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Anaerobic conditions to reduce oxidation of proteins and to accelerate the copper-catalyzed "Click" reaction with a water-soluble bis(triazole) ligand†

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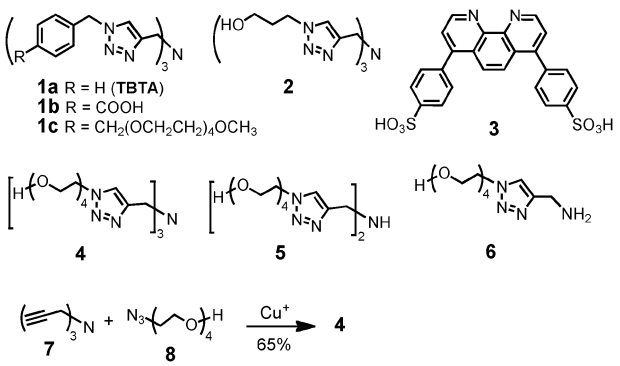
Oxidation of protein (bovine albumin serum) by air still occurred under the copper-catalyzed azide–alkyne cycloaddition (CuAAC) reaction conditions even in the presence of a Cu(I)-stabilizing tris(triazole) ligand. Anaerobic conditions not only avoided the oxidation of the protein, but also greatly accelerated the CuAAC reaction using a water-soluble bis(triazole) Cu(I) ligand.

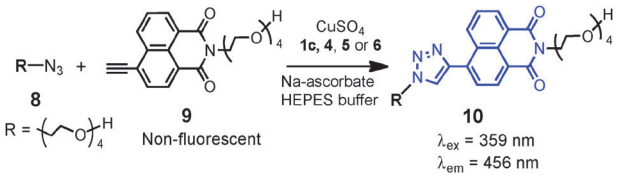
The copper-catalyzed azide–alkyne cycloaddition (CuAAC) reaction[1–4] and its copper-free, strained-alkyne variant[5] is one of the most widely used methods for bioconjugation.[4] The former in general is faster, and does not require the strained alkynes (some of them are commercially available but costly). For the CuAAC reaction in air, sodium ascorbate is commonly used to maintain the catalytically active Cu(I) oxidation state.[2] While this method is practical, the Cu(I)/Cu(II)/ascorbate/O_2 redox system generates reactive oxy species and electrophiles that are harmful to many biological systems.[6] Finn and co-workers recently recommended general conditions for using water-soluble tris-triazole derivatives as co-catalysts that not only greatly accelerated the reaction but also served as a sacrificial reductant to reduce the oxidative damage.[6] In this work, we found that these conditions still could not eliminate the rapid oxidation of bovine serum albumin (BSA) as a model protein. Therefore, for oxidatively labile bio-systems, the reaction may require anaerobic conditions. We show that a water-soluble bis(triazole) Cu(I) ligand is among to date the most efficient co-catalysts for the CuAAC reaction under anaerobic conditions.

A rapid reaction is highly desirable for bioconjugation. Many Cu(I)-ligands have been reported to accelerate the CuAAC reaction, in particular the tris-triazole-based ligands **1a**,[7] and its water-soluble analogues **1b**[6] and **2**,[7] and the bathophenanthroline **3**.[8] The preparation of **2** in pure form required four steps with a low overall yield,[9] using a low molecular weight azide that is not commercially available due to the risk of explosion. In this work, we investigated a series of water-soluble ligands **1c** and **4–6** containing 1–3 triazole moieties. While ligand **1c** was prepared in 3 steps (see ESI†), the ligands **4–6** were readily prepared in one step from the commercially available starting materials **7** and **8** in yields ranging from 65–91% (see ESI†). As an example, the synthesis of **4** is shown in Scheme 1.

A modified fluorogenic assay[10] was used to evaluate the ligands under various reaction conditions (Scheme 2). We introduced an oligo(ethylene glycol) (OEG) chain in the conjugated alkyne **9** to render it water soluble. Upon CuAAC reaction of the non-fluorescent alkyne **9** with an azide, *e.g.* **8**, a highly fluorescent, conjugated triazole product, *e.g.* **10**, was formed, which allowed for convenient monitoring of the reaction and estimating the yields by fluorescence measurement (see ESI†). To facilitate the comparison of efficiency of the ligands, we purposefully chose the slower reaction between **8** and **9**[10] to form the fluorescent product **10**, instead of the rapid reaction between an alkyne and the highly reactive conjugated azide that could be completed within 15 min.[9]



Scheme 1 One-step synthesis of the ligand **4**.



Scheme 2 Using the fluorogenic probes for optimization of the CuAAC reaction conditions.

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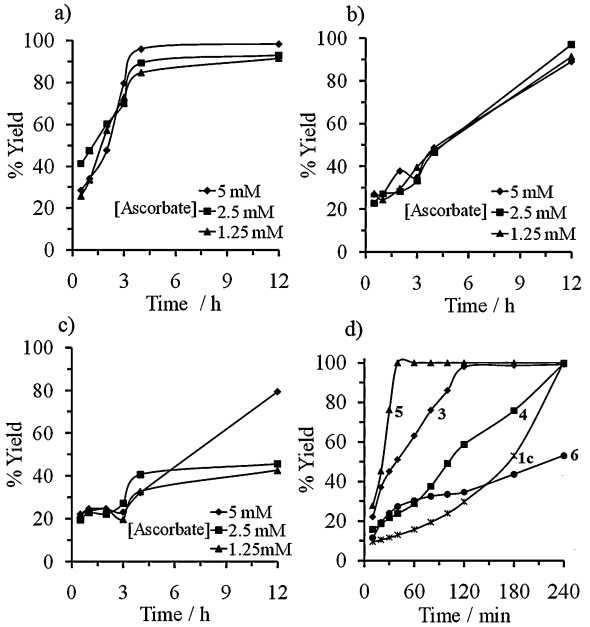

Fig. 1 Yields *vs.* time profiles of the CuAAC reaction of **8** (1 mM) and **9** (0.5 mM) with $CuSO_4$ (125 μM), Cu/ligand ratio of 1 : 2 in 50 mM HEPES buffer, pH 7.2, in air in the presence of (a) ligand **1c**, (b) ligands **4**, (c) ligand **5**, and various ascorbic acid concentrations, and (d) under anaerobic conditions with 1.25 mM ascorbate and ligands **1c**, **3**, **4**, **5**, and **6**.

When the reaction of **8** (1 mM) and **9** (0.5 mM) was performed in air in the presence of $CuSO_4$ (0.125 mM) and the ligand **1c** or **4**[11] (0.25 mM), the rate was not affected by the ascorbate concentration in the range of 1–5 mM (Fig. 1a and b), indicating a good stabilization of the Cu(I) oxidation state by these tris(triazole)methyl-amine ligands that bind Cu(I) in the tetrahedral configuration. Indeed, Fokin and co-workers showed that a similar ligand (**2**) largely increased the redox potential of Cu(I)/Cu(II) by ~300 mV and also decreased the electron transfer kinetics.[7] The most efficient ligand we tested for the reaction in air was the OEG-modified TBTA (**1c**), completing in 4 h in the presence of only 1.25 mM ascorbate. The tris(triazole) ligand **4** without the benzyl substituent was less efficient in air, but easily prepared by a one step reaction (Scheme 1). On the other hand, the mono- and bis(triazole) ligands **5** and **6** performed poorly in air, even with a large excess of ascorbate (*e.g.* Fig. 1c). These ligands greatly promoted the oxidation of Cu(I) to Cu(II) in air, as indicated by the rapid color change of the solution from colorless to blue. The high oxygen sensitivity of Cu(I) complexes with **5** and **6** may be attributed to the stronger electron-donating and less steric hindrance of these ligands as compared to the tristriazole ligand **1**. The resultant Cu(II) species can be reduced back to Cu(I) by ascorbate in the reaction system, but at the expense of generating reactive oxy species (radicals, peroxides and superoxides) and strong electrophiles that are harmful to the biological systems and to the ligand itself.[6] In addition to rapid consumption of the excess of ascorbate, the oxidation of Cu(I) by O_2, which was greatly accelerated by the ligand **5** or **6**, also led to a decrease of the effective concentration of Cu(I), depending on the rates of the redox reactions, thereby decreasing the rate of the CuAAC reaction.

Although the bis(triazole) and the mono(triazole) ligands **5** and **6** greatly accelerate the Cu(I)/Cu(II) redox cycle in the presence of ascorbate and O_2, Fokin and co-workers reported that these ligands also speeded up the CuAAC reaction in oxygen-free conditions.[7] We compared the tris-, bis- and mono(triazole) ligands (**1c**, **4**, **5** and **6**) as well as the water-soluble bathophenanthroline **3**[8] for promoting the CuAAC reaction under oxygen-free conditions. The reaction was performed with **8** (1 mM) and **9** (0.5 mM) in the presence of sodium ascorbate (125 μM), $CuSO_4$ (125 μM) and the ligand with a Cu/ligand ratio of 1 : 2 in 50 mM HEPES buffer, pH 7.2. To eliminate possible errors of quantification using fluorescence, the yields of the reaction at various durations were quantified by HPLC (see ESI†) and plotted in Fig. 1d. The results showed that the rate of the reaction remained low in the presence of the mono(triazole) ligand **6**. Also, the absence of O_2 did not improve the efficiency of the TBTA analogue **1c**, attributable to its excellent stabilization of Cu(I) against oxidation.[7] However, the less hindered tris(triazole) ligand **4** performed better under anaerobic conditions, and was even more efficient than the TBTA analogue **1c** at the beginning of the reaction. This result indicates that the slight decrease of efficiency of **4** than **1c** in air was likely due to the faster oxidation of the less hindered **4**–Cu(I) complex than the **1c**–Cu(I) complex.

The most efficient co-catalyst for the reaction performed in an oxygen-free environment was the bis(triazole) ligand **5**; the reaction was completed in 40 min under the above conditions (Fig. 1d). This ligand was also more efficient than the bathophenanthroline **3**, previously reported to be the most efficient co-catalyst for the CuAAC reaction in aqueous solution under anaerobic conditions.[8] Therefore, among the ligands **3–6**, the bis(triazole) ligand **5** has the best combination of electronic and steric effects for binding the metal with the alkyne and azide leading to triazole formation. We note that despite the inconvenience, running the CuAAC reaction in oxygen-free conditions does bring the following advantages: (1) eliminating the formation of reactive by-products harmful to the biological system (see below), (2) maintaining the catalytically active Cu(I) state, and (3) greatly accelerating the reaction using the most efficient (but oxygen-sensitive) catalyst, such as Cu(I)/**5**.

To evaluate the extent of oxidation in oxidatively labile biological systems during the CuAAC reaction under various conditions, we used an amino-rich protein, bovine albumin serum (BSA), as a model. The formation of carbonyl groups in proteins has been widely used as a marker of oxidative damage of proteins, *e.g.* during oxidative stress in biological systems.[12] We used an enzymatic assay kit (OxiSelect™ protein carbonyl ELISA, Cell Biolabs Inc.)[13] to estimate the amount of carbonyl groups generated on BSA after subjecting it to CuAAC reaction conditions, as detailed in ESI.† Briefly, the BSA samples were immobilized and treated with 2,4-dinitrophenyl-hydrazine (DNPH) that reacted with the active carbonyl groups on the protein. The resultant hydrazone was quantified by binding with the anti-DNP antibody followed by the horseradish peroxidase conjugated secondary antibody that catalyzed a colorimetric reaction (see ESI†). A calibration curve correlating the absorbance (generated by the colorimetric reaction)

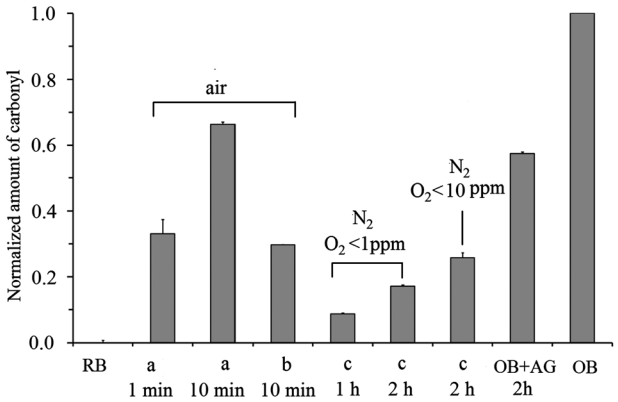

Fig. 2 The normalized amount of carbonyl generated on BSA after being subjected to various CuAAC conditions (a–d) in an aerobic or anaerobic environment for various durations. A fully reduced BSA standard (RB, without carbonyl) and an oxidized BSA standard (OB, with ~0.5 carbonyl per BSA molecule) were used for the normalization. RB (0.5 mg mL^{-1}) was used for the following conditions: (a) 0.1 mM $CuSO_4$, 2.5 mM Na-ascorbate, 0.5 mM **4** in 0.1 M PBS buffer (pH 7.0); (b) 0.1 mM $CuSO_4$, 2.5 mM Na-ascorbate, 5 mM amino guanidine (AG) and 0.5 mM **4** in 0.1 M PBS buffer/DMSO 95 : 5 (pH 7.0); (c) 0.125 mM $CuSO_4$, 1.25 mM Na-ascorbate, 0.25 mM **5** in 50 mM HEPES buffer (pH 7.2). Each data point was the mean of 3 replicates and the error bar represented the standard deviation.

with the amount of carbonyl on BSA was obtained using a series of BSA standards (Cell Biolabs Inc.), including the reduced BSA (RB, containing nearly no active carbonyl groups), partially oxidized BSA, and the fully oxidized BSA (OB, containing ~7.5 nmol carbonyl per mg, corresponding to ~0.5 carbonyl per BSA). The calibration curve was linear from 0–0.5 carbonyl per BSA (see ESI†). The reduced BSA standard (RB) was then subjected to various CuAAC reaction conditions. After rapid removal of the copper and other reagents by centrifuge through size exclusion columns, the samples were immediately subjected to the enzymatic assay. The relative amount of carbonyl generated on the BSA was represented by the normalized absorbance of the sample against a reduced BSA (RB, without carbonyl, Cell Biolabs Inc.) and an oxidized BSA (OB, with ~0.5 carbonyl per BSA molecule). The results are summarized in Fig. 2.

Under similar conditions (condition a, Fig. 2) reported by Hong *et al.*[6] without the use of amino guanidine (AG) and DMSO, over 30% increase of carbonyl groups on BSA occurred in air within 1 minute. The same conditions led to 66% increase of carbonyl groups in 10 minutes. When AG and DMSO were added (condition b, Fig. 2), the increase of carbonyl groups was dropped to 30% after 10 minutes. In a control experiment, treatment of the oxidized BSA standard (OB) with 5 mM amino guanidine (AG) in 0.1 M PBS buffer (pH 7.0) for 2 h resulted in a 40% decrease of the carbonyl groups. This result can be attributed to the condensation of AG with the reactive carbonyl groups on the oxidized BSA.[14,15] It suggests that AG can be used to reduce cross-linking of the oxidized protein causing aggregation. In addition, AG and DMSO might also function as a radical scavenger.[16,17] However, even in the presence of 5 mM AG and 5% DMSO as well as the Cu(I) stabilizing ligand **4** (condition b, Fig. 2), the detectable carbonyl groups (excluding those reacted with AG) on BSA were still increased by 30% within 10 min exposure to air. Therefore, for oxidatively labile biosystems, it may be necessary to perform the current CuAAC reaction under oxygen-free conditions to eliminate oxidation.[18] Indeed, much less oxidation was found under our "oxygen-free" conditions (condition c, Fig. 2) even in the presence of the bis(triazole) ligand **5** that greatly accelerates the oxidation. The observed low degree of oxidation (8% after 1 hour and 17% after 2 hours) was likely due to the presence of trace amounts of oxygen in our anaerobic chamber, and perhaps also due to the incomplete removal of copper species before exposing the samples to air.

In conclusion, we show that a rapid oxidation of oxidatively labile proteins may still occur even when a tris(triazole) ligand is used to stabilize the Cu(I) oxidation state for the CuAAC reaction performed in air. In such cases, the CuAAC reaction and the subsequent removal of copper and ascorbate can be performed under oxygen-free conditions. Although the operation is not convenient, it brings the benefit of a large rate enhancement by using the bis(triazole) ligand **5** and the significant reduction of oxidative degradation of the biological and materials systems highly sensitive to oxidation.

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