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Electrochemically Protected Copper(I)-Catalyzed Azide–Alkyne Cycloaddition

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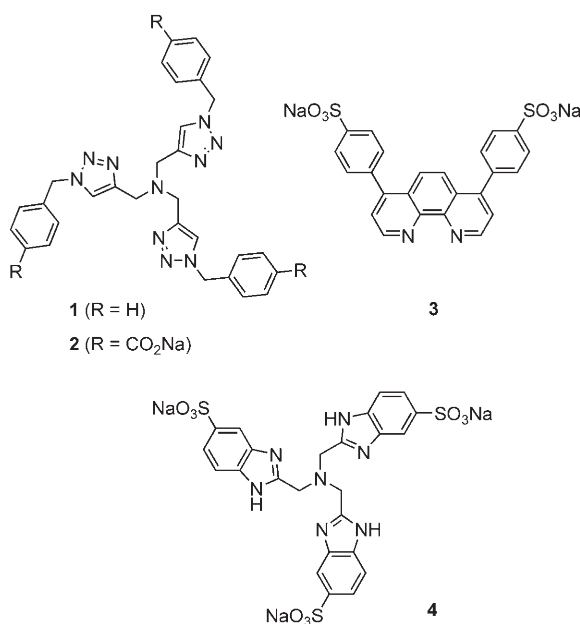
*The copper(I)-catalyzed azide–alkyne cycloaddition (CuAAC) reaction has found broad application in myriad fields. For the most demanding applications that require high yields at low substrate concentrations, highly active but air-sensitive copper complexes must be used. We describe here the use of an electrochemical potential to maintain catalysts in the active Cu^I oxidation state in the presence of air. This simple procedure efficiently achieves excellent yields of CuAAC products from both small-molecule and protein substrates without the use of potentially damaging chemical reducing agents. A new water-soluble carboxylated version of the popular tris(benzyltriazolylmethyl)amine (TBTA) ligand is also described. Cyclic voltammetry revealed reversible or quasi-reversible electrochemical redox behavior of copper complexes of the TBTA derivative (**2**; $E_{1/2}=60$ mV vs. Ag/AgCl), sulfonated bathophenanthroline (**3**; $E_{1/2}=-60$ mV), and sulfonated tris(benzimidazoylmethyl)amine (**4**; $E_{1/2}\approx-70$ mV), and showed catalytic turnover to be rapid relative to the voltammetry time scale. Under the influence of a −200 mV potential that was established by using a reticulated vitreous carbon working electrode, $CuSO_4$ and **3** formed a superior catalyst. Electrochemically protected bioconjugations in air were performed by using bacteriophage Qβ that was derivatized with azide moieties at surface lysine residues. Complete derivatization of more than 600 reactive sites per particle was demonstrated within 12 h of electrolysis with substoichiometric quantities of Cu·3.*

Introduction

The highly efficient and exquisitely selective copper(I)-catalyzed azide–alkyne cycloaddition (CuAAC)—the most widely recognized of the "click" reactions[1,2]—has been rapidly adopted since its discovery[3,4] for application in fields as diverse as surface science,[5–12] dendrimer synthesis,[13–17] polymer ligation,[18–25] combinatorial organic synthesis,[26–29] and bioconjugation.[30–38] Exemplifying its power is our use of the process to create polyvalent virus particles for diagnostic and therapeutic applications, typically requiring hundreds of attachment reactions to occur on each particle under mild and dilute reaction conditions.[39–41]



Scheme 1. Accelerating ligands that are used for CuAAC reactions.

CuAAC requires that the copper catalyst, which is usually prepared with an appropriate chelating ligand, be maintained in the air-sensitive Cu^I oxidation state, usually by the use of an in situ reducing agent such as ascorbate or tris(2-carboxyethyl)phosphine (TCEP). The most commonly used ligand is tris[(1-benzyl-1*H*-1,2,3-triazol-4-yl)methyl]amine (TBTA, **1**, Scheme 1), which was first reported by Fokin and co-workers to accelerate the reaction.[42] Partly because of its poor water solubility, bioconjugations that use **1** are slow and require a large excess of substrates (typically low mM in concentration), as well as mM concentrations of Cu^I and an excess of reducing agent. The latter two components can be detrimental to biomacromolecules: for example, ascorbate-mediated degradation of DNA[43] and copper-mediated generation of reactive oxygen species (via Cu^I or Cu^{II}) are potentially destructive side reac-

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tions,[44–46] and dehydroascorbate and other ascorbate byproducts can react with protein side chains.[47–49] Our initial solution to this problem was the identification of a more potent catalyst by using sulfonated bathophenanthroline ligand **3**,[50] a discovery that we have optimized into a robust bioconjugation protocol.[34] However, a significant drawback of this system is its acute air sensitivity, which requires air-free techniques that can be difficult to execute; for example, bubble degassing or freeze–pump–thawing can cause proteins to denature when an inert-atmosphere glove box is unavailable. Further, despite being "catalytic", efficient bioconjugations are generally only achieved in the presence of excess Cu^I complex. We introduce here a more water-soluble version of TBTA as well as the use of an electrochemical cell to generate and protect catalytically active Cu^I–ligand species for CuAAC bioconjugation and synthetic coupling reactions; this allows demanding reactions to be performed on the bench under mild conditions with minimal effort to exclude air.

Results and Discussion

The poor water solubility of ligand **1** limits its use in CuAAC reactions, particularly for bioconjugation. The *p*-carboxylate version **2** was therefore prepared and found to be substantially more soluble in water. Kinetic comparisons between **1** and **2** revealed the Cu–**2** complex to be slightly more reactive than Cu·**1** in a standard CuAAC reaction in organic solvent (data not shown). We therefore employed **2** as a surrogate for **1** in the bioconjugation experiments described below. We also included sulfonated tris(benzimidazoylmethyl)amine **4**, a fully water-soluble example of the benzimidazole class of chelating ligands, which was recently shown to provide catalysts of high activity.[51]

Cyclic voltammetry at 100 $mV s^{-1}$ in buffer (10 mм HEPES pH 8/20% DMSO/100 mм KPF_6; Figure 1 A) revealed half-wave potentials for Cu^I complexes of **3** and **4** ($E_{1/2} \approx -60$ to -70 mV; all potentials vs. Ag/AgCl) to be approximately 120–130 mV more negative than Cu·**2** ($E_{1/2} = +60$ mV); this is consistent with the electron-donating power of phenanthroline and benzimidazole relative to triazole.[52] A titration of peak current vs. metal/ligand ratio showed that the electroactive species have a 1:2 stoichiometry, which is consistent with the expected coordination environments (Supporting Information). When the chelating ligands are omitted, $CuSO_4$ gives no observable current under these conditions in the examined potential window. Plots of cathodic peak current vs. (scan rate)$^{1/2}$ for the copper–ligand complexes were linear; this indicates diffusion-controlled electron transfer with diffusion coefficients D_o of 1.1×10^{-6} $cm^2 s^2$, 2.4×10^{-6} $cm^2 s^2$, and 2.4×10^{-6} $cm^2 s^2$ for Cu·$\mathbf{2}_2$, Cu·$\mathbf{3}_2$, and Cu·$\mathbf{4}_2$, respectively (Supporting Information).

The electrochemical activity of the copper–ligand complexes suggested that the active Cu^I catalysts for solution-phase CuAAC reactions could be generated by bulk electrolysis, in accordance with studies at electrode surfaces by Collman, Chidsey, and co-workers.[53] The ability of each complex to mediate the reaction was first evaluated by using benzyl azide and phenylacetylene as substrates, with concentrations that approximated those that were typically used for CuAAC bioconjugation reactions. Electrolyses were performed in a two-compartment cell with a glass frit separating the Pt mesh counter electrode from the Ag/AgCl reference and reticulated vitreous carbon working electrodes. Product formation was monitored and quantified by LC–MS with an internal standard.

Each electrochemical reaction was conducted by first electrolyzing a buffered solution of $CuSO_4$ and ligand (1:2 molar ratio) at a potential that was just negative of the cathodic peak in the appropriate cyclic voltammogram to reduce the metal complex to the active Cu^I oxidation state. During this period the current decayed with time and reached a small steady value after 30 min (Supporting Information). For Cu·$\mathbf{3}_2$, the total number of electrons passed during this period (ca. 0.96 C) was found to be approximately four times the molar amount of O_2 that was expected to be dissolved in an aqueous solution of that volume (0.27 mм); this is consistent with the expected four-electron reduction to water that is mediated by the Cu^I complex.[54] Solutions of phenylacetylene and benzyl azide in DMSO were then added, and the reaction was monitored with maintenance of the initial applied potential. No effort was made to exclude air from the reactions other than to cap the working compartments of the cell with septa when not adding or withdrawing material.

Table 1 lists the applied potentials and yields for the electrochemical reactions, and for analogous reactions that were performed by using sodium ascorbate as the reductant. No signifi-

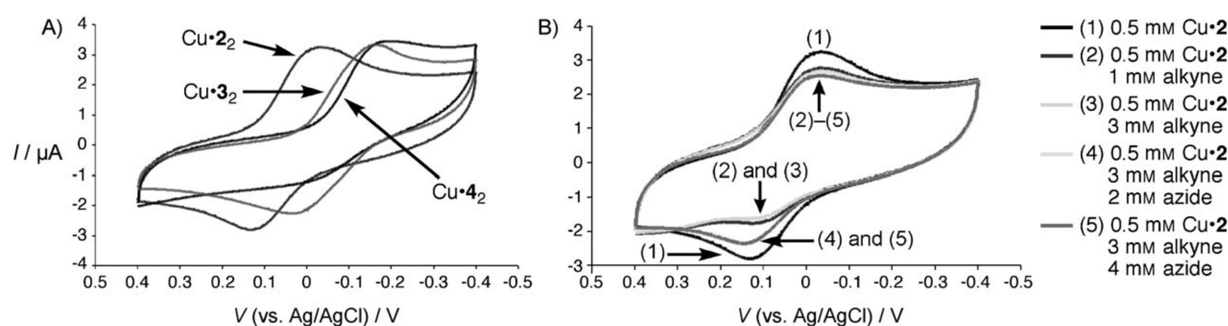

Figure 1. A) Cyclic voltammograms of the copper–ligand complexes (0.5 mм $CuSO_4$, 1 mм ligand) recorded with a glassy carbon electrode at 100 $mV s^{-1}$ in 10 mм HEPES buffer (pH 8.0), containing 20% DMSO and 0.1 м KPF_6. B) Cyclic voltammograms in the presence of the indicated reagents, added in the order indicated; conditions as in part (A).

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Table 1. Results from the reaction shown in Equation (1) as a function of ligand and applied electrochemical potential. The reported yields were obtained by quantitative LC–MS analysis of aliquots removed at the indicated times, and are the average of three independent runs (error ±5%).

Benzyl azide (N_3) 0.9 mM + phenylacetylene 0.15 mM → ($CuSO_4$ (0.1 mM), ligand (0.2 mM); 20% DMSO in 10 mM HEPES, 100 mM KPF_6, pH 8.0, $E_{applied}$ = -50 or -200 mV vs. Ag/AgCl) → 1-benzyl-4-phenyl-1,2,3-triazole (1)

	Reductant	Copper	Ligand	Yield [%] 15 min	Yield [%] 60 min
1	E, none[a]	none	none	1	1
2	none	$CuSO_4$	none	3	3
3	E, none[a]	$CuSO_4$	any	3	3
4	ascorbate[b]	$CuSO_4$	none	4	8
5	ascorbate[b]	$CuSO_4$	**2**	82	97
6	ascorbate[b]	$CuSO_4$	**3**	68	68
7	ascorbate[b]	$CuSO_4$	**4**	98	99
8	E, −50 mV	$CuSO_4$	none	3	5
9	E, −200 mV	$CuSO_4$	none	4	8
10	E, −50 mV	$CuSO_4$	**2**	58	84
11	E, −200 mV	$CuSO_4$	**3**	98	99
12	E, −200 mV	$CuSO_4$	**4**	6	42

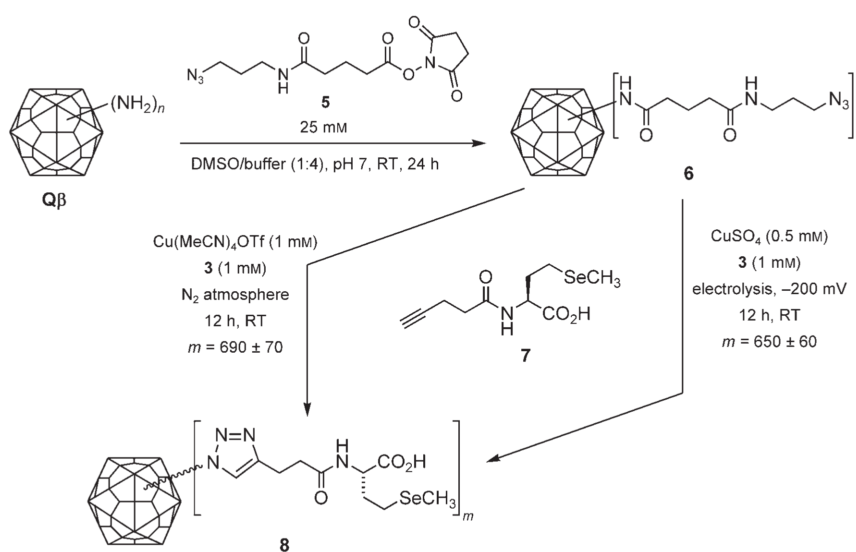
[a] In the electrochemical cell with electrodes disconnected. [b] 1 mM sodium ascorbate.

cant triazole formation occurred in the absence of copper or in the presence of only Cu^{II} (entries 1–3). As noted previously,[50] even in the presence of ascorbate, Cu^{I} without accelerating ligands was insufficiently active to provide substantial amounts of triazole at the dilute concentrations that were tested (entries 4, 8, and 9). Cu·**3** proved to be the most potent system under the electrochemical conditions, giving complete conversion within 15 min (entry 11 vs. 10 and 12). In contrast, ligands **2** and **4** provided better yields in the presence of ascorbate. This is due to the much faster reaction of Cu·**3** with atmospheric oxygen, which depletes the ascorbate pool and deactivates the catalyst within several minutes, as indicated by the yields and the loss of the characteristic color of the Cu^{I} complex of **3**. In separate experiments, Cu·**3** also provided a rare example of catalytic performance under the dilute conditions that are typical of bioconjugation reactions: 200 μM Cu·**3** with 5 mM benzyl azide and 5 mM phenylacetylene produced the 1,4-triazole product in 90% yield after 3 h.

Electrochemical monitoring also proved to be informative about the reaction mechanism. In the presence of ligand **2** and phenylacetylene, the oxidation half-wave of the Cu·**2** complex was diminished relative to the reduction wave (Figure 1B); this suggests that formation of the Cu^{I}–acetylide complex is fast and that the redox potential of this species lies outside of the electrochemical window. When benzyl azide was added, the oxidation wave returned to give the reversible couple characteristic of $[Cu·\mathbf{2}]^{I/II}$, at scan rates up to 500 $mV\,s^{-1}$. This behavior indicates that the triazole-forming catalytic turnover steps are also fast relative to scan rate, and it establishes Cu·**2** as the resting state of the catalyst under these turnover conditions.

We tested the highly active Cu·**3** electrochemical system in a bioconjugation reaction that involved bacteriophage Qβ, an icosahedral virus comprising 180 copies of a 14.1-kDa coat protein.[55] We have previously employed the noninfectious capsid as a robust scaffold for the display of gadolinium complexes, carbohydrates, and other species;[40,41] these were attached with the use of $[Cu·\mathbf{3}_2]$OTf under strictly inert atmosphere conditions in a glove box. The polyvalent CuAAC substrate **6** was prepared by acylation of surface lysine and N-terminal amine groups (4 per subunit; 720 per particle) with a large excess of azido *N*-hydroxysuccinimide ester **5** (Scheme 2). The 4-pentynoyl amide of selenomethionine (**7**) was used as the alkyne component to give polytriazole adduct **8**. After purification, the amount of selenium that was attached to the protein was measured by inductively coupled plasma optical emission spectroscopy (ICP-OES). This technique replaces our normal use of dyes such as fluorescein, which can suffer from batch-to-batch differences in purity of the commercial reagent, occasional changes in molar absorptivity upon protein attachment, and particle instability for some capsids.[56] Although ICP-OES is not as convenient as UV/visible absorption spectroscopy, it is highly sensitive and has the advantage of being unaffected by the chemical environment of the probe element(s).

The benchmark for comparison is our previously established CuAAC bioconjugation method by using the Cu^{I} complex Cu-



Scheme 2. Electrochemically protected CuAAC bioconjugation with substrates **6** (2 $mg\,mL^{-1}$, approximately 400 μM in azide) and **7** (2.5 mM).

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$(MeCN)_4OTf$ (0.5 mM) and **3** (1 mM) in a nitrogen-atmosphere glovebox.[34] Under these conditions, 690±60 molecules of **7** were attached, which represents complete loading of the particle. The electrochemical reaction that is shown in Scheme 2 was initiated by prereduction of a 1:2 mixture of $CuSO_4$/**3** at −200 mV for 30 min to form the active Cu^I catalyst. This species provided a convenient visual report on its oxidation state: because the Cu^I complex of **3** is deep green, whereas the Cu^{II} complex is pale yellow. The virus-azide (**6**) and **7** were then added, and the electrochemical potential (and characteristic color of Cu^I·**3**) was maintained throughout the course of the overnight reaction. The product virus-like particles were purified by ultracentrifugation through a sucrose gradient, the band that corresponded to the intact capsids was isolated, concentrated by ultrapelleting, and resuspended in the desired buffer. Good yields (>70%) of labeled particles were obtained that bore 650±60 SeMet-alkynes per capsid, which is within the experimental error of the glovebox reaction. Notably, when catalytic quantities of Cu·$\mathbf{3}_2$ precatalyst (0.25 mM) were used, comparable loadings were obtained after 12 h (620±60 per particle). The decrease in the required copper–ligand concentration is generally desirable as it facilitates purification and minimizes potentially detrimental protein–catalyst interactions (although we find that the presence of the chelating ligand shields most proteins from copper complexation). The recovered Qβ protein was exclusively in the form of intact icosahedral capsids, as shown by size-exclusion chromatography and transmission electron microscopy (Supporting Information).

Conclusions

The oxidized products from ascorbic acid such as dehydroascorbate are potentially reactive with biomacromolecules.[43,48,49] The use of ascorbate as a reducing agent for the generation of the active Cu^I catalysts for CuAAC reactions will not always be compatible with bioconjugation applications,[57] since the oxidized products of ascorbate are potentially reactive with biomacromolecules. The electrochemical protocol described here provides an efficient and facile way to perform CuAAC reactions when chemical reducing agents cannot be employed, thereby eliminating the need for manipulations under an inert atmosphere it simplifying the product purification. This is especially important for the highly active complex of Cu^I and bathophenanthroline ligand **3**, which provides superior performance in bioconjugation at the cost of extra acute air sensitivity, owing to its more reducing $Cu^{I/II}$ redox potential.[34] We also highlight the use of two other water-soluble accelerating ligands, including a more hydrophilic version of the popular tris(triazolyl)methyl TBTA structure. The successful bulk syntheses of both small-molecule and larger-biomolecule triazole conjugates by using these ligands, under the protective umbrella of a reducing electrochemical potential, provides a broadly applicable method for CuAAC ligation in aqueous environments.

Experimental Section

Ligands and linker 4: Compounds **1**,[42] **4**,[51] and **5**[30] were prepared as previously reported; **3** was commercially available. *Note:* We found occasional batches of **3** to be contaminated with substantial quantities of NMR-silent material (presumably inorganic salts) as received. The problem was detected by quantitative NMR (adding a known quantity of an internal standard), and resolved by recrystallization. Ligand **2** was prepared by the reaction of tripropargylamine with *p*-azidomethylbenzoic acid under similar conditions as reported for **1**. After the azide–alkyne cycloaddition reaction was completed, the highly water soluble carboxylate salt form of the ligand was precipitated by acidification with glacial AcOH. If further purification was desired, the material could be boiled in MeOH or dissolved in aqueous base and reprecipitated with AcOH. Synthetic details can be found in the Supporting Information.

Protein: Expression and purification of the Qβ coat protein from a recombinant plasmid has been previously described.[58] Briefly, a 135-amino-acid version of the Qβ coat protein gene was cloned into the vector pQE-60 and expressed under IPTG control in M15MA cells in SOB media. After expression, collected cells were lysed by sonication and lysozyme treatment, and then centrifuged to remove insoluble cell components. The virus-like particles were precipitated from the resulting supernatant by using 8% PEG 8000. Following further centrifugation, the isolated pellet was resuspended in 0.1 M K_3PO_4 (pH 7.0). The capsid then underwent a final purification by ultracentrifugation through a 10–40% sucrose gradient followed by ultrapelleting and resuspension in 0.1 M K_3PO_4 (pH 7.0). Qβ concentrations were determined by using the Modified Lowry Protein Assay (Pierce).

Qβ that bore azides at surface-exposed lysine residues (**6**) was prepared by incubating a 10 mg mL^{-1} solution of Qβ with 25 mM **5** (35-fold excess with respect to protein subunit) in 0.1 M K_3PO_4 buffer (pH 7) with 20% DMSO for 12 h. The derivatized virus was separated from excess reagent by ultracentrifugation by using a 10–40% sucrose gradient, the protein band that corresponded to intact virions was isolated and concentrated by subsequent ultrapelleting and solvation in 20 mM HEPES buffer (pH 8). Mass recoveries of derivatized virus were typically 60–80%. Size-exclusion fast protein liquid chromatography (FPLC, Superose-6 column) indicated that >95% of the recovered virus was composed of intact particles.

For the reference bioconjugation reaction, a solution of Qβ–azide **6** (2.0 mg mL^{-1}, 140 μM in protein subunits, 560 μM in azide groups) and alkyne **7** (2.5 mM) was prepared under a N_2 atmosphere in a glove box (Vacuum Atmospheres, Inc., Hawthorne, CA, USA). A solution of $Cu(MeCN)_4(OTf)$ and ligand **3** in a 1:2 molar ratio was added to initiate the reaction, which had a final volume of 1 mL, and a final copper concentration of 0.5 mM. The reaction was agitated by gentle tumbling overnight at room temperature under N_2, and the resulting conjugate **8** was purified and characterized as above. Reaction yields were quantified by comparing protein concentration (Lowry assay) to the concentration of Se that was determined by ICP-OES and was calibrated with reference standards (10–10000 ppb Se) in the presence of a constant quantity of $YbCl_3$ as an internal standard. The error limits given for loading values were derived from repeated independent experiments and reflected mostly the uncertainties in protein concentration that was determined by the Lowry assay.

Cyclic voltammetry: Electrochemical measurements were performed with an Epsilon workstation (Bioanalytical Systems, Inc., BAS, West Lafayette, IN, USA), by using glassy carbon working

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(area 0.07 cm^2) and Ag/AgCl reference electrodes. The surface of the working electrode was prepared by polishing with a 0.3 μm alumina slurry followed by brief sonication in H_2O and drying in air. Voltammetry experiments were performed in a two-compartment cell with a Luggin capillary separating the working electrode and platinum wire counter electrode from the reference. All voltammetry experiments were performed under argon in thoroughly degassed buffer (100 mM KPF_6, 10 mM HEPES, pH 8, 20% DMSO).

Bulk electrode-driven CuAAC: Electrolyses were conducted in a two-compartment cell in air with a glass frit separating the platinum gauze counter electrode from the reticulated vitreous carbon working and Ag/AgCl reference electrodes. For CuAAC of phenylacetylene and benzyl azide, reactions were initiated by placing 0.1 mM $CuSO_4$ (10 mL) into the working chamber with ligands **2**, **3**, or **4**, and [D_7]1-benzyl-4-phenyl-1,2,3-triazole (internal standard) in buffer (100 mM KPF_6, 25 mM HEPES, pH 8, 20% DMSO). Electrolyses at the appropriate potential (Table 1) were then conducted for 30 min. Solutions of phenylacetylene and benzyl azide in DMSO were then added to final concentrations of 0.15 mM and 0.9 mM, respectively, and the applied potential was maintained throughout the reaction. Aliquots (5 μL) were taken every 15 min and diluted with EtOH (1.5 mL) for LC–MS analysis on an Agilent 1100 (G1946D) instrument, that was equipped with a 35 mm Agilent Zorbax 1.8 micron SB-C18 column. The elution solvent for the detection of 1-benzyl-4-phenyl-1,2,3-triazole was H_2O/CH_3CN (55:45), with 0.5% trifluoroacetic acid. Detection was performed in single-ion mode (SIM) with [D_7]1-benzyl-4-phenyl-1,2,3-triazole as internal reference for all experiments.

Bioconjugation reactions were conducted in air by first placing into the working chamber $CuSO_4$/**3** (1:2, 8 mL, 0.5 mM with respect to copper) in buffer (100 mM KPF_6, 25 mM HEPES, pH 8). Electrolysis was then performed for 30 min to generate the active Cu^I oxidation state. A total of 2 mL of virus and substrate solutions were added, to give final concentrations of 2 $mg\,mL^{-1}$ virus and 2.5 mM **7**. The chambers of the cell were capped loosely by septa when reagents were not being introduced or withdrawn. The electrochemical potential was applied during the 12 h reaction period, with a low, steady-state passage of current noted throughout. The current was presumably due to the reduction of Cu^{II} to Cu^I, the former was generated by oxidation of reduced metal by oxygen diffusing into the reaction mixture. No traces of reactive oxygen species such as peroxide, superoxide, or singlet oxygen were detected by standard tests; this was consistent with the known electrochemically driven four-electron reduction of O_2 to water by Cu-phenanthroline.[54] Furthermore, no decomposition of ligands, substrates, or products was observed by LC–MS. Virus particles were purified from the reaction mixture and analyzed as described above.

Acknowledgements

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