^I or Cu^I-acetylide, since an electron donating group will increase the electron density on this nitrogen.

We further investigated picolyl azide **4**, since it is the synthetic precursor of the ligase substrate and fluorophore conjugates described later in this work. We repeated the CuAAC reaction, but this time at three different copper concentrations (10, 40, and 100 μM), and in the absence as well as presence of Cu^I ligand THPTA (4 molar equivalents over Cu). Figure 2C shows the timecourses of these six reactions, as well as control reactions using the non-chelating analog of **4**, azide **3**.

As has previously been observed, addition of THPTA has a large effect. For the conventional azide **3**, product is undetectable after 30 min in the absence of THPTA (consistent with Figure 2B), whereas the reactions at the two higher copper concentrations (100 and 40 μM) proceed to completion within 30 min when THPTA is added. Also as expected, reduction of the copper concentration reduces the reaction rate.

Dramatic rate enhancements are seen for all six conditions when azide **3** is substituted by the chelation-competent azide **4** (Figure 2C). First, product can be detected and the reactions even proceed to completion within 30 min for the two higher Cu concentrations (100 and 40 μM), when THPTA is absent, in striking contrast to azide **3**. Second, when THPTA is added, azide **4** reacts to completion within 5 min at all three copper concentrations. In other words, the use of chelating azide **4** far offsets the reduction in CuAAC reaction rate caused by lowering Cu concentration. The effect is so strong that the reaction rate of chelating azide **4** at the lowest Cu concentration of 10 μM exceeds the reaction rate of the non-chelating azide **3** at the highest Cu concentration (100 μM). It is also noteworthy that the use of picolyl azide **4** over the conventional azide **3** can more than offset the effect of omitting the accelerating ligand THPTA. Figure 2C shows that the reaction rates with picolyl azide **4** at all three Cu concentrations in the *absence* of THPTA are at least as high as the reaction rates of conventional azide **3** in the presence of THPTA.

Based on the dramatic effects observed *in vitro*, we wished to test the utility of copper-chelating azides in the cellular setting. To target the picolyl azide moiety to specific cellular proteins, we turned to our lab's PRIME (Probe Incorporation Mediated by Enzymes^[17]) protein labeling method. A panel of *E. coli* lipoic acid ligase (LplA) mutants was prepared, each with a mutation at the gatekeeper residue, Trp37^[17–19]. We synthesized a picolyl azide derivative that matches the substrate requirements for LplA, i.e., with a carboxylic acid joined by four methylenes to the picolyl azide moiety (picolyl azide **8**; structure in Figures 1B and 3; synthesis in Figure S2). *In vitro* screening by HPLC showed that among six LplA mutants (W37G, A, V, I, L, S), W37V LplA was most efficient at recognizing picolyl azide **8** and catalyzing its covalent and ATP-dependent ligation to LplA's 13 amino acid recognition sequence, LAP (LplA acceptor peptide)^[20] (Figure S3).

To test LplA-catalyzed picolyl azide targeting on cells, we prepared HEK mammalian cells expressing LAP-tagged cyan fluorescent protein targeted to the cell surface. To these transfected cells, picolyl azide **8**, W37V LplA, and ATP were added for 20 min. LAP-conjugated picolyl azide was then detected by CuAAC with Alexa Fluor® 647-alkyne. Figures 3 and S4 show that labeling was easily detectable and specific to transfected cells.

To quantitatively examine the contribution of chelation assistance to labeling efficiency in the context of the cell-surface fluorescence labeling experiment, we repeated the labeling under several more conditions. We varied the copper concentration (10, 40, and 100 μM ; same as in Figure 2C). We supplied either the THPTA ligand used in our *in vitro* experiments (Figure 2), or BTAA ligand, which was recently reported by Wu et al. to be superior to THPTA, giving the fastest and most cell-compatible CuAAC labeling to date^[9;21].

As a control, we compared to LAP-expressing cells labeled with LplA and 8-azidooctanoic acid, an alkyl azide that is incapable of chelation assistance^[22]. Since this enzymatic ligation may have different kinetics than picolyl azide **8** ligation catalyzed by ^{W37V}LplA, we compared their labeling yields on cells quantitatively after 20 min (Figure S5). Although picolyl azide **8** ligation proceeds faster, the difference in yield is at most 1.5-fold over that of 8-azidooctanoic acid ligation. This correction factor can therefore be applied to the multivariate comparison performed in Figure S4 (cell images) and Figure 3 (quantitation of these cell images).

Several trends are apparent from Figure 3. First, for the conventional azide, 8-azidooctanoic acid, reduction of Cu concentration reduces the cell labeling signal, as expected. Second, BTAA does indeed give higher signals than THPTA, but not as much as previously reported^[9], and not at the lowest tested Cu concentration of 10 μ M. Third, replacement of 8-azidooctanoic acid on LAP with the chelation-competent picolyl azide **8** boosts cell signal across the board 4- to 38-fold, or 2.7- to 25-fold when differences in picolyl azide versus alkyl azide enzymatic ligation efficiencies (Figure S5) are taken into account. The signal enhancements are greatest at the higher Cu concentrations of 40 and 100 μ M. Like the *in vitro* data shown in Figure 2C, the signal enhancement caused by picolyl azide *more than* offsets the decrease in CuAAC rate caused by lowering the Cu concentration. For instance, the signal with picolyl azide at 10 μ M Cu (+THPTA) is still 1.6-fold (corrected value) greater than the signal with alkyl azide at 100 μ M Cu (+THPTA). Comparisons in the presence of BTAA show that picolyl azide at 40 μ M Cu gives 3.9-fold (corrected value) greater signal than alkyl azide at 100 μ M Cu. This experiment also shows that the rate enhancement caused by picolyl azide (compared to non-chelating alkyl azide) is much greater than the rate enhancement due to switching from a previous-generation ligand (THPTA) to the newest-generation ligand (BTAA). Overall, the best cell labeling results are obtained using picolyl azide in combination with BTAA ligand and either 40 or 100 μ M CuSO₄.

Using these optimized labeling conditions, we tested the site-specificity of cell surface protein labeling using LplA and CuAAC. In Figure 4, HEK cells expressing the transmembrane construct LAP-neurexin-1 β (LAP is extracellular) were labeled first with ^{W37V}LplA and picolyl azide **8**, then with Alexa Fluor[®] 647-alkyne in the presence of 40 μ M CuSO₄ and 200 μ M BTAA ligand. Transfected cells (expressing the nuclear YFP marker) were strongly labeled with a ring of Alexa Fluor[®] 647 fluorescence, whereas neighboring untransfected cells were unlabeled. Negative controls with ATP omitted, wild-type LplA in place of ^{W37V}LplA, or an alanine mutation in LAP eliminated Alexa Fluor[®] 647 labeling. The use of the picolyl azide ligase in combination with chelation-assisted CuAAC thus seems clearly advantageous, dramatically increasing signal without sacrificing specificity.

For maximal versatility, we also developed a two-step site-specific protein labeling scheme based on LplA ligation of an alkyne, followed by CuAAC derivatization with picolyl azide-fluorophore conjugates (Figure S6). The alkyne substrate for LplA is 10-undecynoic acid, and it is best ligated by ^{W37V}LplA. Figure S7 compares this labeling scheme to the original, reverse-order one. Picolyl azide ligation, followed by fluorophore-alkyne, gives ~2.4-fold greater signal on average than alkyne ligation followed by fluorophore-picolyl azide. This may reflect higher efficiency for the enzymatic ligation of picolyl azide **8** compared to 10-undecynoic acid.

Since strain-promoted cycloaddition is frequently used as an alternative to CuAAC in the cellular context, including by us^[22], we compared it beside our newly optimized CuAAC, in terms of both labeling signal and cell toxicity. Figure S8A shows that picolyl azide ligation

to LAP on cells, followed by CuAAC at 50 μM CuSO_4 is a far more sensitive labeling method than alkyl azide ligation to LAP followed by dibenzocyclooctyne^[23]. We also compared the toxicity of these labeling conditions using the CellTiter-Glo assay, which measures cellular ATP levels. Figure S8B shows that both CuAAC and strain-promoted cycloaddition conditions are equally non-toxic.

A demanding test of the biocompatibility of our new CuAAC is to perform it on neuron cultures. These delicate cells show morphological changes in the presence of even low concentrations of toxic substances. We transfected day 5 hippocampal rat neurons with plasmids encoding Homer1b-GFP (a post-synaptic marker) and LAP-neurologin-1 (a post-synaptic transmembrane adhesion protein with an extracellular LAP tag). At day 11, picolyl azide **8** was ligated to LAP for 20 min, then CuAAC was performed with THPTA and either 50 μM or 300 μM CuSO_4 . Figure 4C shows that both conditions produce specific labeling, with Alexa Fluor[®] 647 on LAP-neurologin-1 colocalization with Homer1b-GFP marker. However, the high copper condition causes focal swelling in the neurons (white arrows), a sign of toxicity. The use of chelation-assisted CuAAC allows for reduction of Cu to concentrations non-toxic to neurons, without much sacrifice in the signal intensity.

A faster and more biocompatible CuAAC labeling protocol also benefits the detection of metabolically labelled proteins and RNA. To demonstrate this, we used either conventional CuAAC or chelation-assisted CuAAC to image cellular RNAs and proteins metabolically labeled with 5-ethynyl uridine (EU)^[13] or L-homopropargylglycine (Hpg)^[14], respectively (Figure 5). Detection of these alkynes on fixed cells was accomplished with a 1.8–2.7-fold improvement in signal to noise ratio on average with Alexa Fluor[®] 647-picolyl azide compared to Alexa Fluor[®] 647-alkyl azide.

In summary, the use of copper-chelating azides dramatically accelerates the CuAAC reaction under conditions relevant to biomolecular labeling. We see this advance as complementary to advances in ligand design, which have led to CuAAC rate acceleration and reduced cell toxicity^[2;9]. Our *in vitro* data show that the picolyl azide effect is so strong that it more than compensates for the effect of omitting THPTA ligand, or reducing the Cu concentration 10-fold from 100 μM to 10 μM . On living cells, our experiments showed that use of picolyl azide instead of a conventional non-chelating azide increased specific protein signal by as much as 25-fold.

By engineering a lipoic acid ligase mutant capable of ligating picolyl azide **8** to LAP fusion proteins, we have made it straightforward to use chelation-assisted CuAAC to tag specific cell surface proteins with bright and photostable fluorophores such as the Alexa Fluors. We also demonstrated the utility of picolyl azide for sensitive detection of metabolically labelled proteins and RNAs in cells. In summary, the CuAAC protocol reported here, utilizing a copper-chelating organic azide, a newest-generation Cu^{I} ligand (BTTAA), and low Cu concentrations (10–100 μM) may represent the fastest and most biocompatible version of CuAAC to date.

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Figure 1.

Chelation-assisted Cu^{I} -catalyzed click for site-specific and metabolic labeling of biomolecules. **A)** Generic reaction scheme for Cu^{I} -catalyzed, picolyl azide-alkyne cycloaddition (chelation-assisted CuAAC), characterized in this work. **B)** Site-specific probe targeting to cell surface proteins via PRIME and chelation-assisted CuAAC. An engineered PRIME ligase (Trp37 $\rightarrow$ Val LplA) first ligates a picolyl azide derivative (picolyl azide **8**), onto LplA Acceptor Peptide (LAP), which is genetically fused to a protein of interest (POI). Picolyl azide-modified proteins are then derivatized with a terminal alkyne-probe conjugate (red circle), via live cell-compatible chelation-assisted CuAAC. BTAA^[9] and THPTA^[2]

are Cu(I) tris-triazole ligands. **C)** Labeling of newly synthesized RNAs (top) and proteins (bottom) in cells via alkynyl metabolites and chelation-assisted CuAAC. EU is a uridine surrogate^[13] and Hpg is a methionine surrogate^[14]. Alkyne-labeled RNAs and proteins are derivatized after cell fixation with picolyl azide-fluorophore conjugates (red circle).

A)

B)

azide	structure	conversion (%) after 10 min	conversion (%) after 30 min	azide	structure	conversion (%) after 10 min	conversion (%) after 30 min
1		< 1	< 1	5		13 ± 1	43 ± 1
2		43 ± 3	81 ± 3	6		52 ± 5	97 ± 2
3		< 1	< 1	7		12 ± 2	31 ± 3
4		14 ± 2	38 ± 3

Figure 2.

In vitro analysis of CuAAC rates with chelating azides. **A)** A fluorogenic click reaction with 7-ethynyl coumarin^[16] was used to measure CuAAC reaction progress. **B)** Various chelating azide structures tested and their CuAAC reaction yields after 10 min and 30 min. Reactions were run with 10 μM CuSO_4 and no Cu(I) ligand. Complete reaction traces are shown in Figure S1. **C)** Kinetic comparison of chelating azide **4** and its non-chelating benzyl counterpart **3** at different copper concentrations. CuAAC product was detected using the assay in A). Solid lines indicate reactions in which the Cu(I) ligand THPTA was added (1 equivalent over the copper concentration). Measurements were performed in triplicate. Error bars, $\pm$ s.d.

Figure 3.

Comparison of protein labeling signals on live cells using PRIME and CuAAC, with and without chelating azides. Two-step site-specific protein labeling was performed as in Figure 1B on HEK cells expressing LAP-tagged cyan fluorescent protein fused to the transmembrane domain of the PDGF receptor (LAP-CFP-TM). In the first step, either ^{W37V}LpIA was used to target picolyl azide **8** to LAP, or wild-type LpIA was used to target non-chelating 8-azidooctanoic acid. The efficiencies of these two ligation reactions are compared in Figure S5. In the second step, CuAAC was performed for 5 min with Alexa Fluor® 647-alkyne and CuSO₄ (10, 40, or 100 μM), with either THPTA or BTAA ligand (in 5-fold excess relative to the CuSO₄ concentration). Cells were imaged live immediately and representative images are shown in Figure S4. To quantify labeling signals, the mean Alexa Fluor® 647 and mean CFP intensities were calculated for >90 cells for each condition, ratioed to normalize for variations in LAP-CFP-TM expression level, and averaged. Error bars, ± s.e.m.

Figure 4.

Site-specific labeling of cell surface proteins with an engineered picolyl azide ligase and chelation-assisted CuAAC. **A)** Labeling of LAP-neurexin-1 β on the surface of live HEK cells using PRIME and CuAAC. First, picolyl azide **8** was ligated to LAP using 10 μ M ^{W37V}LpIA and 1 mM ATP for 20 min. Second, the cell media was replaced with 20 μ M Alexa Fluor® 647-alkyne, 40 μ M CuSO₄, and 200 μ M BTTAA for 5 min. Negative controls are shown with ATP omitted from the first step, wild-type LpIA used in place of ^{W37V}LpIA, and a Lys $\rightarrow$ Ala mutation in LAP. Histone2B-YFP was used as a transfection marker. **B)** Labeling of LAP-neuroigin-1 on the surface of living hippocampal rat neurons. 11 day-old neuron cultures expressing LAP-neuroigin-1 and GFP-Homer1b were labeled with picolyl azide **8** as in A), then Alexa Fluor® 647-alkyne via chelation-assisted CuAAC. Cells were imaged live after brief rinsing. CuAAC labeling was performed with two Cu concentrations indicated, in the presence of 5:1 THPTA:Cu molar ratio and 50 μ M Tempol. Alexa Fluor®

647 images in the second column correspond to the boxed regions **1** and **2**, shown at higher zoom. White arrows denote regions of focal swelling when 300 μM CuSO_4 is used. All scale bars, 10 μm .

Figure 5.

Metabolic labeling of cellular RNAs and proteins, and detection by chelation-assisted CuAAC. **A)** RNA labeling and imaging as shown in Figure 1C. *Left:* A375 cells were incubated with 200 μ M 5-ethynyl uridine (EU) for 90 min, then fixed. Detection was performed with either Alexa Fluor 647[®]-picolyl azide (first column) or Alexa Fluor 647[®]-alkyl azide (second column). 2 mM CuSO₄ and 8 mM THPTA were used. Thereafter, cellular DNA was stained with Hoechst 33342. A negative control with EU omitted is shown (third column). *Right:* Graph showing mean Alexa Fluor 647 intensities, for >3500 single cells for each condition. **B)** Same as A, except that instead of RNA, proteins were metabolically labeled with 50 μ M homopropargylglycine (Hpg) for 90 min, before fixation and detection with Alexa Fluor 647[®] (picolyl azide or alkyl azide conjugate). Error bars, $\pm$ s.e.m.