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COMMUNICATION

Optimized Aqueous Kinugasa Reactions for Bioorthogonal Chemistry Applications

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Kinugasa reactions hold potential for bioorthogonal chemistry in that the reagents can be biocompatible. Unlike other bioorthogonal reaction products, β-lactams are potentially reactive, which can be useful for synthesizing new biomaterials. A limiting factor for applications consists of slow reaction rates. Herein we report an optimized aqueous copper(I)-catalyzed alkyne-nitrone cycloaddition involving rearrangement (CuANCR) with rate accelerations made possible by the use of surfactant micelles. We have investigated the factors that accelerate the aqueous CuANCR reaction and demonstrate enhanced modification of a model membrane-associated peptide. We discovered that lipids/surfactants and alkyne structure have a significant impact on the reaction rate, with biological lipids and electron-poor alkynes showing greater reactivity. These new findings have implications for the use of CuANCR for modifying integral membrane proteins as well as live cell labelling and other bioorthogonal applications.

Bioorthogonal chemistry provides invaluable tools to chemical biology research for studying specific biological processes inside cells that are otherwise challenging to probe.[1–6] A number of bioorthogonal reactions have been developed and are now used as techniques to efficiently, selectively and covalently link two reactive groups.[7–13] One such reaction is copper(I)-catalyzed azide-alkyne cycloaddition (CuAAC) (Fig. 1a),[14,15] which has been and continues to be utilized extensively in a myriad of biological labelling applications and is used to perform labelling of cellular targets.[16–20] Concerns around copper toxicity are addressable with careful choice of copper(I) ligand,[21] through traceless reagents bearing catalytic copper,[22] or development of alternative copper-catalyzed reactions.[23,24] However, alternatives are still desired to expand the variety of reacting partners and bring additional reactions for use in bioorthogonal applications, allowing for the discovery of more efficiently catalyzed reactions.[25–27] The classical Kinugasa reaction,[28] although notoriously sensitive and challenging, represents a unique prototype reaction for development and optimization for bioorthogonal chemistry applications. Since its initial discovery, the Kinugasa reaction has received considerable recognition as a reaction that produces β-lactams in a variety of conditions.[28,29] β-lactams are of significant interest because of their use as antibiotics,[30,31] and their general properties as electrophiles.[32] The Kinugasa reaction has been developed from original reports using pyridine as a base and solvent,[28,33] to using polar aprotic solvents as well as polar protic/aprotic solvent mixtures,[29] and reactions in aqueous conditions,[34,35] with the potential to be applied to biological systems for bioorthogonal labelling.[2,3,21,36] Yet, examples of the Kinugasa reaction in aqueous conditions are few, with our group previously reporting one of the first examples.[34] Thus, it can be considered a prototype reaction that still needs development and optimization in order to operate as a useful bioorthogonal reaction.

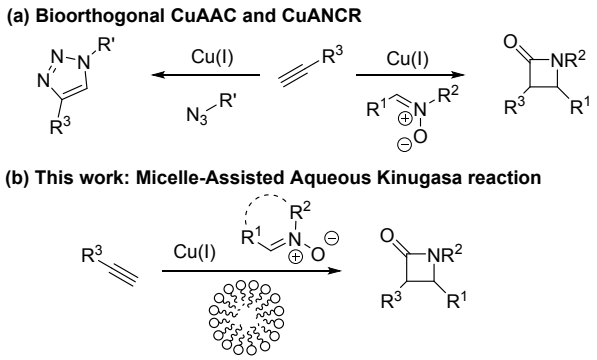

Fig 1. a) Comparing Kinugasa/CuANCR with CuAAC bioorthogonal chemistry. b) Lipid acceleration through micelles.

In addition to applications of biological labelling, bioorthogonal chemistry allows for the production and functionalization of nanoparticles and biomaterials.[37,38] CuAAC chemistry has been used extensively for modification of material surfaces,[39] attachment of proteins to materials[40] and nanomaterial synthesis.[41] The Kinugasa reaction could be applied to similar applications, while producing potentially bioactive β-lactam products directly on biomaterial surfaces. The development of

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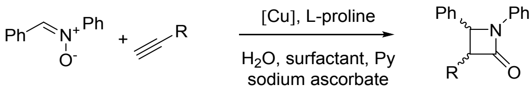
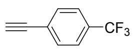
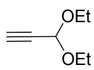
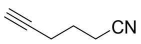
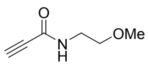
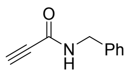
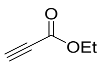
biomaterials with antibacterial properties is vital due to bacterial colonization being a major cause of implant failure.[42] β-lactam antibiotic doping has been proposed to produce such materials[43]; thus the Kinugasa reaction could allow for direct functionalization of such bioorthogonal implant materials.

Nitrones are excellent tunable bioorthogonal reactants, which have already been demonstrated in strain-promoted alkyne-nitrone cycloaddition (SPANC) reactions.[44,45] We recently discovered a version of the Kinugasa reaction, copper(I)-catalyzed alkyne-nitrone cycloaddition involving rearrangement (CuANCR), that can be used as a bioorthogonal reaction for live-cell labelling.[46] However, reaction rates were slow. Herein, we optimize reaction conditions for the aqueous CuANCR and use surfactant micelles[47] to accelerate aqueous reaction rates and yields.

Table 1. Screen of alkynes in micelle-assisted Kinugasa reactions



Entry	Alkyne	Conversion[a] (Isolated Yield %)
1		38 (22[b])
2		76 (16[b])
3		85 (20[b])
4		90 (32[b])
5		95 (69)
6		81 (64)
7		91 (65)

[a] Reactions were conducted in 20 mL Argon degassed H_2O containing 10 mM SDS. Sodium ascorbate (0.2 mmol), pyridine (0.1mmol), L-proline (0.05 mmol) and $CuSO_4$ (0.025 mmol) were added successively. Following the addition of alkyne (0.05 mmol), C,N-diphenylnitrone (0.05 mmol) was added and stirred for 30 minutes at 25 °C. NMR yields are reported following extraction with 3x20 mL EtOAc, using 1,4-Dimethoxybenzene as internal standard. [b] Conducted with 3.5 mM CTAB in the absence of L-proline.

Initially, we sought to optimize the alkyne portion of the reaction. Alkynes have been activated towards [3+2] cycloaddition by conjugation to electron-withdrawing groups, such as esters, through lowering of the energy of their LUMO.[48] Therefore, other alkynes bearing electron withdrawing groups were tested (Table 1, Entries 2-7). The use of propiolamides (Table 1, Entries 5-6) and propiolic esters (Table S1, Entry 7) led to increases in observed yield, as did the inclusion of micellar catalysts.[30] The scope of acyclic nitrones was also evaluated and the reaction was found to be amendable to a wide range of aryl substitution (See Table S2). Furthermore, it should be noted that the reported yields of β-lactam products after extraction might not fully represent the total reaction yield, given potential product loss in the extraction process with surfactants/micelles. Additionally, the β-lactam yields do not report on other potential covalently linked side products which would also lead to successful bioconjugation.[36] Thus, percent conversion is thought to be more representative of reaction progression.

Given difficulties in product isolation and quantification due to use of surfactants, the determination of reaction kinetics proves challenging. Therefore, to further evaluate the reaction efficiency and ultimately relative rates, we performed studies utilizing our recently reported alkyne-tagged magnetic bead assay.[46] The latter represents a simple and convenient approach to assessing reaction progression under biological lipid/micellar catalysis, which otherwise interferes with most conventional spectroscopic approaches. Briefly, we employed a biotin-tagged cyclic nitrone (biotin-CMPO) that was reacted with alkyne-functionalized magnetic beads in the presence of $CuSO_4$ and sodium ascorbate, while varying ligand type and presence of different biomimetic lipids. After magnetic separation, covalently linked biotin conjugated to alkyne-tagged magnetic beads via CuANCR, were detected by FITC-streptavidin staining and fluorescence microscopy. An initial comparison between L-histidine and L-proline as bioorthogonal copper ligands led us to choose L-proline to perform further on-bead assays of a variety of lipid micelles, due to higher observed labelling efficiency (Fig. 2, S1) and due to L-proline having been previously shown to be a competent copper ligand.[49,50]

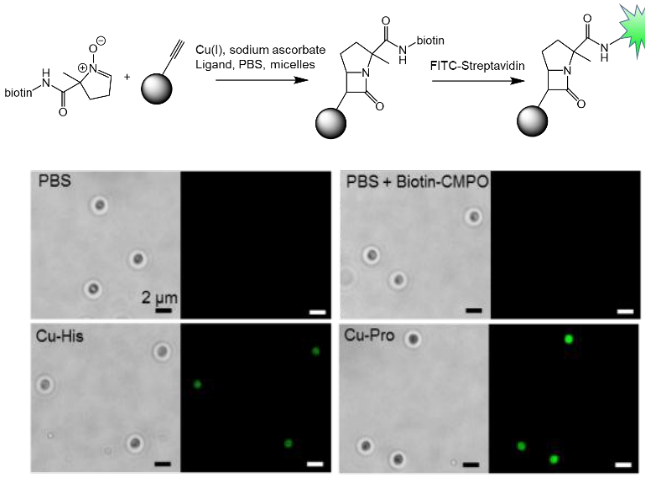


Fig. 2 Effect of ligand on CuANCR labelling of alkyne-tagged magnetic beads. Alkyne beads were incubated for 30 minutes at 37 °C in PBS, PBS with biotin-CMPO or PBS containing 100 μM $CuSO_4$, 200 μM ligand, 2 mM sodium ascorbate and 50 μM biotin-CMPO. Beads were washed in PBS then stained with FITC-streptavidin prior to imaging. Scale bars = 2 μm.

Next, we sought to evaluate the effects of biomimetic lipids on the efficiency of CuANCR reactions. We have previously reported that CuANCR is an effective approach for cell labelling.[46] We hypothesized that cell surface components, such as cellular lipids, glycans, glycoproteins and the membrane itself increase the efficiency of the CuANCR labelling reaction, as previously seen by others.[51] To test this hypothesis, we performed a screen of biomimetic lipids with a hydrophilic sugar group or with quaternary ammonium and phosphate groups (Table S3). We chose an initial lipid concentration of 1.5 mM, corresponding to the highest critical micellar concentration (CMC) amongst all screened lipids (Table S3), to ensure that CuANCR is conducted in a micellar environment. The background fluorescence, determined for beads incubated without biotin-CMPO and with Fos-choline 16 (arbitrarily chosen amongst screened surfactant), was subtracted from all

samples. All examined lipids contributed to a more efficient CuANCR labelling of alkyne beads (Fig. 3), and maltopyranoside-functionalized lipids increased the efficiency about 2-fold. These results confirmed our hypothesis that biomimetic lipids can significantly accelerate CuANCR reactions.

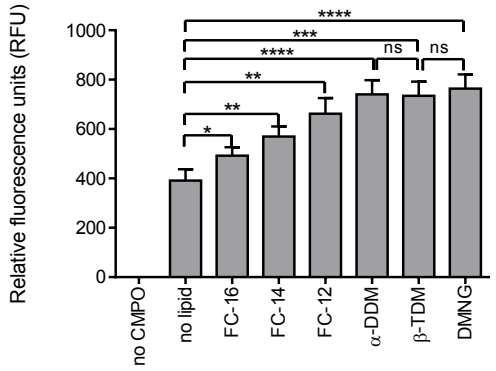


Fig. 3 Lipid screen for micelle-assisted Kinugasa/CuANCR reaction. Alkyne-tagged magnetic bead assay was used. Beads were labelled via CuANCR reaction in 100 μM CuSO4, 2 mM sodium ascorbate, 200 μM L-proline and 50 μM biotin-CMPO, in presence of 1.5 mM of indicated lipid. Reaction was carried out in PBS for 30 minutes at 37 °C, after which beads were washed in PBS and incubated with 5 μg/mL streptavidin-FITC, then washed. Above background fluorescence for at least five beads from two separate field views (10 beads total) was used to determine the average fluorescence of beads for each condition. * p<0.1, ** p< 0.01, *** p< 0.001, **** p< 0.0001.

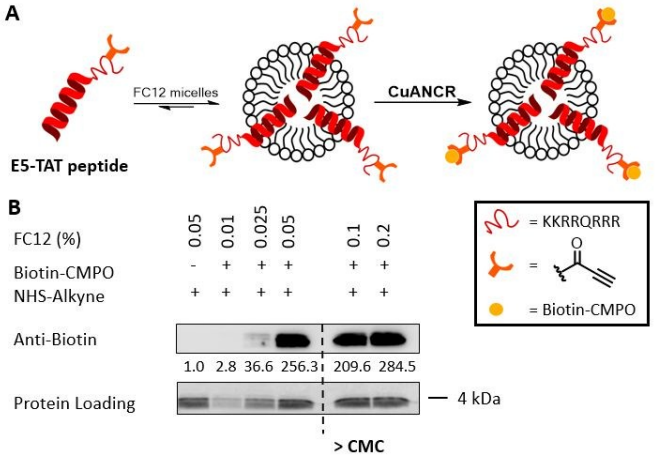


Fig. 4 Biotin conjugation of E5-TAT membrane peptide using CuANCR. (A) Schematic representation depicting the two step biotin conjugation of E5-TAT peptide under micellar assistance. Alkyne functionalization of E5-TAT (20 μM) was carried out using N-hydroxysuccinimide ester alkyne (300 μM). Biotin labelling was achieved by treating modified peptide with $CuSO_4 \cdot H_2O$ (20 μM), sodium ascorbate (300 μM), L-proline (40 μM) and biotin-CMPO (300 μM). (B) Top panel represents the western blot analysis of biotin labelled E5-TAT samples under varying percentages (%W/V) of detergent (FC12) ranging from (0.01-0.2%, CMC=0.05%). Bottom panel represents the TGX-Stain free blot as a loading control. The presented relative biotin conjugation values consist of densitometry analysis normalized to protein loading.

To further optimize CuANCR reactions for future bioorthogonal applications, we selected an optimal lipid to examine the effects on the rates of reactions. We chose β-TDM, which has the lowest CMC amongst the three maltopyranoside lipids, for further CuANCR condition optimization. We then focused on optimal conditions of this biological lipid for CuANCR, focusing on sub-micellar and micellar conditions. A concentration screen showed that at CMC of β-TDM in water (10 μM), the CuANCR labelling of alkyne beads is roughly as efficient as in 10-fold or 100-fold excess of β-TDM (Fig. S3, S4, S5). However, in absence of β-TDM and in sub-micellar concentrations of β-TDM, the labelling efficiency drops to or below 50% in comparison, showing the importance of the presence of β-TDM micelles for optimal CuANCR labelling. This suggests that a micellar environment, and not individual lipid species are responsible for catalysis.

Next, we investigated the kinetics of CuANCR labelling in micellar β-TDM, seeking insight in the possible mechanism of micelle catalysis of CuANCR (Fig. S6). After 5 minutes of CuANCR labelling, there is a significant difference between micelle-assisted CuANCR, and surfactant-free reaction. Under pseudo-first order kinetics with 50 μM biotin-CMPO nitrone in 10-fold excess over alkyne, we were able to estimate a $k_{obs\text{-}micelle}/k_{uncat}$ ratio of ≥100, suggesting that presence of micelles contributes to a faster rate of CuANCR, possibly by solubilizing hydrophobic reactants and effectively increasing their local concentration, or orienting the reactants for optimal reaction trajectory. We envision membrane and biological lipids to play a similar role for bioorthogonal applications; allowing for greater labelling efficiencies and enhanced reaction rates on cells.

To demonstrate the applicability of micelle-assisted CuANCR chemistry, we performed biotin labelling of a small membrane-associated peptide from the class of cell-penetrating peptides, E5-TAT.[52,53] Membrane proteins represent an important class of protein acting as membrane receptors, integral structural components and transporters, amongst other functions. Their isolation and functionalization can be challenging requiring hydrophobic environments to maintain proper structure and function. Micellar conditions represent a suitable system in which these membrane proteins can be solubilized and modified.[54] E5-TAT is a small membrane-associated peptide that contains a fusion between HIV-TAT protein transduction domain (PTD) and a variant of the influenza HA2 domain that inserts into lipid membranes and destabilizes them at low pH.[52,53] It was chosen as a model peptide to which an alkyne handle could be appended through lysine residues on the cationic TAT sequence (KKRRQRRR). To accomplish labelling, the peptide samples were treated with an N-hydroxysuccinimide ester alkyne for 1 hour under micellar conditions (Fig. 4). The alkyne modified peptides were purified by FPLC and modification of expected lysine residues (K26, K27) was confirmed by mass spectrometry analysis (see Fig. S8 and Tables S4-S5). The modified peptides were then submitted to CuANCR conditions for 1 hour to conjugate a biotin moiety, using Biotin-CMPO as the nitrone reacting partner. After the two-step labelling protocol, the peptide samples were analyzed by western blot with results showing a significant difference in the relative biotin conjugation between the micelle-assisted CuANCR conditions (≥CMC) and the surfactant-free conditions (>250 fold) (See Fig. S7 for non-modified peptide control). These results emphasize the importance of micellar conditions not only for peptide solubility, but as well for achieving robust peptide modification.

Conclusions

In conclusion we have found that lipids, biological ligands and electron poor alkynes help accelerate and increase the yield of the CuANCR reaction developed from the prototype Kinugasa reaction. The use of micelle catalysis consists of a cost-effective platform on which bioorthogonal reaction development can take place. We have also shown that membrane protein modification is possible through CuANCR chemistry, highlighting the bioorthogonal nature

of the reaction and value for rate acceleration in micellar environments for selective membrane protein modifications. We envision that new findings from optimized CuANCR reactions could be applied in a bioorthogonal manner for *in vitro* labelling to live cell labelling. The development of the CuANCR reaction could also allow for the synthesis of bioactive β-lactam products in biological system via membrane lipids, allowing for further functionalization and engineering of cell membranes. Further studies are under way to develop applications of CuANCR and expand upon the potential chemistry of the produced β-lactams.

Conflicts of interest

There are no conflicts to declare.

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Keywords: Kinugasa Reaction • Micelle catalysis • Bioorthogonal • Copper-catalyzed alkyne-nitrone cyclization involving rearrangement • β-lactam • Membrane-associated peptide modification

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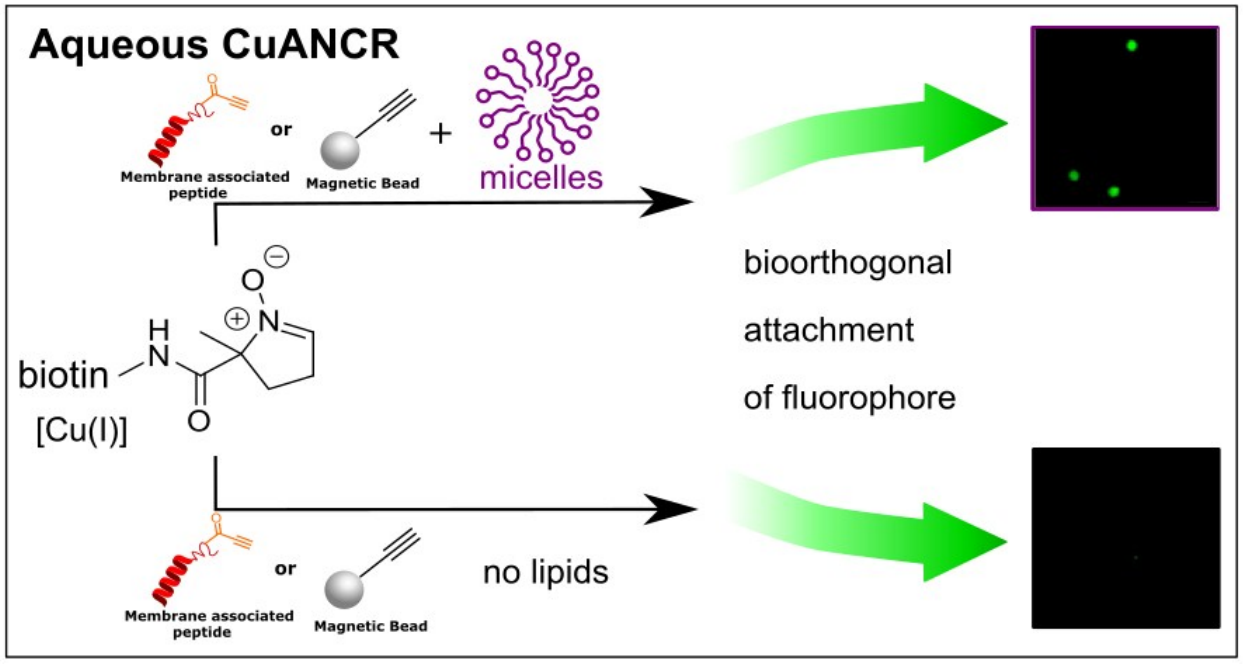

We present optimized micelle-assisted aqueous copper(I)-catalyzed alkyne-nitrone cycloaddition involving rearrangement (CuANCR) reactions applicable to bioorthogonal applications, namely membrane-associated peptide modification.