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Sulfated Ligands for the Copper(I)-Catalyzed Azide–Alkyne Cycloaddition

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On the occasion of the 10th anniversary of click chemistry

Abstract: The copper(I)-catalyzed azide–alkyne cycloaddition (CuAAC), the prototypical reaction of click chemistry, is accelerated by tris(triazolylmethyl)amine-based ligands. Herein, we compare two new ligands in this family—3-[4-({bis[(1-*tert*-butyl-1*H*-1,2,3-triazol-4-yl)methyl]amino}methyl)-1*H*-1,2,3-triazol-1-yl]propanol (BTTP) and the corresponding sulfated ligand 3-[4-({bis[(1-*tert*-butyl-1*H*-1,2,3-triazol-4-yl)methyl]amino}methyl)-1*H*-1,2,3-triazol-1-yl]propyl hydrogen sulfate (BTTPS)—for three bioconjugation applications: 1) labeling of alkyne-tagged glycoproteins in crude cell lysates, 2) labeling of alkyne- or azide-tagged glycoproteins on the surface of live mammalian cells, and 3) labeling of azides in surface proteins of live *Escherichia coli*. Although BTTPS exhibits faster kinetics than BTTP in accelerating the CuAAC reaction in in vitro kinetic measurements, its labeling efficiency is slightly lower than BTTP in modifying biomolecules with a significant amount of negative charges due to electrostatic repulsion. Nevertheless, the negative charge conferred by the sulfate at physiological conditions significantly reduced the cellular internalization of the coordinated copper(I), thus making BTTPS–Cu^{I} a better choice for live-cell labeling.

Keywords: bioconjugation · click chemistry · copper · cycloaddition · glycoconjugates

Introduction

The past 30 years have witnessed a growing synergy between advanced organic chemistry and biomedical research.[1] Featuring exquisite selectivity and bioorthogonality, the copper(I)-catalyzed azide–alkyne cycloaddition (CuAAC),[2–4] the prototypical reaction of click chemistry,[5] is one of the prominent chemical transformations that have brought new technique advances in numerous biological fields.[6,7] For example, CuAAC has offered simpler and highly specific methods for bioorthogonal conjugation in the covalent labeling of biomolecules.[8–11] It also allows straightforward derivatization of natural products to generate new antibiotic activities.[12,13] However, the current copper(I) catalyst formulation has two major problems: toxicity, which hinders its use in living systems,[14,15] and slow kinetics,[16] which hampers its use in quantitative functionalization of biomolecules of limited quantities.[17]

Our initial screening of a small library consisting of 14 water-soluble tris(triazolylmethyl)amine-based ligands led to the discovery of a potent ligand—2-[4-({bis[(1-*tert*-butyl-1*H*-1,2,3-triazol-4-yl)methyl]amino}methyl)-1*H*-1,2,3-triazol-1-yl]ethyl hydrogen sulfate (BTTES)—that dramatically accelerated the rate of the azide–alkyne cycloaddition by coordinating with the in situ generated copper(I), and also rendered the CuAAC nontoxic.[18] The new catalyst formulation is BTTES–$CuSO_4$: for live-cell labeling, [ligand]/[$CuSO_4$] = 6:1, [$CuSO_4$] = 50–75 μM and 2.5 mM sodium ascorbate to reduce copper(II) to copper(I) in situ (Scheme 1). Although this catalytic system holds great promise for biocompatible applications, further improvement of its activity and lowering of the copper loading are desirable for broader biomedical applications. Building upon this discovery, we performed

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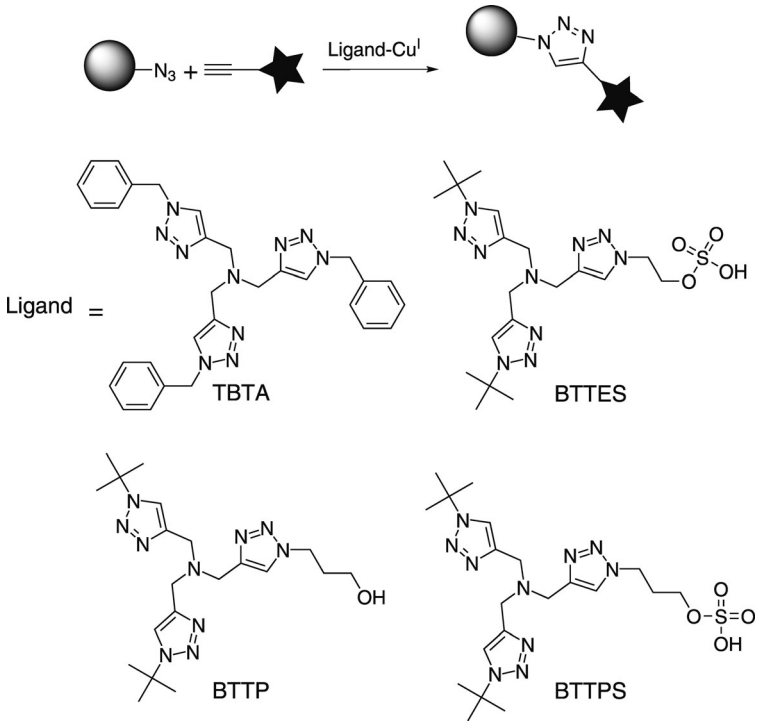

Scheme 1. CuAAC is accelerated by the copper(I)-stabilizing ligands. TBTA = tris[(1-benzyl-1*H*-1,2,3-triazol-4-yl)methyl]amine, BTTPS = 3-[4-({bis[(1-*tert*-butyl-1*H*-1,2,3-triazol-4-yl)methyl]amino}methyl)-1*H*-1,2,3-triazol-1-yl]propyl hydrogen sulfate, BTTP = 3-[4-({bis[(1-*tert*-butyl-1*H*-1,2,3-triazol-4-yl)methyl]amino}methyl)-1*H*-1,2,3-triazol-1-yl]propanol.

a structure–activity relationship study, in which we identified a new ligand (BTTPS) that shows better kinetics in accelerating the CuAAC.

Herein, we compare the activity of BTTPS and the corresponding unsulfated ligand (BTTP) in a set of bioconjugation studies: 1) labeling of alkyne-tagged glycoproteins in crude cell lysates, 2) labeling of azide- and alkyne-tagged glycoproteins on the surface of live mammalian cells, and 3) labeling of azide-bearing proteins on the surface of live *Escherichia coli*. We discovered that both ligands were highly efficient for all these bioconjugation applications with biocompatibility of the catalyst further improved by ligand sulfation.

Kinetic Evaluation of the Ligand-Accelerated CuAAC

The relative reactivity of the copper(I) catalysts in the form of TBTA–Cu^I, BTTES–Cu^I, BTTP–Cu^I, and BTTPS–Cu^I complexes was determined in a fluorogenic assay by treating propargyl alcohol (50 μM) with 3-azido-7-hydroxycoumarin (100 μM) (Figure 1a).[19] In all studies, $CuSO_4 \cdot 5H_2O$ was used as the copper source to form complexes with the ligands. The active catalysts were generated in situ by using a 2.5 mM solution of sodium ascorbate. BTTPS showed the highest activity of the four tris(triazolylmethyl)amine-based ligands evaluated in accelerating the CuAAC; BTTP and BTTES showed comparable rate acceleration and TBTA exhibited the lowest activity. BTTPS–Cu^I gave the largest slope of the reaction curve, yielding more than 50% cycloaddition product within the first 30 min when a 75 μM solution of $CuSO_4$ was used and the ligand/$CuSO_4$ ratio was 6:1. Under the same conditions, quantitative conversion was realized with increasing concentration of propargyl alcohol (300 μM). By contrast, the TBTA-mediated reaction was significantly slower, thereby yielding less than 20% cycloaddition product (Figure 1b).

Labeling of Glycoproteins in Crude Cell Lysates

To compare the efficacy of BTTPS- and BTTP-mediated CuAAC in labeling glycoproteins, we sought to investigate bioconjugation of affinity probes to alkyne-tagged sialylated glycoproteins in crude cell lysates, which is the first step in enriching these proteins for glycoproteomic analysis. We cultured Jurkat cells, a human T lymphocyte cell line, in medium supplemented with peracetylated *N*-(4-pentynoyl)mannosamine (Ac_4ManNAl) to introduce terminal alkynes onto the cell-surface sialylated glycoconjugates.[20] After three days, we lysed the cells and reacted the cell lysates with either biotin–azide or FLAG–azide by the CuAAC. In these labeling reactions, 100 μM of biotin-azide or FLAG–azide was used as the coupling partner, and the ratio of azide, ligand, $CuSO_4$, and sodium ascorbate was held at 1:5:2.5:25–a labeling condition optimized in our lab. After reaction for one hour, we probed the modified cell lysates with anti-biotin or anti-FLAG Western blots. Robust labeling was observed in both cases for lysates isolated from cells treated with Ac_4ManNAl, whereas no signals were detectable for lysates obtained from cells cultured in the absence of the sugar (Figure 2). Consistent with the kinetic measurements, stronger signals were detected for BTTPS–Cu^I-treated lysates than the BTTP–Cu^I-treated counterparts when biotin was used as the probe (Figure 2a). Interestingly, when FLAG was used as the probe, the BTTP–Cu^I catalyst afforded stronger signals (Figure 2b). At neutral pH, the FLAG peptide has a large amount of negative charges, which may interfere with the approach of BTTPS functionalized with a negatively charged arm, thus lowering the labeling efficiency.

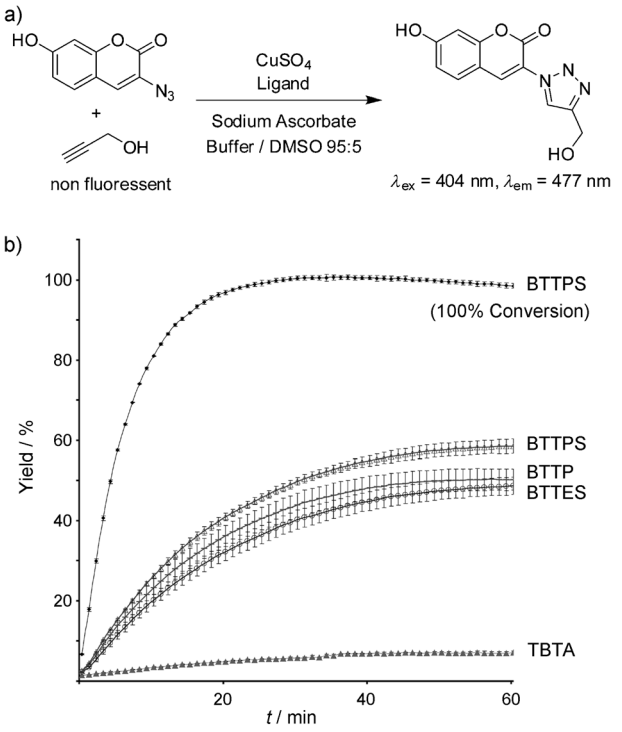

Figure 1. Comparison of CuAAC kinetics in the presence of various accelerating ligands. a) A fluorogenic assay for the qualitative measurement of CuAAC kinetics. b) Conversion–time profiles of CuAAC in the presence of various ligands. Reaction conditions: propargyl alcohol (50 μM), 3-azido-7-hydroxycoumarin (100 μM), $CuSO_4$ (75 μM), 0.1 M potassium phosphate buffer (pH 7.0)/dimethylsulfoxide (DMSO) 95:5, sodium ascorbate (2.5 mM), room temperature ([ligand]/[$CuSO_4$] = 6:1). The reaction yields were calculated against 100% conversion curve, which was obtained under the same reaction conditions using BTTPS with increasing concentration of propargyl alcohol (300 μM). Error bars represent the standard deviation of three replicate experiments.

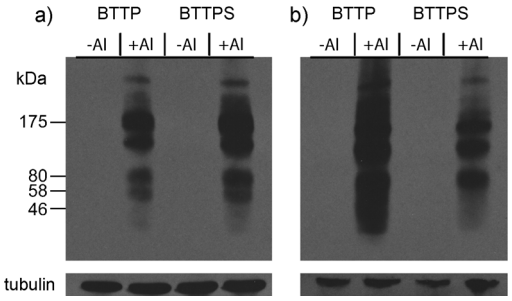

Figure 2. Comparison of the efficiency of the BTTPS–Cu^I- and BTTP–Cu^I-mediated azide–alkyne cycloaddition in labeling glycoproteins in crude cell lysates. Cell lysates prepared from Ac_4ManNAl-treated or untreated Jurkat cells were reacted with a) biotin–azide (100 μM) or b) FLAG–azide (100 μM) in the presence of sodium ascorbate (2.5 mM), and $CuSO_4$ (250 μM) premixed with ligands BTTPS or BTTP (500 μM). Reactions were allowed to proceed for 1 h at room temperature, and were analyzed by Western blots using a horseradish peroxidase (HRP)-conjugated anti-biotin antibody (a, top panel) or an HRP-conjugated anti-FLAG antibody (b, top panel). Anti-tubulin Western blots were perform to confirm equal protein loadings (bottom panel).

Labeling of Live Mammalian Cells

In our ligand design, sulfation of BTTP is used to generate a negatively charged ligand, BTTPS, to minimize the cellular internalization of the coordinated copper. To evaluate if sulfation confers the ligand with the desired property, we compared BTTPS–Cu^I and BTTP–Cu^I in live-cell labeling experiments. We metabolically labeled Jurkat cells with Ac_4ManNAl and reacted the treated cells with biotin–azide (50 μM) in the presence of BTTPS–Cu^I or BTTP–Cu^I for 5–15 min at room temperature (catalyst formulation: [ligand]/[$CuSO_4$] = 6:1, [$CuSO_4$] = 75 μM). After the reaction was quenched with bathocuprioine disulfate (BCS), the biotinylated cells were incubated with Alexa Fluor 488-streptavidin. The cells were then stained with 7-aminoactinomycin D (7-AAD), which is a fluorescent molecule with strong affinity for double-stranded DNA, and analyzed by flow cytometry. 7-AAD does not pass through intact membrane, but it readily enters damaged cells with compromised membranes. Therefore, healthy and damaged cells can be easily distinguished. As shown in Figure 3a, cell-associated Alexa Fluor 488 fluorescence was detected for both BTTPS–Cu^I or BTTP–Cu^I treated cells, and the fluorescence increased along with increasing reaction time. Although both catalytic systems showed comparable labeling efficiency, BTTPS–Cu^I was significantly better in protecting cells from the copper(I)-associated toxicity, especially for labeling with extended reaction time (15 min). More than 60% of cells were still undamaged after treatment with BTTPS–Cu^I (7-AAD negative), whereas only 26% of cells treated with BTTP–Cu^I remained normal (Figure 3b). Furthermore, we noticed that the labeling temperature also played a significant role in modulating the copper-associated toxicity. When both Jurkat cells and the labeling reagents were precooled to 4°C before triggering the CuAAC, more than 85% of cells remained viable after a BTTPS–Cu^I-mediated reaction for 15 min with high labeling efficiency achieved (mean fluorescence intensity 2200, for reactions performed at 4°C to room temperature, versus 3679, for reactions performed at room temperature).

Our previous studies showed that significant labeling of Ac_4ManNAl-treated Jurkat cells was achieved with BTTES–Cu^I-mediated click chemistry when 50–75 μM $CuSO_4$ was used as the copper source. With the observation that BTTPS confers CuAAC with faster kinetics than BTTES, we were eager to test if efficient cell labeling could be achieved with BTTPS when using a lower copper loading. To this end, we treated alkyne-bearing Jurkat cells with biotin–azide (50 μM) in the presence of BTTPS–Cu^I for one minute at room temperature (catalyst formulation: [ligand]/[$CuSO_4$] = 6:1, [$CuSO_4$] = 20–75 μM). After the reaction was quenched with BCS, the biotinylated cells were incubated with Alexa Fluor 488-streptavidin, stained with 7-AAD, and analyzed by flow cytometry. As shown in Figure 3c, significant labeling was realized with as little as 30 μM copper loading.

The BTTPS–Cu^I catalyst is equally active in detecting cell-surface azides in Jurkat cells metabolically treated with

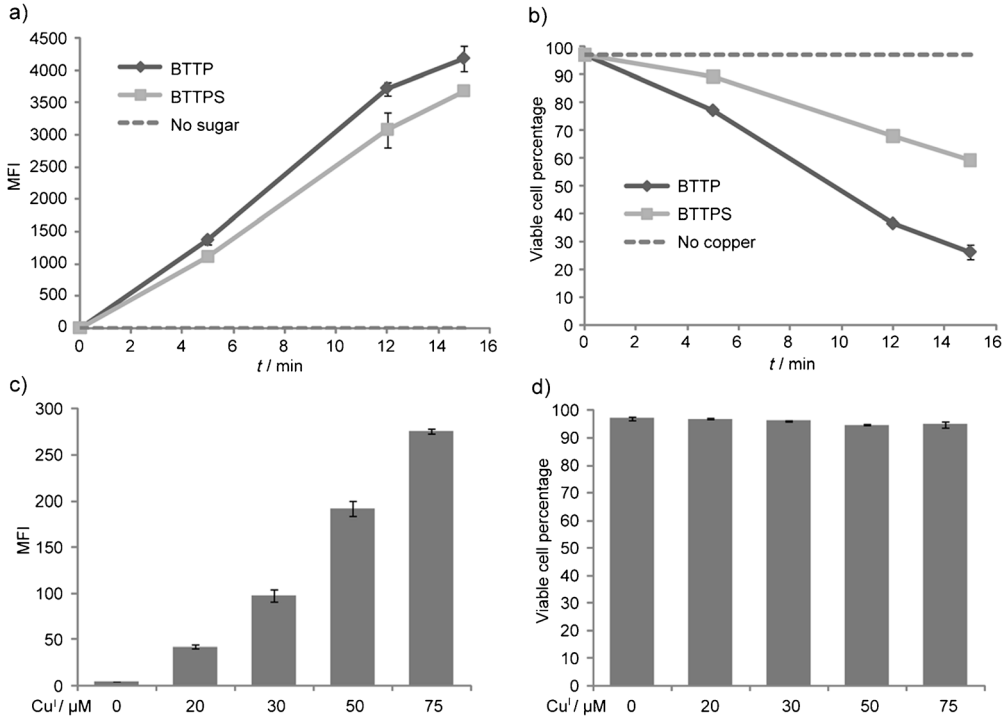

Figure 3. Comparison of the efficiency of the BTTPS–Cu^I- and BTTP–Cu^I-mediated azide–alkyne cycloaddition in labeling sialylated glycoconjugates in live cells. Jurkat cells were cultured in the presence or absence of Ac_4ManNAl for 3 days. Cells were then treated with biotin azide (50 μM) in the presence of sodium ascorbate (2.5 mM), and $CuSO_4$ premixed with ligands BTTPS or BTTP ([ligand]/[$CuSO_4$] = 6:1). Reactions were quenched with BCS, stained with Alexa Fluor 488-streptavidin, 7-AAD, and analyzed by flow cytometry. a) Mean fluorescence intensity (MFI) of cells treated with the BTTPS–Cu^I or BTTP–Cu^I catalyst ([$CuSO_4$] = 75 μM) in the course of 5–15 min reactions. b) Percentage of viable cells without cell-membrane damage after the click reactions with the BTTPS–Cu^I or BTTP–Cu^I catalyst ([$CuSO_4$] = 75 μM) in the course of 5–15 min reactions. c) MFI of cells treated with the BTTPS–Cu^I catalyst ([$CuSO_4$] = 20–75 μM) in a reaction for 1 min. d) Percentage of viable cells without cell-membrane damage after the click reactions with the BTTPS–Cu^I catalyst ([Cu] = 20–75 μM) in a reaction for 1 min.

peracetylated *N*-azidoacetylmannosamine (Ac_4ManNAz).[21] To evaluate if the faster kinetic behavior of BTTPS–Cu^I relative to BTTES–Cu^I could be transferred in vivo, we compared these two catalysts directly in biotinylation of azido sialic acid (SiaNAz)-bearing Jurkat cells. Under exactly the same labeling conditions, about 15% higher labeling efficiency was achieved when using the BTTPS–Cu^I catalyst (Figure 4).

We next evaluated the use of BTTP- and BTTPS-mediated CuAAC for direct fluorescence imaging of glycans on live-cell surfaces. HeLa cells, which are a human epithelial carcinoma cell line, were incubated with 50 μM Ac_4ManNAz to metabolically incorporate the corresponding SiaNAz into cell-surface glycoconjugates. The resulting HeLa cells with azides on their cell surface were treated with Alexa Fluor 488-alkyne (50 μM) in the presence of BTTP–Cu^I or BTTPS–Cu^I ([ligand]/[$CuSO_4$] = 6:1, [$CuSO_4$] = 50 μM). After five minutes, the reaction was quenched with BCS. As shown by confocal fluorescence microscopy, robust Alexa Fluor 488 fluorescence was detected in the cell membrane (Figure 5). Under exactly the same imaging conditions, comparable labeling efficiency was achieved by using BTTPS–Cu^I and BTTP–Cu^I. No clear cytotoxicity was observed after the copper(I) treatment, as confirmed by trypan blue stain (data not shown).

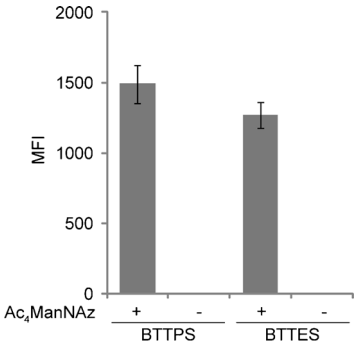

Figure 4. Comparison of the efficiency of BTTPS–Cu^I and BTTES–Cu^I in labeling azido sialic acids in live cells. Jurkat cells were cultured in the presence or absence of Ac_4ManNAz for 3 days. Cells were then treated with biotin alkyne (50 μM) in the presence of sodium ascorbate (2.5 mM), and $CuSO_4 \cdot 5H_2O$ premixed with ligands BTTPS or BTTES ([$CuSO_4$] = 75 μM, [ligand]/[$CuSO_4$] = 6:1). After 3 min, the reactions were quenched with BCS, stained with Alexa Fluor 488-streptavidin, 7-AAD, and analyzed by flow cytometry.

Labeling of Azide-Bearing Surface Proteins in *E. Coli*

The development of genetically encoded unnatural amino acid technology based on amber nonsense codons[22] and the complementary metabolic approach[23] allows the incorpora-

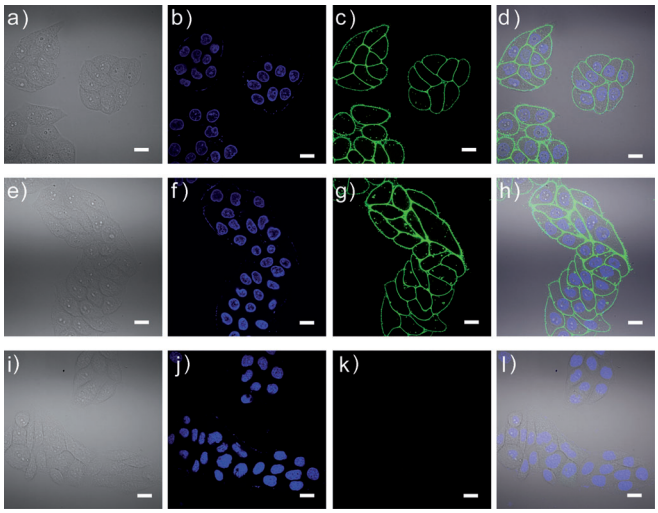

Figure 5. Fluorescent imaging of SiaNAz-containing glycans on cell surfaces by using biocompatible CuAAC. HeLa cells were incubated with (a–h) or without (i–l) 50 μM Ac_4ManNAz for 3 days. The cells were subsequently labeled with Alexa Fluor 488-alkyne by using BTTP–Cu^I and BTTPS–Cu^I-catalyzed AAC for 5 min with 50 μM $CuSO_4$, [ligand]/[$CuSO_4$]=1:6, sodium ascorbate 2.5 mM (a–d) ligand: BTTP, e–l) ligand: BTTPS). The cell nuclei were stained with Hoechst 33342 prior to microscopy analysis. First column: bright field; second column: Hoechst 33342 channel; third column: Alexa Fluor 488 channel; and fourth column: overlay. Scale bars: 20 μm.

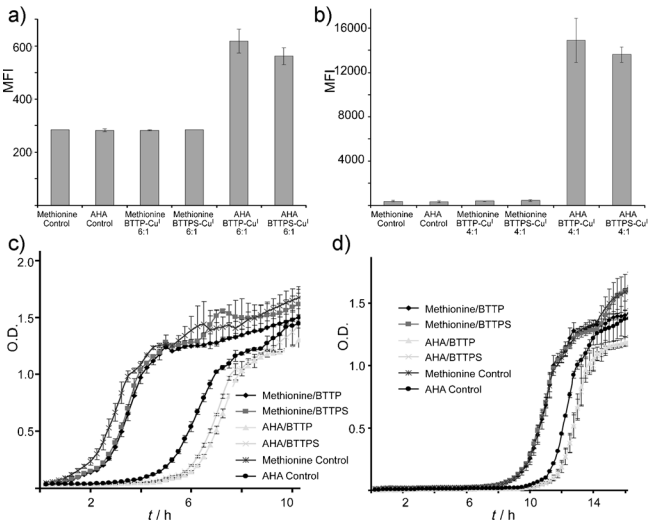

Figure 6. Labeling *E. coli* by using CuAAC. *E. coli* bearing OmpC were cultured in the presence or absence of AHA until an optical density (O.D.) of 1 was reached, and then treated with biotin–azide (50 μM) in the presence of sodium ascorbate (2.5 mM), and $CuSO_4$ (75 μM) premixed with ligands BTTPS or BTTP at room temperature. Reactions were quenched by BCS after 10 min, and probed with Alexa Fluor 488-streptavidin and analyzed by flow cytometry. MFI of *E. coli* reacted with a) BTTPS–Cu^I or BTTP–Cu^I ([ligand]/[$CuSO_4$]=6:1 complex) and b) BTTPS–Cu^I or BTTP–Cu^I ([ligand]/[$CuSO_4$]=4:1 complex). The corresponding *E. coli* growth curves after the click reactions are shown in c) and d), respectively.

tion of functional groups beyond those found in the 20 canonical amino acids in proteins of live cells. As demonstrated elegantly by the Tirrell group, azide- or alkyne-bearing groups can be introduced onto the surface of *E. coli* and be further derivatized by the CuAAC.[24,25] However, bacteria subjected to the overexpression of outer-membrane protein C (OmpC) containing azidohomoalanine (AHA) and then treated with canonical CuAAC reagents (i.e., $CuSO_4$, TBTA,[26] tris(2-carboxyethyl)phosphine) were unable to divide after being transferred to rich medium.[24,25] To evaluate if the BTTP–Cu^I or BTTPS–Cu^I catalyst could improve the viability of the treated bacteria, we reacted *E. coli* cells expressing OmpC with biotin–alkyne. Here, two [ligand]/[$CuSO_4$] ratios, 6:1 and 4:1, were used. After reaction for ten minutes, the bacteria were stained with Alexa Fluor 488-streptavidin and subjected to flow cytometry analysis. As expected, bacteria expressing OmpC and induced in the presence of 20 natural amino acids had the same mean fluorescence as the unlabeled cells. In contrast, bacteria induced in the presence of AHA showed a 2.2-fold increase in the fluorescence of Alexa Fluor 488 over the background when the 6:1 ligand–Cu^I complex was used (Figure 6a). When the [ligand]/[$CuSO_4$] ratio decreased to 4:1, more than 30-fold fluorescence over the background was observed (Figure 6b). Notably, with the canonical CuAAC conditions, the comparable labeling level was only achieved after reaction for 16 hours.[25] In our *E. Coli* labeling experiments, BTTP–Cu^I gave a 10% stronger signal than BTTPS–Cu^I (Figure 6b). In phosphate buffered saline (PBS; pH 7.4), the surface of *E. coli* is known to be negatively charged, which may have repulsive interactions with the negatively charged BTTPS, thus lowering the labeling efficiency of the corresponding BTTPS–Cu^I complex.[27]

To evaluate if the copper catalysts caused any long-term perturbations to the treated bacteria, we cultured the copper-treated and untreated *E. coli* in nutrient-rich medium and followed their growth by O.D. reading. As shown in Figure 6c and d, the bacteria bearing AHA showed about a four-hour delay in their cell proliferation compared with the bacteria growing with all 20 natural amino acids through this series of experiments. Noteworthy, copper treatment barely had any influence on the bacteria growing with 20 natural amino acids—the copper(I)-treated and untreated bacteria showed similar proliferation trends. However, after bacteria bearing AHA were subjected to CuAAC, a one-hour delay in cell proliferation was observed. Nevertheless, both the copper(I)-treated and untreated bacteria reached same cell density 15 hours after being transferred to the rich medium, thus suggesting only minor toxicity was imparted to the labeled *E. coli*.

Conclusion

Our systematic investigation of BTTPS–Cu^I and BTTP–Cu^I in this series of labeling studies showed that both catalysts were highly efficient for bioconjugation. Consistent with the criteria of our ligand design, the negatively charged sulfate minimized the membrane permeability of the coordinated

copper(I) at physiological conditions and significantly retained cell viability post-copper(I) treatment. Therefore, the BTTPS–Cu^{I} catalyst is the clear choice for live-cell surface-labeling experiments. Importantly, a ligand/$CuSO_4$ ratio greater than one is critical to reduce copper(I)-associated toxicity, a phenomenon discovered by us and the Finn group previously.[18,28] However, the negative charge conferred by the sulfate functionality may impose electrostatic repulsion with probes or biomolecules bearing significant negative charges, thus reducing the catalytic efficiency of the corresponding BTTPS–Cu^{I} complex. For these reasons, BTTP–Cu^{I} may be a better choice for in vitro labeling of negatively charged biomolecules.

Noteworthy, this study has identified highly reliable copper(I) catalyst formulations that can be easily scaled up and adopted for various bioconjugation applications. The new catalysts reported herein also solved longstanding problems of canonical CuAAC, including toxicity and poor labeling efficiency at low-substrate concentration, thus opening new possibilities for bioorthogonal click chemistry-based molecular imaging and proteomic analysis.

Experimental Section

Metabolic Labeling of Jurkat Cell and Detection by CuAAC Click Chemistry and Flow Cytometry Analysis

Jurkat cells were cultured for 3 days in untreated RPMI 1640 medium or medium containing 50 μM Ac_4ManNAl (or Ac_4ManNAz). The cells then were distributed into a 96-well round-bottomed tissue culture plate (0.4–0.5 million cells per well), pelleted (300×*g*, 3 min), and washed twice with labeling buffer (200 μL, PBS, pH 7.4, containing 1% fetal bovine serum (FBS)). Cells were then resuspended in labeling buffer (92 μL), followed by addition of 100 μM biotin–alkyne (or biotin–azide to react with SiaNAl), BTTP or BTTPS–Cu complex ([ligand]/[$CuSO_4$]=6:1) and 2.5 mM sodium ascorbate in labeling buffer at room temperature. The reactions were quenched by adding bathocuproine disulfonate (BCS) copper chelator (2 μL, 50 mM). Then the cells were pelleted, washed three times with labeling buffer, and resuspended in the same buffer containing 1 μg mL^{-1} streptavidin-Alexa Fluor 488 and incubated in the dark at 4 °C for 30 min. Following incubation, cells were washed three times with labeling buffer and resuspended in FACS buffer (400 μL; Hank's Balanced Salt Solution, pH 7.4, 1% FBS, 2 μg mL^{-1} 7-AAD, 0.2% NaN_3) for flow cytometry analysis. Flow cytometry experiments were performed on a Becton Dickinson FACScan flow cytometer using a 488 nm argon laser. At least 15000 cells were recorded for each sample. Flow cytometry data were analyzed by using FlowJo software. MFI was calculated for live cells.

Metabolic Labeling of Jurkat Cell Glycoproteins and Detection by CuAAC Click Chemistry and Western Blot

Jurkat cells were incubated for 3 days in RPMI medium or RPMI medium containing 50 μM Ac_4ManNAl. The cells were washed with PBS, harvested by centrifugation (300*g*, 3 min), and homogenized in lysis buffer (100 mM sodium phosphate, 150 mM NaCl, 1% NP-40, pH 7.4) containing protease inhibitors (Roche complete tablets, ethylenediaminetetraacetic acid (EDTA) free) by 10 freeze–thaw cycles. Insoluble debris was removed by centrifugation (10000*g*, 10 min) and the soluble protein concentration was determined by using the DC protein assay kit. To label the sialylated glycoproteins, protein was resuspended at a concentration of 0.69 mg mL^{-1} and treated with 100 μM FLAG–azide or 100 μM biotin–azide in a 100 μL reaction vessel containing premixed ligand–Cu complex ([$CuSO_4$]=250 μM, [ligand]/[$CuSO_4$]=2:1) and 2.5 mM freshly prepared sodium ascorbate. Ligands used included BTTP and BTTPS. The samples were lightly vortexed and allowed to react for 1 h (25 °C, 800 rpm in eppendorf Theromomixer R). Reacted samples (8.3 μg each) were heated in SDS-loading buffer (40% glycerol, 200 mM Tris-HCl pH 6.8, 8% SDS, 0.4% bromophenol blue) at 95 °C for 5 min and resolved on 4–20% Criterion XT Precast Gels. The samples were transferred to nitrocellulose and incubated for 1 h at room temperature in blocking buffer (5% non-fat milk in TBST (tris-buffered saline with 0.1% Tween-20, pH 7.5)). The blocked membrane was incubated for 1 h at room temperature with an HRP anti-FLAG antibody (1:3000 dilution) or an HRP anti-biotin antibody (1:100000 dilution) in blocking buffer, washed three times with TBST. The membranes were developed by using SuperSignal West Pico chemiluminescent substrate and imaged on film. To confirm equal protein loading, the primary antibody-treated membranes were stripped (http://www.abcam.com/ps/pdf/protocols/Stripping%20for%20reprobing.pdf). The membranes were then incubated for 1 h at room temperature in blocking buffer and then incubated with monoclonal anti-tubulin clone AA13 (1:1000 dilution) for 1 h at room temperature. After being washed three times with TBST, the membranes were incubated with goat anti-mouse HRP (1:60000 dilution) for 1 h at room temperature, then washed three times with TBST. The membranes were incubated with SuperSignal West Pico chemiluminescent substrate and imaged on film.

HeLa Cell Labeling by CuAAC Chemistry and Imaging with Confocal Microscopy

HeLa cells were cultured in Dulbecco's modified eagle medium (DMEM) supplemented with or without Ac_4ManNAz (50 μM) on Lab-Tek Chambered cover glass for 3 days. The cells were washed three times with PBS (100 μL) and treated with Alexa Flour 488-alkyne (50 μM) in a 100 μL reaction vessel containing premixed ligand—Cu complex ([$CuSO_4$]=50 μM, [ligand]/[$CuSO_4$]=6:1) and 2.5 mM freshly prepared sodium ascorbate for 5 min at room temperature. The reaction was quenched with 1 mM BCS. The cells were washed three times with PBS and stained with Hoechst 33342 at 4 °C for 5 min. A laser scanning confocal microscope (Nikon, A1R-si) 60× was used for imaging Alexa Flour 488 on the HeLa cell surfaces.

Metabolic Labeling of E. Coli and Detection with CuAAC Chemistry and Flow Cytometry Analysis

A single colony of M15MA[pQE-60/OmpC] was grown in M9 minimal medium supplemented with all 20 natural amino acids and with carbenicillin (100 mg L^{-1}) and kanamycin (35 mg L^{-1}) until $O.D._{600}=1$. The bacteria were pelleted (2500×*g*, 10 min), resuspended in M9 medium (50 mL; supplemented with 19 amino acids), and agitated at 37 °C for 10 min. The cells were pelleted, resuspended in the same volume M9 medium (supplemented with19 amino acids), and divided into two equal portions (≈25 mL each): methionine (40 mg L^{-1}) was added to one and AHA (40 mg L^{-1}) was added to the other. Isopropyl β-D-1-thiogalactopyranoside (IPTG; 1 mM) was added to each culture and then shaken at 37 °C for 3 h. The *E. Coli* cultures (≈25 mL each) were centrifuged at 2500×*g* for 5 min and washed once with PBS (12.5 mL). The cells were centrifuged again and resuspended in PBS (2.5 mL). In a 96-well round-bottomed tissue culture plate, biotin–alkyne (100 μM) and premixed ligand–Cu complex ([$CuSO_4$]=75 μM, [ligand]/[$CuSO_4$]=4:1 or 6:1) were added to each well containing an aliquot of these bacteria (200 μL). After 10 min, BCS was added (1 mM) to the bacteria to quench the reaction. Bacteria were then washed three times with PBS (200 μL), then were resuspended in PBS (200 μL), and divided into two portions. Portion one was diluted with PBS to 1 mL, from which 10 μL was taken and diluted with M9 medium containing all 20 amino acids (190 μL; 40 mg L^{-1} each) as well as carbenicillin (100 mg L^{-1}) and kanamycin (35 mg L^{-1}), and shaken at 37 °C. $O.D._{600}$ was measured every 15 min for an 18 h period by using a Synergy Hybrid plate reader. Portion two was incubated with Alexa Fluor 488-streptavidin (final concentration 1 μg mL^{-1}) at 4 °C for 25 min. Bacteria were then washed three times with PBS (200 μL), resuspended in PBS (200 μL), and analyzed by flow cytometry. Flow cytometry experiments were performed on an Eclipse iCyt flow cytometer

using a 488 nm argon laser. At least 20000 cells were recorded for each sample. Flow cytometry data were analyzed by using FlowJo software.

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