

Fast, Cell-Compatible Click Chemistry with Copper-Chelating Azides for Biomolecular Labeling**

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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 the cellular context because of toxicity caused by the Cu^I-mediated generation of reactive oxygen species (ROS) from O₂.^[2] One way 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 more than tenfold slower than terminal alkynes in the presence of Cu^I ($k_{\text{obs}} \approx 1 \text{ M}^{-1} \text{ s}^{-1}$ for (aza)dibenzocyclooctyne^[6] compared to $k_{\text{obs}} \approx 10\text{--}100 \text{ M}^{-1} \text{ s}^{-1}$ per 10–100 μM Cu^I/Cu^{II} for CuAAC^[7]). A second approach to improve cell compatibility is to use water-soluble ligands such as tris-(hydroxypropyltriazolylmethyl)amine (THPTA),^[8] bis[(*tert*-butyltriazolyl)methyl]-(2-carboxymethyltriazolyl)methylamine (BTAA),^[9] or bis(L-histidine)^[10] for Cu^I. 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, decreasing the copper concentration lowers the toxicity of CuAAC to cells, but this is accompanied by a large decrease in reaction kinetics.^[9] We reasoned that it might be possible to compensate for this decrease by using an azide reaction partner that contains an internal copper-chelating moiety (Figure 1 A), which would raise the effective copper concentration at the reaction site. This concept has been explored for azide–alkyne reactions in organic solvents, with Cu^{II} rather than Cu^I species, and at very high copper (10 mM) and

reactant (200–400 mM) concentrations,^[11,12] but never before under conditions relevant to biomolecular labeling. The goal of our study was to examine the effect of substrate chelation assistance on CuAAC kinetics and biocompatibility.

The rate-determining step of CuAAC is postulated to be the formation of the metallacycle from the Cu^I acetylide and the organic azide.^[15] We decided 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 proximal pyridine nitrogen atoms to chelate the Cu^I ion (picolyl azides **2** and **4**), as well as their nonchelating carbocyclic analogues, **1** and **3** (Figure 2).

CuAAC reaction timecourses were measured using 7-ethynylcoumarin, a fluorogenic alkyne whose quantum yield (QY) increases from 1 % to 25 % upon reaction with azides^[4] (Figure 2 A). Assays were first performed with 10 μM CuSO₄ in the absence of Cu^I ligands. Reaction timecourses are shown in Figure S1 (see Supporting Information) and values for percent conversion into product after 10 and 30 minutes are given in Figure 2 B. Whereas the conventional azides **1** and **3** give no detectable product after 30 minutes under these conditions, the picolyl azides **2** and **4** give 81 % and 38 % product yields, respectively, after 30 minutes. We examined a few other picolyl azide derivatives as well. The methyl ester **5** gives results similar to the acid **4**. Substitution of the aromatic ring with an electron-donating methoxy group (azide **6**) further accelerates the CuAAC reaction, while an electron-withdrawing chloride substituent (azide **7**) reacts slower than the other picolyl azide derivatives. These observations are consistent with a mechanism in which rate acceleration is caused by coordination of the pyridyl nitrogen atom to Cu^I or Cu^I acetylide, since an electron-donating group will increase the electron density on this nitrogen atom, improving coordination.

We further investigated picolyl azide **4**, because 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), either in the absence or the presence of Cu^I ligand THPTA (4 molar equivalents relative to copper). Figure 2 C shows the timecourses of these six reactions, as well as control reactions using the non-chelating analogue of **4**, azide **3**.

As has previously been observed, the addition of THPTA increases the CuAAC reaction rate. For the conventional azide **3**, product is undetectable after 30 minutes in the absence of THPTA (consistent with Figure 2 B), whereas the reactions at 100 and 40 μM copper proceed to completion

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[**] We thank Carolyn Kwa, Daniel Liu, and Ken Loh for assistance with neuron culture, Jennifer Yao for LpIA enzymes, and Peng Zou for critical reading of the manuscript. Prof. M. G. Finn (Scripps) provided the initial batch of THPTA ligand. Funding was provided by the NIH (R01 GM072670), the Dreyfus Foundation, and the American Chemical Society. C.U. was supported by the C.P. Chu and Y. Lai summer graduate fellowship (MIT).

Supporting information for this article (figures and experimental details) is available on the WWW under <http://dx.doi.org/10.1002/anie.201108181>.

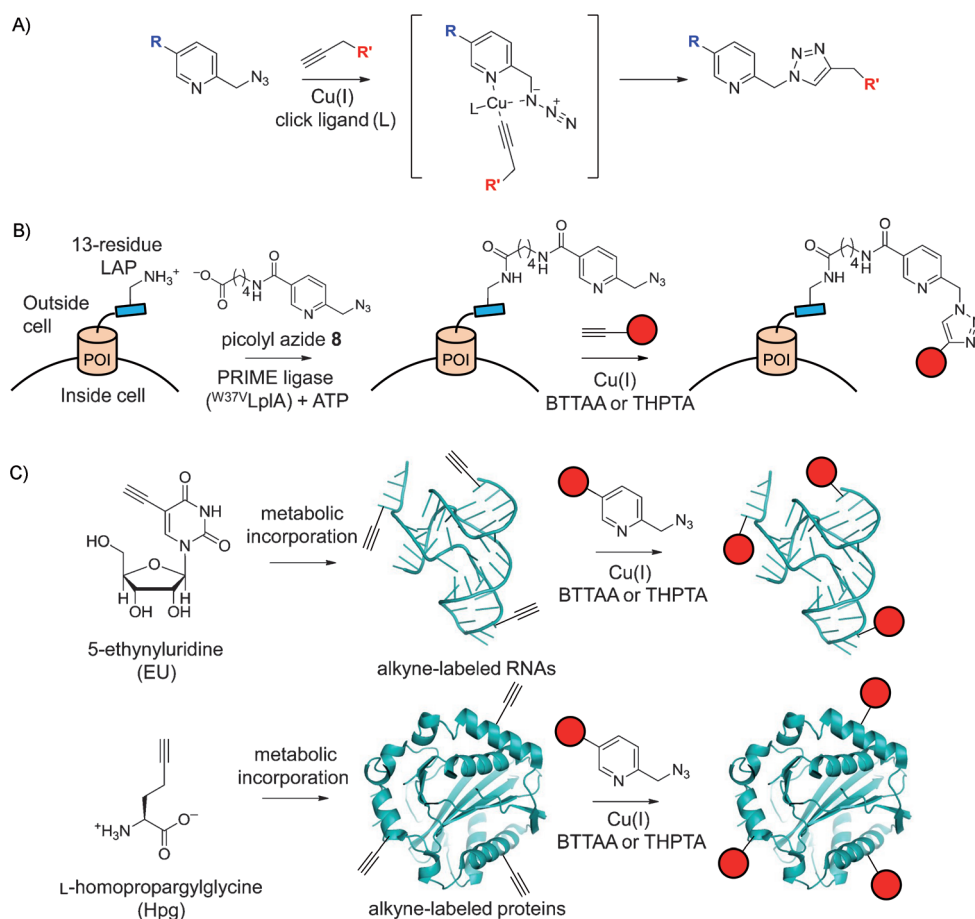


Figure 1. Chelation-assisted Cu^I-catalyzed click reaction for site-specific and metabolic labeling of biomolecules. A) Generic reaction scheme for Cu^I-catalyzed, picolyl azide-alkyne cycloaddition (chelation-assisted CuAAC). B) Site-specific probe targeting to cell-surface proteins by PRIME and chelation-assisted CuAAC. An engineered PRIME ligase (LplA Trp37→Val) first ligates a picolyl azide derivative (picolyl azide **8**) onto LplA acceptor peptide (LAP, blue), 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), by 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 using 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).

within 30 minutes when THPTA is added. As expected, lowering the copper concentration lowers 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). Without THPTA, reactions proceed to completion within 30 minutes for the two higher Cu concentrations (100 and 40 μM), in striking contrast to azide **3**. When THPTA is added, azide **4** reacts to completion within 5 minutes at all three copper concentrations. In other words, the use of chelating azide **4** far offsets the decrease in the CuAAC reaction rate caused by lowering the Cu concentration. The effect is so strong that the reaction rate of chelating azide **4** at the lowest Cu concentration (10 μM) exceeds the reaction rate of the nonchelating azide **3** at the highest Cu concentration (100 μM). It is also noteworthy that the use of picolyl azide **4** instead of 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 tested the utility of copper-chelating azides to fluorescently label proteins in a cellular setting. To target the picolyl azide moiety to specific cellular proteins, we turned to our 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 gate-keeper 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 methylene groups to the picolyl azide moiety (picolyl azide **8**; structure in Figure 1B; synthesis in Figure S2 of the Supporting Information). *In vitro* screening by HPLC of six LplA mutants (W37G, A, V, I, L, S) showed that the valine mutant (^{W37V}LplA) was most efficient at recognizing picolyl azide **8** and catalyzing its covalent, ATP-dependent ligation to the 13 amino acid recognition sequence of

LplA, namely LAP (LplA acceptor peptide; Supporting Information Figure S3).^[20]

To test LplA-catalyzed picolyl azide targeting on cells, we prepared human embryonic kidney (HEK) cells expressing LAP-tagged cyan fluorescent protein (CFP) targeted to the cell surface. Picolyl azide **8**, ^{W37V}LplA, and ATP were added for 20 minutes to these transfected cells. LAP-conjugated picolyl azide was then detected by CuAAC with Alexa Fluor 647-alkyne. Figure 3 and Figure 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 cell-surface fluorescence experiments, we repeated the labeling under several more conditions. We varied the copper concentration (10, 40, and 100 μM ; same as in Figure 2C). We added either THPTA ligand used in our *in vitro* experiments (Figure 2) or BTAA ligand, which was recently reported by Wu and co-

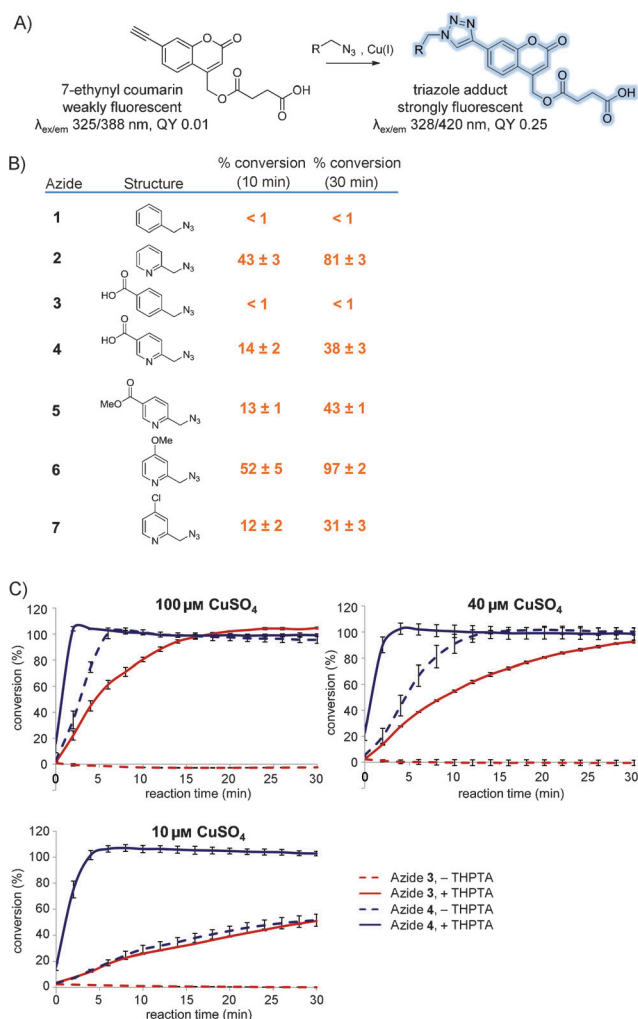


Figure 2. In vitro analysis of CuAAC rates with chelating azides. A) Fluorogenic click reaction with 7-ethynylcoumarin^[16] used to measure CuAAC reaction progress. B) Various chelating azide structures tested and their CuAAC reaction yields after 10 min and 30 min. Reactions were run with 10 μM CuSO_4 and no Cu^I ligand. Complete reaction traces are shown in Figure S1 of the Supporting Information. C) Kinetic comparison of chelating azide **4** and its nonchelating 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 in fourfold molar excess over CuSO_4 . Measurements were performed in triplicate. Error bars, $\pm$ standard deviation (s.d.).

workers to be superior to THPTA and to give the fastest and most cell-compatible CuAAC labeling to date.^[9,21]

As a control, we compared LAP-expressing cells labeled with LpIA 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 LpIA, we compared their labeling yields on cells after 20 minutes (Supporting Information, Figure S5). Although picolyl azide **8** ligation is faster, the difference in yield after 20 min of labeling is at most 1.5-fold over that of 8-azidooctanoic acid ligation. This correction factor can therefore be applied to the multivariate compar-

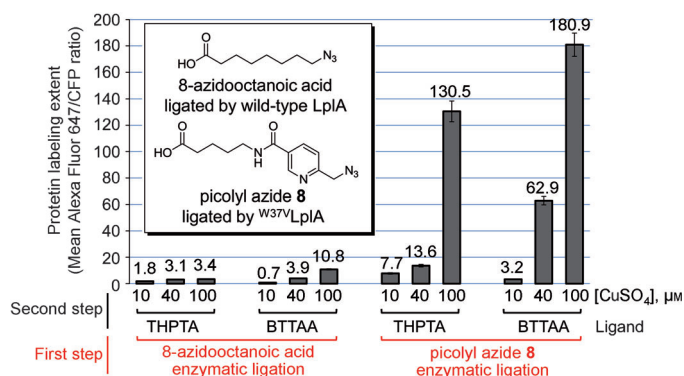


Figure 3. Comparison of protein labeling signals on live cells using PRIME and CuAAC, with and without chelating azide. Two-step, site-specific protein labeling was performed as depicted in Figure 1 B on HEK cells expressing LAP-tagged cyan fluorescent protein fused to the transmembrane domain of the 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 nonchelating 8-azidooctanoic acid. In the second step, CuAAC was performed for 5 min with Alexa Fluor 647-alkyne and CuSO_4 (10, 40, or 100 μM), with either THPTA or BTAA ligand (in fivefold excess over CuSO_4). Live cells were imaged 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 and averaged. Error bars, $\pm$ standard error of the mean (s.e.m.).

ison shown 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, decreasing the copper 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 copper concentration of 10 μM . Third, replacement of 8-azidooctanoic acid on LAP with the chelation-competent picolyl azide **8** boosts cell signal at all Cu concentrations 4- to 38-fold. When differences in picolyl azide versus alkyl azide enzymatic ligation efficiencies are taken into account, the difference is 2.7- to 25-fold (Supporting Information, Figure S5). The signal enhancements are greatest at the higher Cu concentrations of 40 and 100 μM . Similar to the in vitro data shown in Figure 2C, the signal enhancement caused by picolyl azide more than offsets the decrease in the CuAAC rate caused by lowering the Cu concentration. For example, the signal with picolyl azide at 10 μM Cu (+ THPTA) is still 1.6-fold (corrected value) greater than the signal with alkyl azide at 100 μM Cu (+ THPTA). Comparisons in the presence of BTAA ligand show that picolyl azide at 40 μM Cu gives a 3.9-fold (corrected value) greater signal than alkyl azide at 100 μM Cu. This experiment also shows that the rate enhancement caused by picolyl azide (compared to the nonchelating 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 **8** in combination with BTAA ligand and either 40 or 100 μM CuSO_4 .

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

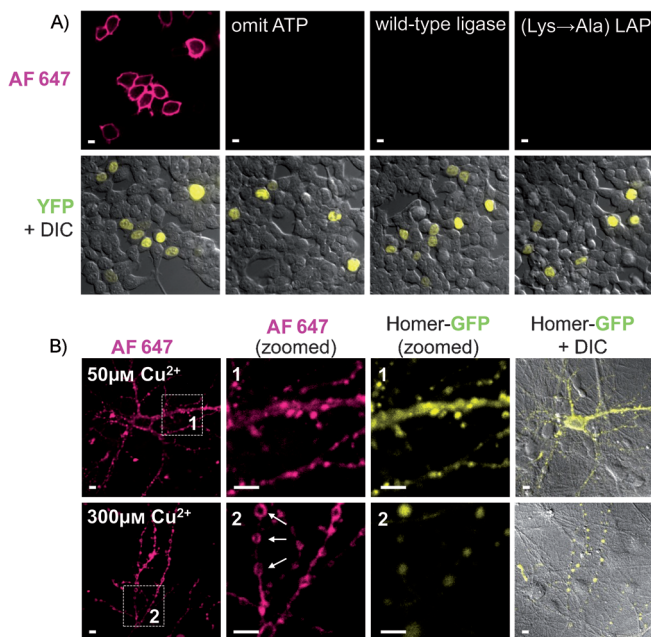


Figure 4. Site-specific labeling of cell-surface proteins with an engineered picolyl azide ligase and chelation-assisted CuAAC. A) Labeling of LAP-neurexin-1 β on the surface of live HEK cells. First, picolyl azide **8** was ligated to LAP using 10 μ M ^{W37V}LplA and 1 mM ATP for 20 min. Second, the cell media was replaced with 20 μ M Alexa Fluor 647-alkyne, 40 μ M CuSO₄, and 200 μ M BTAA for 5 min. Negative controls are shown with ATP omitted, wild-type LplA in place of ^{W37V}LplA, and a Lys→Ala mutation in LAP. Histone2B-YFP was used as a transfection marker. DIC = differential interference contrast image, AF 647 = Alexa Fluor 647. B) Labeling of LAP-neuroigin-1 on the surface of living hippocampal rat neurons. 11 day-old neuron cultures expressing LAP-neuroigin-1 and Homer1b-GFP were labeled with picolyl azide **8** as in (A), followed by Alexa Fluor 647-alkyne. Cells were imaged live after brief rinsing. CuAAC labeling was performed at the two copper concentrations indicated, in the presence of 5:1 THPTA/Cu molar ratio and 50 μ M 4-hydroxy-2,2,6,6-tetramethylpiperidine (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 μ M CuSO₄ is used. All scale bars, 10 μ m. GFP = green fluorescent protein.

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 a nuclear yellow fluorescent protein (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 did not result in Alexa Fluor 647 labeling. The use of the picolyl azide ligase in combination with chelation-assisted CuAAC thus seems clearly advantageous, dramatically increasing the signal without sacrificing specificity.

For maximal versatility, we also developed a similar but reverse-order site-specific protein labeling scheme based on LplA ligation of an alkyne, followed by CuAAC derivatization with picolyl azide-fluorophore conjugates (Supporting Information, Figure S6). The alkyne substrate for LplA is 10-undecynoic acid, and it is best ligated by ^{W37V}LplA. Figure S7 compares the original labeling scheme to this reverse-order scheme. Picolyl azide ligation, followed by CuAAC with fluorophore-alkyne, gives an approximately 2.4-fold greater signal on average than alkyne ligation followed by CuAAC with 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 to our newly optimized CuAAC, in terms of both labeling signal and cell toxicity. Figure S8A in the Supporting Information shows that picolyl azide ligation to LAP on cells, followed by CuAAC at 50 μ M CuSO₄ gives far greater signal than alkyl azide ligation to LAP followed by addition of 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 our CuAAC and strain-promoted cycloaddition conditions are equally nontoxic.

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 five-day-old hippocampal rat neuron cultures with plasmids for Homer1b-GFP (a postsynaptic marker) and LAP-neuroigin-1 (a postsynaptic transmembrane adhesion protein with an extracellular LAP tag). On day 11, picolyl azide **8** was ligated to LAP for 20 minutes, then CuAAC was performed with THPTA and either 50 μ M or 300 μ M CuSO₄. Figure 4B shows that both conditions produce specific labeling, with Alexa Fluor 647 on neuroigin-1 colocalized with Homer1b-GFP marker. However, the high copper concentration causes focal swelling in the neurons (white arrows), which is a sign of toxicity. The use of chelation-assisted CuAAC allows the concentration of Cu to be decreased to levels minimally toxic to neurons, with little sacrifice to the signal intensity.

A faster and more biocompatible CuAAC labeling protocol also benefits the detection of metabolically labeled 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-ethynyluridine (EU)^[13] or L-homopropargylglycine (Hpg),^[14] respectively (Figure 1C and Figure 5). Detection of these alkynes on fixed cells was accomplished with a 1.8–2.7-fold improvement in average signal intensity 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 also led to CuAAC rate acceleration and reduced cell toxicity.^[2,9]

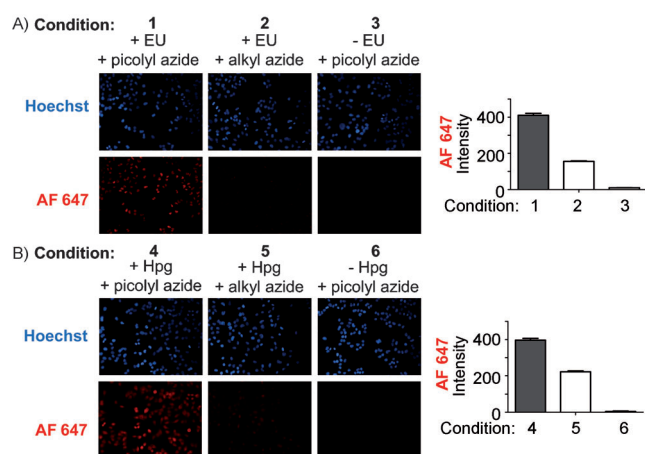


Figure 5. Metabolic labeling of cellular RNAs and proteins, and detection by chelation-assisted CuAAC. A) RNA labeling and imaging as shown in Figure 1 C. Left: A375 cells were incubated with 200 μM 5-ethynyluridine (EU) for 90 min, then fixed. Detection was performed with either Alexa Fluor 647-picolyl azide (first column) or Alexa Fluor 647-alkyl azide (second column). 2 mM CuSO_4 and 8 mM THPTA were used. Cellular DNA was stained with Hoechst 33342. A negative control with EU omitted is shown in the third column. Right: Graph showing mean Alexa Fluor 647 intensities, for > 3500 cells for each condition. B) Same as (A), except that instead of RNA, proteins were metabolically labeled with 50 μM 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. AF 647 = Alexa Fluor 647.

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 decreasing the Cu concentration from 100 μM to 10 μM . Our experiments on living cells show that the use of picolyl azide, instead of a conventional nonchelating azide, increases the specific protein labeling signal by as much as 25-fold.

By engineering a lipoic acid ligase mutant capable of conjugating 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 the sensitive detection of metabolically labeled proteins and RNAs in cells. In summary, the CuAAC protocol reported here, in which a copper-chelating organic azide, a newest-generation Cu^I ligand (BTAA), and low Cu concentrations (10–100 μM) are utilized, may represent the fastest biocompatible version of CuAAC to date.

Received: November 21, 2011

Revised: February 13, 2012

Published online: May 3, 2012

Keywords: click chemistry · fluorescent probes · lipoic acid ligase · metabolic labeling · protein engineering

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