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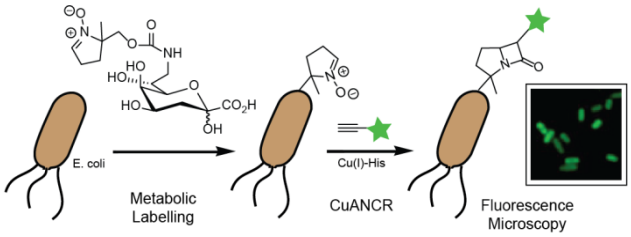

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Copper-catalysed cycloaddition reactions of nitrones and alkynes for bioorthogonal labelling of living cells

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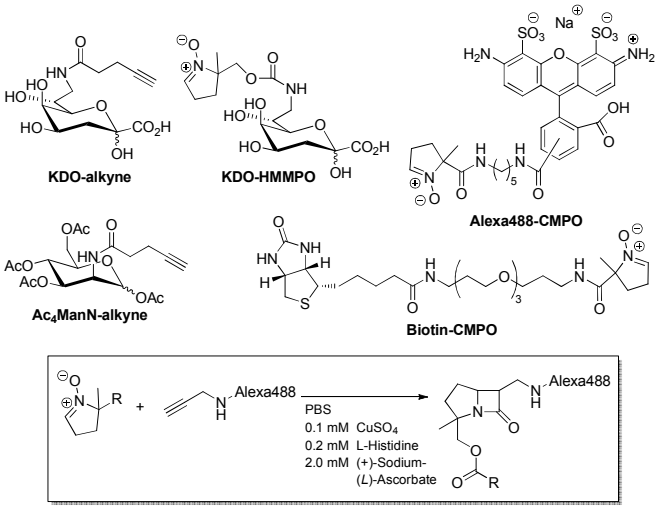
An adapted biocompatible version of the Kinugasa reaction, the copper-catalysed alkyne-nitrone cycloaddition followed by rearrangement (CuANCR), was developed for live-cell labelling. CuANCR labelling was demonstrated for both mammalian and bacterial cells, with the nitrone group metabolically incorporated by Huh-7 human hepatoma cells and *E. coli*, respectively.

Bioorthogonal labelling strategies, which involve covalent attachment of chemical reporters to biomolecules of interest, have been applied to numerous biological processes that have otherwise been inaccessible in the past.[1] Labelling strategies typically involve the introduction of a non-native, biocompatible reactive group into a biomolecule and then selective ligation with a reporter conjugated to the cognate reactive group.[2] Reactions that have been adapted in the manner contribute to the bioorthogonal toolbox, which continues to expand with the development of unique reactive handles such as nitrones,[3] tetrazines,[4] and alkenes,[5] for example. Bioorthogonal reactions for bioconjugation applications include both metal catalysed (e.g. Cu, Pd)[6] and strain promoted reactions.[3d, 3e, 4a, 7] However, copper-catalysed azide-alkyne cycloaddition (CuAAC)[6d, 6e] remains as one of the most popular.

One of the main drawbacks to CuAAC arises from the biological consequences of copper including toxicity, although this is certainly ligand dependent.[8] Recently, a number of reports have demonstrated significant reductions in copper toxicity such that CuAAC can be applied to living systems. Improvements in ligand properties[8b, 9] that accelerate the rate of cycloaddition in aerobic conditions, avoid the production of deleterious reactive oxygen species, sequester free copper ions and stabilize the Cu(I) state provide better opportunities for the applications of CuAAC in living systems. Another approach is to utilize a reporter azide bearing an internal Cu(I) chelating moiety, which minimizes copper concentrations to biocompatible levels, and without sacrificing reaction rate.[10] Overall, bioorthogonal labelling by CuAAC has revealed a wealth of biological information through protein,[11] DNA,[12] RNA,[13] lipid,[14] glycan[9b-d] and activity-based probes.[15] Yet despite the progress for the use of copper-catalysis in biological experiments, few reactions other than CuAAC have been utilized for bioorthogonal labelling.



Scheme 1 Structures of endocyclic nitrones and alkyne-functionalized compounds used in this study (see Electronic Supplementary Information (ESI) for synthesis and characterization), and a general CuANCR reaction scheme (Box).

One reaction that has potential applications in copper catalysed bioorthogonal reactions is the Cu(I)-catalysed formation of a β-lactam ring from a terminal alkyne and nitrone, also known as the Kinugasa reaction.[16] Recently we and others have shown that this reaction can be conducted efficiently in aqueous conditions with micelle-promotion[17] and Cu(I) catalysis (e.g. sodium ascorbate to reduce Cu(II) salts).[18] Furthermore endocyclic nitrones are attractive for bioorthogonal chemistry as they combine enhanced stability in acidic and basic conditions, and exhibit fast reaction kinetics in strain-promoted alkyne-nitrone cycloadditions (SPANC).[3a-d] Herein, we demonstrate that copper-catalysed alkyne-nitrone cycloadditions followed by rearrangement (CuANCR) utilizing stable endocyclic nitrones can efficiently label surfaces of living cells. We further demonstrate for the first time metabolic incorporation and detection of endocyclic nitrones, highlighting their stability in living cells, and potential for diverse biological applications.

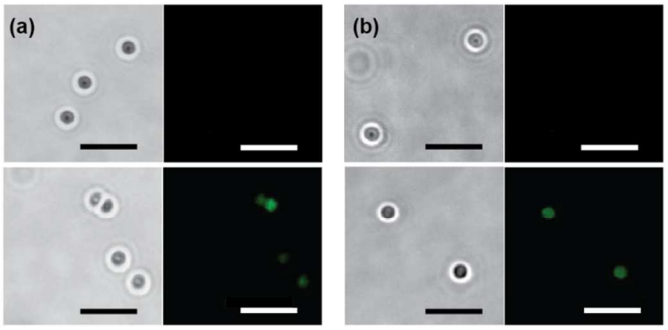


Fig. 1 In vitro labelling of alkyne functionalized magnetic beads via CuANCR. Alkyne beads were treated with 50 μM (a) Alexa488-CMPO or (b) Biotin-CMPO. CMPO reporters were incubated in PBS (top) or PBS containing 0.1 mM CuSO4, 0.2 mM L-histidine, 2 mM sodium ascorbate (bottom) for 30 minutes at 37°C. Beads were washed in PBS then either imaged directly in (a) or stained with FITC-streptavidin prior to imaging in (b). Scale bars = 5 μm.

To determine if conditions optimized for live-cell labelling by CuAAC could be applied to CuANCR, alkyne-functionalized magnetic beads were treated with nitrone-tagged reporters and detected by fluorescence microscopy. We previously established L-histidine as a minimal-toxicity copper ligand suitable for click reactions on living cells.[8b] To test these conditions, Alexa488-CMPO (Scheme 1) was synthesized according to ESI, which would allow for rapid screening through fluorescence labelling of alkyne-functionalized magnetic beads. Alkyne beads were incubated for 30 minutes with 50 μM Alexa488-CMPO in PBS alone or with Cu(I)-histidine click conditions (0.1 mM CuSO4, 0.2 mM L-histidine, 2 mM sodium ascorbate in PBS). After subsequent washes in PBS, beads were fluorescently labelled only in the presence of the Cu(I)-histidine complex (Fig. 1A). Similar treatment of the alkyne beads with 50 μM Biotin-CMPO (Scheme 1), followed by FITC-streptavidin staining, confirmed covalent attachment of the reporter molecule bearing an endocyclic nitrone to the terminal alkyne-functionalized beads (Fig. 1B). Furthermore, labelling of the alkyne beads increased in a dose-dependent manner, as shown in Fig. S1 (ESI).

Having established labelling by CuANCR *in vitro*, we then tested the ability of CuANCR to detect metabolic labelling on mammalian cells. Huh-7 human hepatoma cells were cultured for 72 hours in the presence or absence of an alkyne-tagged mannosamine derivative (Ac_4ManN-alkyne), which is known to get incorporated into sialylated glycans.[19] Live cells were washed in PBS, treated with 50 μM Biotin-CMPO for 30 minutes using the CuANCR conditions described above, and then stained with FITC-streptavidin for detection of alkyne tagged surface glycans by fluorescence microscopy. As shown in Fig. 2, only cells cultured with Ac_4ManN-alkyne were fluorescently labelled, establishing CuANCR as a bioorthogonal labelling strategy for mammalian cells.

To expand upon potential applications of CuANCR beyond mammalian cells, we utilized this labelling method to detect surface glycans of bacterial cells. It was previously shown that gram negative bacteria could metabolize an azide-functionalized derivative of 3-deoxy-D-manno-octulosonic acid (KDO), which is targeted to the inner core of lipopolysaccharides (LPS) embedded in the outer membrane.[20] To establish that CuANCR labelling can also function in this context, we incubated BL21 *E. coli* overnight in minimal medium containing an alkyne-functionalized 3-deoxy-D-manno-octulosonic acid (KDO-alkyne, Scheme 1, synthesized according to ESI). The cells were subsequently washed in PBS, and then treated with 50 μM Alexa488-CMPO by CuANCR. *E. coli* incubated only in the presence of KDO-alkyne were fluorescently

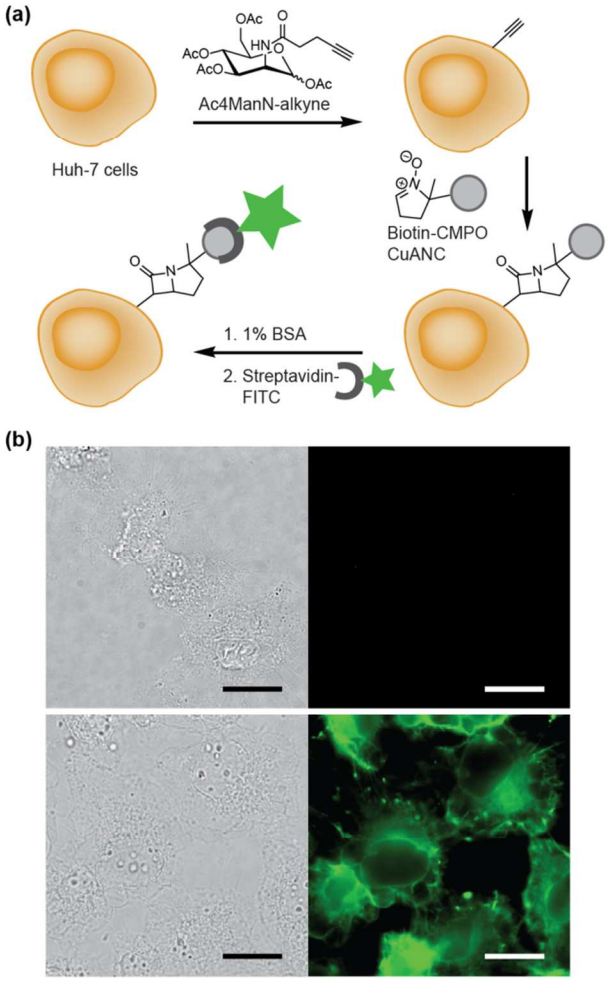


Fig. 2 *In situ* surface labelling of Huh-7 cells by CuANCR. (a) Huh-7 cells were cultured in the presence or absence of 50 μM Ac_4ManN-alkyne for 72 hours prior to treatment with 50 μM Biotin-CMPO in PBS containing 0.1 mM CuSO4, 0.2 mM L-histidine, 2 mM sodium ascorbate for 30 minutes at 37°C. Cells were blocked with 1% BSA in PBS, then stained with 5 μg/ml FITC-streptavidin and fixed prior to detection by fluorescence microscopy. (b) Fluorescence microscopy images of Huh-7 cells treated as described in (a), cultured in the absence (top) or presence (bottom) of 50 μM Ac_4ManN-alkyne. Scale bars = 25 μm.

labelled (Fig. 3A), showing CuANCR conditions are also suitable for treatment of living bacterial cells.

To date, the nitrone group has shown great promise in biological applications, such as protein and cell surface labelling via SPANC.[3b, 3e, 21] However, metabolic incorporation of nitrone-bearing biomolecular precursors has proven challenging, notably, when targeting the sialic acid pathway.[3e] Since LPS biosynthesis tolerates incorporation of KDO-alkyne (Fig. 3A), which is more bulky due to an amide group in the tag (Scheme 1), we sought to determine if the endocyclic nitrone group could be tolerated as well. *E. coli* cells were cultured overnight in the presence of KDO-HMMPO, synthesized according to ESI, and then treated with 50 μM Alexa488-alkyne using CuANCR conditions identical to those described above. *E. coli* incubated with KDO-HMMPO, bearing a slightly different nitrone than the CMPO of biotin and Alexa488 (Scheme 1), were fluorescently labelled, indicating metabolic incorporation of the nitrone group (Fig. 3B). To confirm incorporation of KDO-HMMPO into *E. coli* LPS specifically, LPS were extracted from *E. coli* that were incubated with KDO, KDO-azide or KDO-HMMPO and subsequently treated with 50 μM

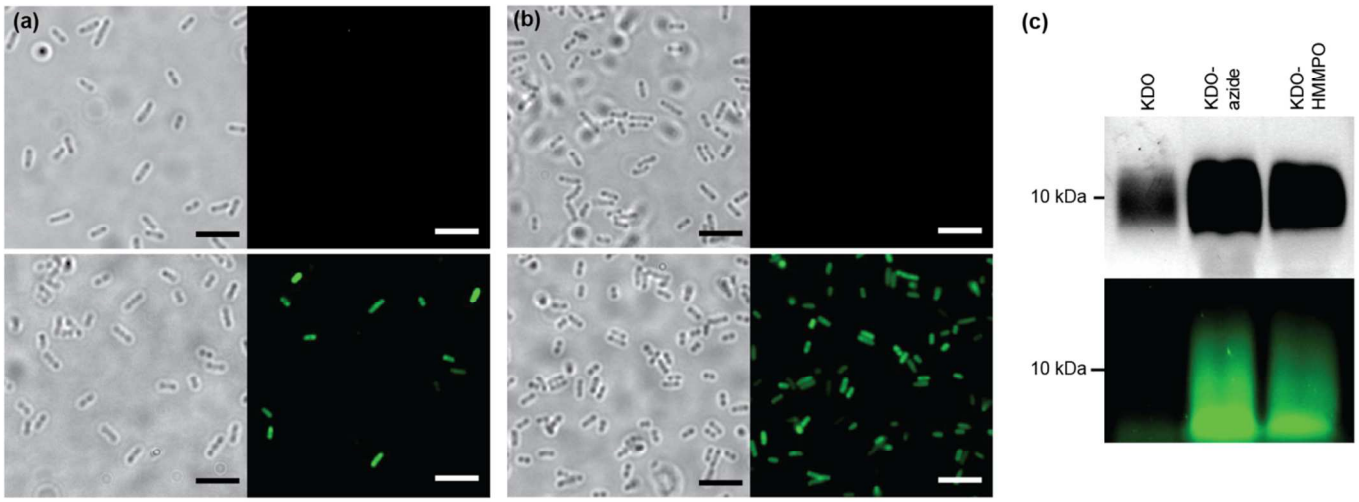


Fig. 3 Metabolic labelling of *E. coli* by functionalized KDO derivatives. (a) Metabolic labelling of *E. coli* with KDO-alkyne. *E. coli* cells were cultured in M9 in 4 mM KDO (top) or 4 mM KDO-alkyne (bottom), reacted with 50 μM Alexa488-CMPO by CuANCR, and then live cells were imaged by fluorescence microscopy. (b) Metabolic labelling of *E. coli* with KDO-HMMPO. *E. coli* cells were cultured with 4 mM KDO (top) or 4 mM KDO-HMMPO (bottom) in M9 and reacted with 50 μM Alexa488-alkyne by CuANCR. (c) Extracted LPS of metabolically labelled *E. coli* after treatment with 50 μM Alexa488-alkyne by CuAAC/CuANCR. Fluorescence gel scan is shown below, with LPS silver stain shown above. Scale bars = 5 μm.

Alexa488-alkyne via CuAAC/CuANCR conditions. Extracted core LPS of BL21 *E. coli* were then analysed by SDS-PAGE in-gel fluorescence and LPS silver staining,[22] revealing only cells incubated with KDO bearing an azide or nitrone functional group were fluorescently labelled (Fig. 3C). Taken together, these results indicate KDO-HMMPO is metabolized by *E. coli* and incorporated into LPS molecules decorating their surfaces. This is the first example, to the best of our knowledge, of metabolic labelling using an endocyclic nitrone.

Conclusions

In summary, we have shown that Cu(I)-histidine catalysed cycloaddition of terminal alkynes with endocyclic nitrones is possible for the labelling of living cells. In addition, we showed for the first time metabolic incorporation of a biomolecular precursor bearing a nitrone group. The CuANCR bioconjugation approach presented here has demonstrated utility in labelling magnetic beads *in vitro*, and mammalian and bacterial cells *in situ*, highlighting its potential for a broad array of future applications. Finally, CuANCR represents a new bioorthogonal reaction that is catalysed by Cu(I) complexes that should function interchangeably and complementary to CuAAC reactions. We are currently assessing both the broader applicability as well as the possibility of alternative ligands for use in CuANCR, since the diverse range of potential nitrone tags may show different dependencies towards lower toxicity ligands and faster chemistry. CuANCR should also be applicable to multiplex labelling using copper catalysis, for which there are relatively few options.

Notes and references

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