Bioconjugation

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Increasing the Efficacy of Bioorthogonal Click Reactions for Bioconjugation: A Comparative Study**

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Dedicated to Professor Carolyn Bertozzi

The recent discovery of bioorthogonal click chemistry has created a new field in chemical biology in which biomolecules that are not directly encoded in the genome can be monitored in living systems.[1] By hijacking a cell's biosynthetic machinery, a metabolic precursor functionalized with a bioorthogonal chemical tag is incorporated into target biomolecules, including glycans,[2] lipids,[3] proteins,[4] and nucleic acids.[5] Subsequently, a tailor-designed click reaction is employed to conjugate a complementary biophysical probe, which enables visualization[2] or enrichment of the target biomolecules for molecular identification.[6,7] Though both applications require exquisite selectivity to maximize signal to noise ratio, each has specific criteria to meet. For dynamic imaging studies, not only must the employed reaction proceed rapidly under physiological conditions to allow monitoring of events that take place on the minute time scale, it must also be nontoxic and noninterfering with the surrounding cellular milieu.[2,8] Contrarily, molecular identification applications, for example, proteomics analysis, prioritize sensitivity over biocompatibility—in most cases, only limited amounts of samples are available for analysis and the targets of interest may be low in abundance. Thus, reactions that feature fast kinetics at low substrate concentration (e.g., micromolar) are preferred. In view of these requirements, the choice of a tailored chemical tag and chemistry for a specific bioconjugation process is not trivial.

To date, the azide group is the most utilized bioorthogonal chemical tag for biomolecule–conjugate experiments because of its small size and inertness to most components in a biological environment.[9] Three bioorthogonal click reactions have been reported for labeling azide-tagged biomolecules. The Staudinger ligation, introduced by Saxon and Bertozzi in 2000, covalently links the azide and an ester-functionalized triphenylphosphine by an amide bond.[10] Though highly specific for the azide group, this reaction suffers from slow reaction kinetics and competing oxidation of the phosphine reagents.[11] By contrast, the Cu^{I}-catalyzed azide–alkyne cycloaddition (CuAAC), promoted by the Cu^{I}-stabilizing ligand BTTES (Scheme 1),[12] and the strain-promoted azide–alkyne cycloaddition,[13] inherit the bio-benign characteristics of the Staudinger ligation but are further endowed with improved kinetics. With the discovery of BTTES, not only did we confer the canonical CuAAC[14,15] with biocompatibility, but also dramatically boosted its reactivity. Similarly, by increasing the strain energy of cyclooctyne probes, Bertozzi

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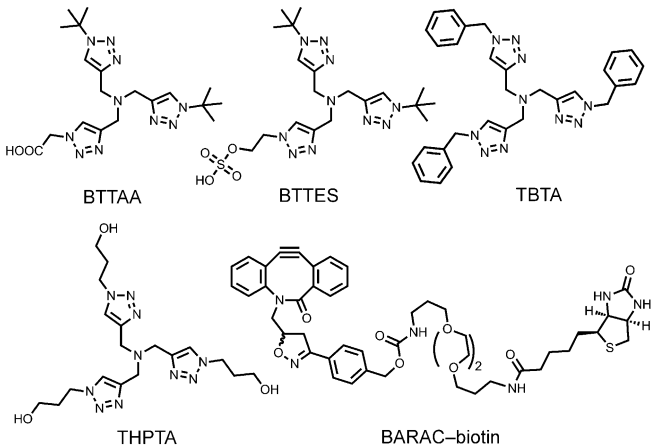

Scheme 1. Structural formulas of tris(triazolylmethyl)amine-based ligands and BARAC-biotin. BTTAA = 2-[4-{(bis[(1-tert-butyl-1*H*-1,2,3-triazol-4-yl)methyl]amino)methyl}-1*H*-1,2,3-triazol-1-yl]acetic acid, BTTES = 2-[4-{(bis[(1-tert-butyl-1*H*-1,2,3-triazol-4-yl)methyl]amino)-methyl}-1*H*-1,2,3-triazol-1-yl]ethyl hydrogen sulfate, TBTA = tris[(1-benzyl-1*H*-1,2,3-triazol-4-yl)methyl]amine, THPTA = tris[(1-hydroxypropyl-1*H*-1,2,3-triazol-4-yl)methyl]amine, BARAC = biarylazacyclooctynone.

and co-workers developed a biarylazacyclooctynone (BARAC) reagent, which showed significantly enhanced kinetics in live cell labeling experiments compared to the unfunctionalized cyclooctyne probes.[16] Though both reactions show great promise for molecular identification or imaging studies, no direct comparison of the bio-benign CuAAC and the BARAC-mediated cycloaddition, often referred to as copper-free click chemistry, has been performed to provide a framework for choosing the optimal reaction for these specific applications. We compared these two bioorthogonal reactions in four applications: detection of purified recombinant glycoproteins and glycoproteins in crude cell lysates, and labeling of glycans on the surface of live cells and zebrafish embryos.

The canonical CuAAC protocol using TBTA and THPTA (Scheme 1) as Cu^I-stabilizing ligands is associated with slow kinetics in aqueous solution.[12,17b] In our recent studies, we discovered that BTTES, a tris(triazolylmethyl)amine-based ligand for Cu^I ions, promoted the cycloaddition reaction rapidly in living systems.[12] BTTES contains two *tert*-butyl groups and a hydrogen sulfate group, thus conferring the ligand an ideal balance between reactivity and solubility. When coordinating with the in situ generated Cu^I ions, the bulky *tert*-butyl groups are believed to prevent the polymerization of copper acetylides and thereby the formation of unreactive species.[18] In the meantime, the hydrogen sulfate group that ionizes to a negatively charged sulfate at physiological pH values secures the solubility of the BTTES–Cu^I complex in aqueous mixtures and prevents cellular internalization of the coordinated copper ions. We found a Cu^I concentration of 50–75 μM sufficient to achieve robust labeling in mammalian cells and zebrafish embryos. However, minor developmental defects were observed when zebrafish embryos were treated under these conditions: approximately 10% of embryos exhibited impaired posterior body development characterized by a shorter anterior–posterior axis. To improve labeling efficiency and biocompatibility of the CuAAC, a faster and less toxic catalyst is required.

We began our current study with ligand optimization by varying the ionizable substituent on the tris(triazolylmethyl)-amine while retaining the two *tert*-butyl groups. We replaced the ethyl hydrogen sulfate group of BTTES with an acetic acid group to produce a new ligand BTTAA (Scheme 1, see the Supporting Information for synthetic details). At physiological pH values, the acetic acid of BTTAA ionizes into acetate. The acetate functionality not only bears a negative charge, but it may also serve as an additional weak donor to coordinate with Cu^I ions, thus increasing the electron density of the metal center and facilitating the formation of the strained copper metallacycle and copper triazolide intermediate.[18b] Consequently, additional acceleration of the rate of the cycloaddition may be achieved.

The relative reactivity of the canonical Cu^I catalysts in the form of TBTA–Cu^I[17] and THPTA–Cu^I[19] and the new Cu^I catalysts in the form of BTTES–Cu^I and BTTAA–Cu^I complexes was determined in a fluorogenic assay by reacting propargyl alcohol with 3-azido-7-hydroxycoumarin (Figure 1a).[20] The azidocoumarin has very weak fluorescence that is quenched by the lone pair of electrons from the internal nitrogen atom of the azide. The cycloaddition localizes the lone pair of electrons to the triazole ring, thus activating its fluorescence. Among the four ligands evaluated, BTTAA showed the highest activity in accelerating the CuAAC, followed by BTTES and THPTA, with TBTA showing the lowest activity. More than 45% of the cycloaddition product was formed using a Cu^I concentration of 50 μM within the first 30 min when the ligand/Cu^I ratio was 6:1. By contrast, the THPTA- and TBTA-mediated reactions were significantly slower and resulted in lower than 15% of cycloaddition products (Figure 1b).

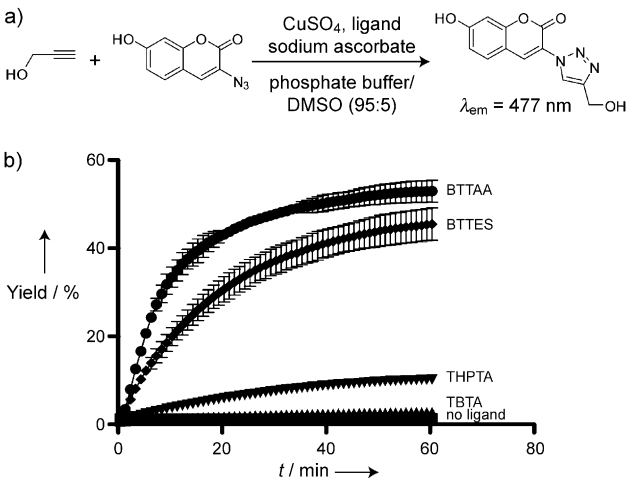

Figure 1. Comparison of CuAAC rates in the presence of various accelerating ligands. a) A fluorogenic assay for the qualitative measurement of the CuAAC rate. b) Conversion–time profiles of the CuAAC in the presence of various ligands. Reaction conditions: propargyl alcohol (50 μM), 3-azido-7-hydroxycoumarin (100 μM), $CuSO_4$ (50 μM) ([ligand]/[$CuSO_4$] = 6:1), potassium phosphate buffer (0.1 M, pH 7.0)/DMSO = 95:5, sodium ascorbate (2.5 mM), room temperature. Error bars represent the standard deviation of three experiments.

To evaluate the activity of the new ligand–Cu^I complex and compare the click reactions in the context of biomolecular labeling experiments, the first system we chose to explore was a recombinant glycoprotein: Programmed Death 1-Immunoglobulin G Fc fusion (PD1-Fc). When expressed in mammalian cells, the Fc region of the recombinant protein is glycosylated and terminated with sialic acids. We cultured HEK-293 cells stably expressing PD1-Fc fusion protein for 4 days in a medium containing peracetylated *N*-azidoacetylmannosamine (Ac_4ManNAz; 50 μM), which is a sialic acid metabolic precursor. The target protein was then isolated using protein G (a genetically engineered protein that contains Fc binding domains of protein G) agarose. To probe for the presence of the sialic acid associated azide, the protein was reacted with biotin–alkyne by the CuAAC or BARAC–biotin by a copper-free cycloaddition. For the CuAAC, a biotin–alkyne concentration of 100 μM was used as the coupling partner and the biotin–alkyne/ligand/$CuSO_4$/sodium ascorbate ratio was maintained at 1:5:2.5:25, a labeling condition optimized by us. The copper-free click chemistry was performed with BARAC–biotin at a concentration of 100 μM. The reactions were allowed to proceed for 1 hour and the labeled protein was analyzed by SDS-PAGE

and Western blot. As quantified by the image-processing program ImageJ, reactions mediated by BTTES–Cu^I and BTTAA–Cu^I provided signals that were 2.6 and 2.1 times stronger, respectively, than the signal afforded by THPTA–Cu^I for the fusion protein isolated from Ac_4ManNAz-treated culture (Figure 2a). By contrast, no detectable signal was observed for the reactions mediated by TBTA–Cu^I and BARAC.

To evaluate the efficacy of the CuAAC and BARAC-mediated copper-free cycloaddition in a more complex system, we investigated bioconjugation of a biotin affinity probe to azide-tagged sialyl glycoproteins in crude cell lysates as this is the first step in enriching these proteins for glycoproteomic analysis. We introduced azides to the cell-surface sialyl glycoconjugates of Jurkat cells, a human T lymphocyte cell line, by culturing the cells with Ac_4ManNAz. After 3 days, we lysed the cells in phosphate buffer with 1% nonyl phenoxypolyethoxylethanol (NP40) to solubilize the membrane proteins. The cell lysates were then reacted with biotin–alkyne through the CuAAC or BARAC–biotin copper-free cycloaddition using the same conditions specified for the labeling of the recombinant PD1-Fc.

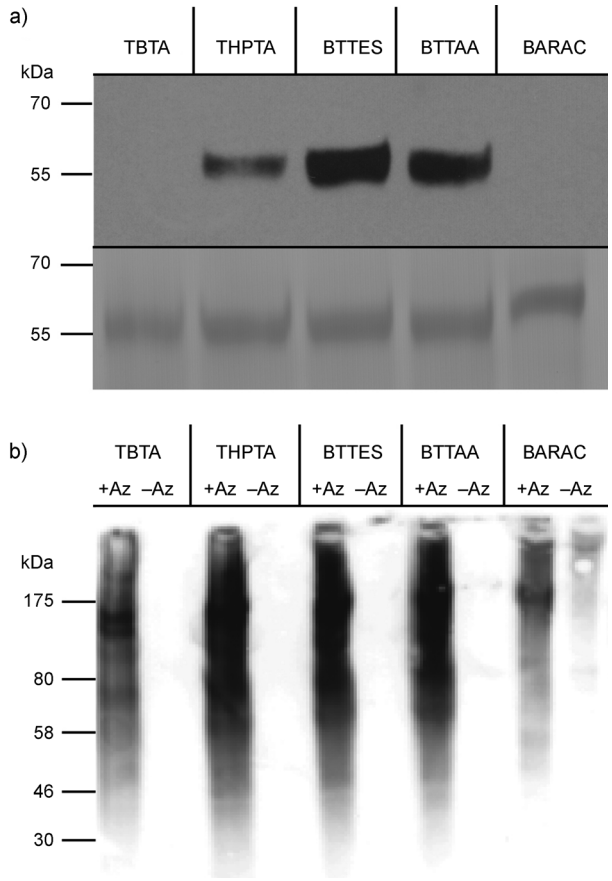

Figure 2. Comparison of the efficiency of the CuAAC and BARAC-mediated copper-free cycloaddition in labeling recombinant proteins and crude cell lysates. a) Western blot analysis of PD1-Fc isolated from HEK cells treated with Ac_4ManNAz (top panel). Total protein loading was confirmed by Coomassie staining (bottom panel). b) Western blot analysis of Ac_4ManNAz-treated or untreated Jurkat cell lysates. Reaction conditions for CuAAC: biotin–alkyne (100 μM), $CuSO_4$ (250 μM) premixed with various tris(triazolylmethyl)amine ligands (500 μM), sodium ascorbate (2.5 mM); copper-free click reaction: BARAC–biotin (100 μM). Reactions were allowed to proceed for 1 h at room temperature and analyzed by Western blot using a horseradish peroxidase conjugated anti-biotin antibody.

Consistent with the observation by Cravatt and co-workers, we discovered that a significant portion of proteins precipitated out of the reaction buffer when the lysates were treated with TBTA–Cu^I (see Figure S1 in the Supporting Information).[21] By contrast, the reaction mixture remained homogeneous for the lysates treated with Cu^I complexed with the other three ligands. As revealed by anti-biotin Western blot (Figure 2b), the reaction mediated by the three water-soluble ligands provided significantly stronger glycoprotein labeling in lysates from cells treated with Ac_4ManNAz than that achieved using the TBTA–Cu^I catalyst or BARAC-mediated cycloaddition. Importantly, the Cu^I-mediated reactions exhibited remarkable selectivity with no background signals detectable for cell lysates harvested in the absence of the sugar. However, nonspecific biotinylation, which is presumably generated by the reaction of the cyclooctyne with cysteine residues in the protein lysates,[22] was observed for the BARAC-based copper-free click chemistry (Figure 2b).

One advantage of CuAAC over the BARAC-based cycloaddition is that the former reaction can also be used to detect biomolecules tagged with terminal alkyne residues. We compared the four Cu^I catalysts for labeling peracetylated *N*-(4-pentynoyl)mannosamine (Ac_4ManNAl) treated cell lysates. Similarly, the strongest labeling signal was obtained using the BTTAA–Cu^I catalyst whereas the weakest signal was observed with TBTA–Cu^I (see Figure S2 in the Supporting Information).

We subsequently compared the efficacy of the CuAAC and copper-free cycloaddition for labeling the azide-tagged glycoconjugates in live cells. We cultured Jurkat cells in medium supplemented with Ac_4ManNAz, and then reacted the cells bearing the corresponding azido sialic acid (SiaNAz) with biotin–alkyne (45 μM) catalyzed by various concentrations of the four Cu^I catalysts (catalyst formulation: [ligand]/[Cu] = 6:1, 2.5 mM sodium ascorbate, optimized for live cell labeling)[12] or BARAC–biotin (45 μM) for 3 min at room temperature. The biotinylated cells were then analyzed by flow cytometry using Alexa Fluor 488/streptavidin. As shown in Figure 3, reactions mediated by the BTTAA–Cu^I catalyst provided the strongest labeling of the SiaNAz-bearing cells and resulted in a cell-associated Alexa Fluor 488 signal three to four times higher than the signal achieved with the BTTES–Cu^I catalyst. A Cu^I concentration as low as 30 μM was sufficient to yield a significant signal. Both catalysts gave highly selective labeling with minimal background labeling observed in the absence of Cu^I ions. By contrast, cell treatment with the THPTA–Cu^I catalyst resulted in labeling that was six times weaker, even at twice the concentration of Cu^I (60 μM), and barely any labeling was detectable for cells treated with the TBTA–Cu^I. Interestingly, the BARAC-mediated cycloaddition showed a comparable level of efficiency for labeling cell-surface SiaNAz as the BTTES-promoted CuAAC ([Cu] = 50 μM).

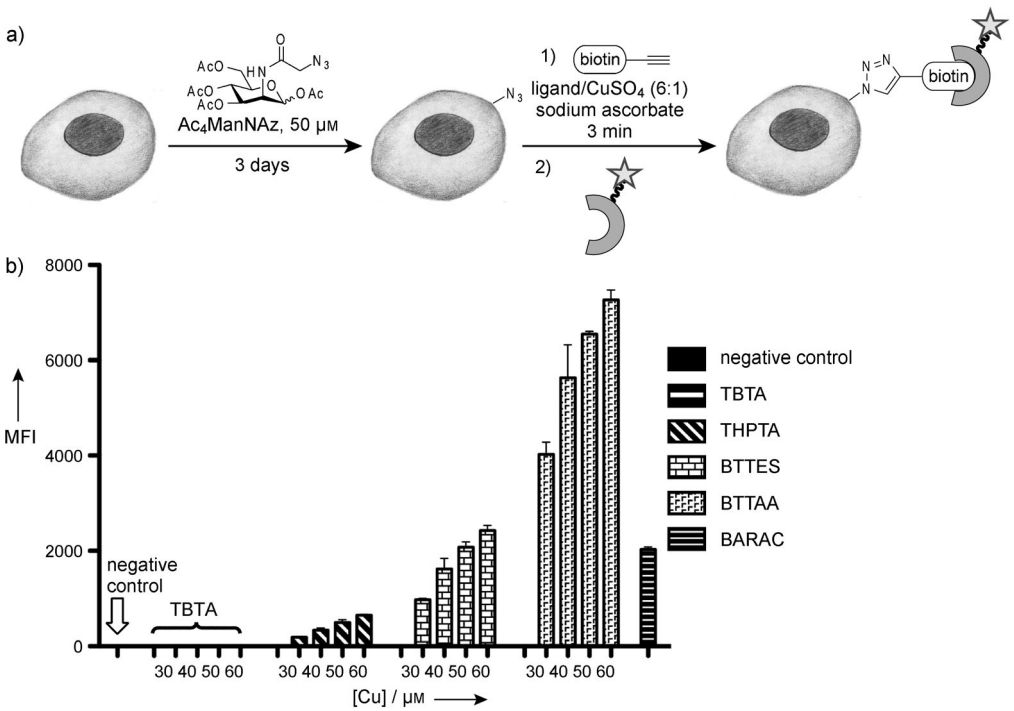

Figure 3. Relative labeling efficiency of the bioorthogonal click reactions on live cells. a) Schematic representation of metabolic labeling and detection of cell-surface sialic acids using Ac_4ManNAz and click chemistry. b) Flow cytometry analysis of cell surface labeling described in (a) using Jurkat cells. Cells were treated with biotin–alkyne (45 μM) in the presence of CuAAC catalysts ([$CuSO_4$] = 30–60 μM, [ligand]/[$CuSO_4$] = 6:1) and sodium ascorbate (2.5 mM) or BARAC (45 μM) for 3 min, then probed with Alexa Fluor 488/streptavidin. Error bars represent the standard deviation of three experiments.

To evaluate if the copper catalysts cause any long-term perturbations to the treated cells, we labeled Jurkat cells bearing SiaNAz residues with biotin–alkyne in the presence of the four catalysts ([Cu] = 30 and 50 μM) for 3 min. The reactions were then quenched with bathocuproine sulphonate (BCS), a biocompatible copper chelator. For negative controls, unreacted cells cultured in the absence and presence of Ac_4ManNAz were included. For a positive toxicity control we treated SiaNAz-bearing cells with Cu^I ions in the absence of the ligands. All cells were cultured for 4 days post-reaction.

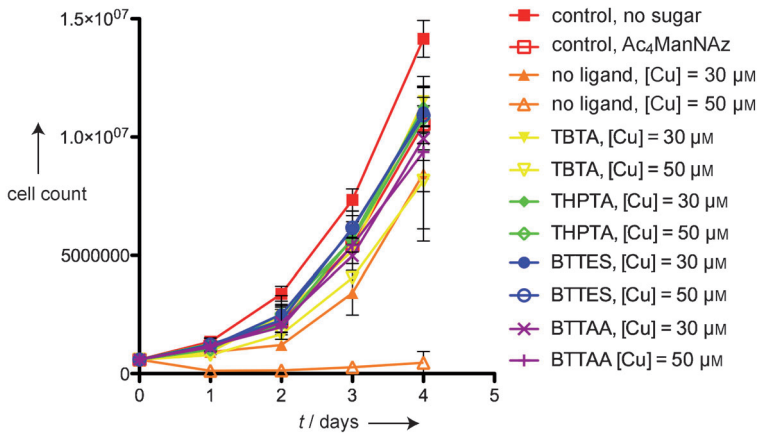

Figure 4. Cell proliferation assays indicating that tris(triazolylmethyl)amine-based ligands protect Jurkat cells from Cu^I-induced long-term perturbation.

Viable cells, based on a trypan blue assay, were counted each day. As shown in Figure 4, cells treated with the BTTAA–, BTTES– and THPTA–Cu^I catalysts proliferated at rates similar to the untreated cells cultured with Ac_4ManNAz. However, at a Cu^I concentration of 50 μM, cells treated with the TBTA–Cu^I catalyst showed a slower rate of proliferation than the cells treated with the other three catalysts, and more than 50% cells underwent cell lysis at a Cu^I concentration of 75 μM (data not shown). Noteworthy, incubation with Cu^I at a concentration of 30 μM for 3 min in the absence of the ligands only induced minor toxicity to cells as revealed by the 20% slower proliferation rate of the treated cells on days 3 and 4. By contrast, when treated with a Cu^I concentration of 50 μM and no ligand, more than 90% of the cells underwent cell lysis within 24 hours and division of the remaining cells was significantly impaired. Combining this proliferation assay with the live cell labeling results, we concluded that the BTTAA–Cu^I catalyst is the optimal choice for cell surface azide–alkyne ligation.

After establishing the relative reactivity of the Cu^I catalysts and BARAC on cultured cells, we extended our comparison to the labeling of glycans in living systems. We chose zebrafish as a vertebrate model for this purpose. Zebrafish is transparent in the first 24 hours of its developmental program, which allows the labeled glycans to be detected by molecular imaging techniques.[23] Bertozzi and co-workers demonstrated that peracetylated *N*-azidoacetylgalactosamine (Ac_4GalNAz) can be used to metabolically label O-linked glycans in zebrafish embryos by microinjecting or bathing the embryos in a medium supplemented with this azido sugar.[24,25] By following their protocol, we microinjected embryos at the one-cell stage with Ac_4GalNAz. Microinjection into the yolk sack at this stage allows the sugar to diffuse into all daughter cells during the developmental program. At 24 hours post-fertilization (24 hpf), we reacted the Ac_4GalNAz-treated embryos with biotin–alkyne (50 μM) catalyzed by BTTAA–Cu^I ([Cu] = 45 μM) or BARAC–biotin (50 μM) for 5 min to detect the membrane-associ-

ated azides in cell surface O-linked glycans. Following the reactions, we incubated the biotinylated embryos with Alexa Fluor 488/streptavidin and compared the efficacy of the BTTAA-mediated CuAAC and copper-free click chemistry using fluorescence confocal microscopy. As shown in Figure 5a, significant labeling was achieved with the BTTAA–Cu^{I} catalyst, whereas only weak labeling was detectable with BARAC–biotin.

Our previous work showed that an alkyne-bearing fucose analogue, FucAl, can be successfully incorporated into the glycans of zebrafish embryos. We also compared the efficiency of the BTTAA– and BTTES–Cu^{I} catalysts for labeling these alkyne-tagged fucosides in live zebrafish embryos. To accomplish this task, we microinjected one-cell embryos with an alkyne-derivatized GDP-fucose analogue (GDP-FucAl). At 9 hpf, we reacted the embryos with Alexa Fluor 488/azide in the presence of BTTAA–Cu^{I} or BTTES–Cu^{I} for 3 min ([Cu] = 40 μM), and acquired fluorescent images using confocal microscopy. As quantified using ImageJ, BTTAA–Cu^{I} provided a signal that was 2.5 times stronger than that achieved using the BTTES–Cu^{I} catalyst (Figure 5b). After the labeling reactions, we followed the development of the BTTAA-treated embryos for 5 days and observed no developmental defects ($n = 30$, see Figure S5 in the Supporting Information), which confirmed the biocompatibility of the new Cu^{I} catalyst formulation.

In summary, the parallel comparison of the bioorthogonal click reactions, namely the strain-promoted copper-free cycloaddition and the ligand-accelerated CuAAC, verifies the great potential of the latter as a highly effective ligation tool for broad biological applications. With the discovery of a new accelerating ligand for CuAAC, not only are kinetics that are faster than those of the known catalysts achieved, but more importantly, it allows for effective bioconjugation with suppressed cell cytotoxicity by further lowering Cu^{I} loading in the catalyst formulation. Although CuAAC requires multiple reagents to promote the reaction, which is more complicated compared to the copper-free click chemistry where only one single reagent is used, the reaction conditions optimized here are the most effective in four biological settings, that is, labeling of recombinant glycoproteins, glycoproteins in crude cell lysates and on live cell surfaces, and in the enveloping layer of zebrafish embryos. An additional advantage of the bio-benign CuAAC is that it liberates the bioconjugation from the limitation where ligations could only be accomplished with azide-tagged biomolecules. Terminal alkyne residues can now also be incorporated into biomolecules and detected in vivo. Overall, the reported ligand-accelerated CuAAC represents a powerful and highly adaptive bioconjugation tool for biologists, which holds great promise for further improvement with the discovery of more versatile catalyst systems.

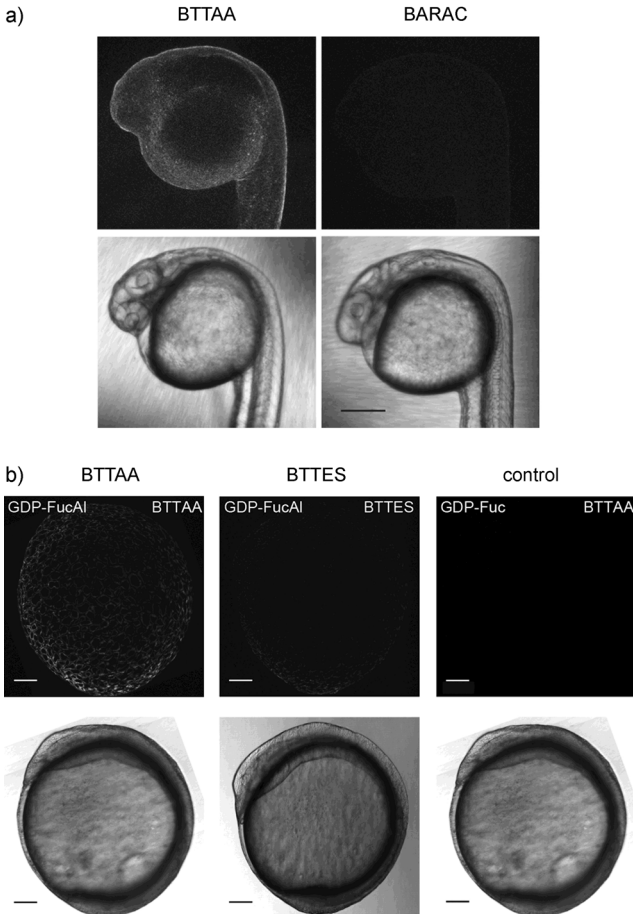

Figure 5. Relative efficiencies of CuAAC and copper-free click chemistry in labeling zebrafish glycans. a) One-cell embryos were microinjected with a single dose of Ac_4GalNAz. At 24 hpf, the embryos were treated with biotin–alkyne (50 μM) catalyzed by the BTTAA–Cu^{I} catalysts ([$CuSO_4$] = 45 μM) or BARAC–biotin (50 μM) for 5 min, then probed with Alexa Fluor 488/streptavidin and imaged using confocal microscopy. b) One-cell embryos were microinjected with a single dose of GDP-FucAl or GDP-Fuc (control) and allowed to develop to 9 hpf. The embryos were then treated with Alexa Fluor 488/azide (50 μM) catalyzed by BTTAA–Cu^{I} or BTTES–Cu^{I} ([$CuSO_4$] = 40 μM, [ligand]/[$CuSO_4$] = 6:1) for 3 min and imaged using confocal microscopy. Scale bar: a) 200 μm; b) 100 μm. Top: 488 channel; bottom: brightfield. GDP-FucAl = guanosine 5′-diphospho-6-ethynyl-β-L-fucose, GDP-Fuc = guanosine 5′-diphospho-β-L-fucose.

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