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Bioconjugation by Copper(I)-Catalyzed Azide-Alkyne [3 + 2] Cycloaddition

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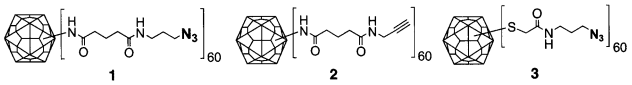
Azides and alkynes are highly energetic functional groups with particularly narrow distributions of reactivity. Thanks to their weak acid−base properties, they are nearly inert toward biological molecules and toward the reaction conditions found inside living cells. At the same time, azide and alkyne groups are easy to introduce into organic compounds by both nucleophilic and electrophilic processes. One may therefore envision their incorporation into biological molecules by organic synthesis and chemical conjugation (or via biosynthetic pathways using designed precursors[1]) to create unique points of addressable reactivity in large and complex targets.[2] The irreversible Huisgen cycloaddition of azides and alkynes,[3] thermodynamically favorable by approximately 30−35 kcal/mol, is here applied to a prototypical biomolecular target.

We have previously induced an enzyme (acetylcholinesterase) to lower the activation barrier for an azide-alkyne cycloaddition and thereby synthesize its own femtomolar triazole inhibitor.[4] In that case, the rate of the reaction in the absence of enzyme was negligible, allowing for selectivity of the shape of the protein binding pocket to be obtained. For the quite different purpose of addressing labeled positions on a large protein structure, the ligation reaction must be rapid and highly selective near room temperature. These requirements are met by our recent findings that copper(I) species in aqueous solution are powerful catalysts for the formation of 1,2,3-triazoles from azides and terminal alkynes.[5]

Cowpea mosaic virus (CPMV) was used as the protein component for our studies. As a biomolecular scaffold, it is superb, being a structurally rigid assembly of 60 identical copies of a two-protein asymmetric unit around a single-stranded RNA genome, conveniently available in gram quantities. We have reported on the derivatization of this species by a combination of chemical and genetic methods, highlighting its resemblance to synthetic dendrimers.[6] Thus, the exterior surface of the coat protein of CPMV was decorated with azides or alkynes at either reactive lysine or cysteine residues, giving labeled particles **1**−**3**.



The efficiency of coupling to virus-azide and virus-alkyne conjugates was assayed with fluorescein derivatives containing complementary groups for the desired cycloaddition (Scheme 1). Table 1 presents a survey of reaction conditions involving **1**−**3** and the dye-alkynes **4** and **5**. Coupling does not occur in the absence of copper (entry 1) or reducing agent (entry 2), demonstrating the requirement for Cu in the +1 oxidation state.[5a] Although elemental copper serves as a convenient reductant for Cu(II) in synthetic organic applications of the process, here its use with the $CuSO_4$ precursor gives a sluggish reaction (entry 3). Addition of tris(triazolyl)amine **6** greatly enhances the reaction rate,[7] such that all of the virus-borne azide groups are converted to corresponding triazoles (entry 4). Tris(carboxyethyl)phosphine (TCEP), a water-soluble reducing agent used to protect cysteine residues in proteins from oxidative coupling, also allows the reaction to proceed in the presence of **6** (entry 5) but not in its absence (entries 6 and 7). The potential role of **6** is discussed below.

Scheme 1

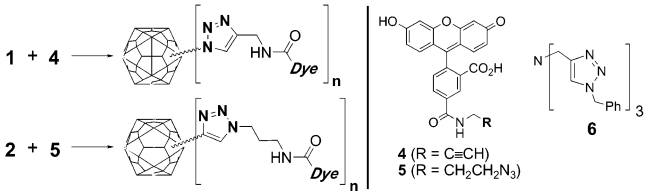


Table 1. [3 + 2] Cycloaddition Reactions of Virus-Azides/Alkynes **1**−**3** with Dye-Alkyne **4** and Dye-Alkyne **5**[e]

entry	reagents[a]	$CuSO_4$ (mM)	**6** (mM)	TCEP (mM)	Cu wire	loading[b]	yield[c]
1	**1** + **4**		2.0	2.0	−	<1 (<2%)	94%
2	**1** + **4**	1.0	2.0		−	<1 (<2%)	80%
3	**1** + **4**	1.0			+	23 (22%)	87%
4	**1** + **4**	1.0	2.0		+	60 (100%)	94%
5	**1** + **4**	1.0	2.0	2.0	−	60 (100%)	96%
6	**1** + **4**	1.0		2.0	+	17 (29%)	95%
7	**1** + **4**	1.0		2.0	−	10 (17%)	94%
8	**1** + **4**	1.0	2.0	2.0	+	60 (100%)	86%
9	**1** + **4**	1.0	2.0		+	22 (37%)[d]	87%
10	**1** + **4**				−	2 (3%)	100%
11	**2** + **5**	1.0	2.0		+	10 (17%)	96%
12	**2** + **5**	2.0	2.0	5.0	−	48 (80%)	80%
13	**2** + **5**	2.0	2.0	5.0	+	43 (71%)	75%

[a] Replacing virus **1** with **3** gave identical results within experimental error for all reactions. [b] Number of dye molecules attached per virion (percentage); average of three independent reactions, error range is ±10% of the reported value (for example, 60 ± 6 or 10 ± 1 dyes per particle). [c] Overall recovery of derivatized virus; error is ±5%. [d] pH 7.0. [e] **1**−**3** at 2.0 mg/mL (360 nM in virus particles, 21.4 μM in viral protein and reactive groups); **4** at 2.5 mM, 117 equiv); **5** at 5.0 mM, 234 equiv; reactions in pH 8.0 potassium phosphate buffer containing 5% *tert*-butyl alcohol, 4 °C for 16 h.

Lowering the pH from 8.0 to 7.0 slows the reaction (entry 9 vs 4), suggesting that a threshold concentration of Cu(I)-acetylide intermediate is required. Switching the "polarity" of the process − in which a virus-alkyne (**2**) is addressed by an excess of azide **5** − is less favorable (entry 11 vs 4). However, when higher concentrations of the other components are used, the reaction rate is increased to a useful range (entries 12 and 13). The dependence of the process on the concentration of Cu(II) in solution is shown in Figure 1. Under the otherwise standard conditions described in Table 1, coupling activity is supported better by copper wire than by TCEP as the reducing agent, but the reaction still requires a minimum of 0.5 mM $CuSO_4$ to maintain high efficiency.

Preservation of the folding state of the protein is a consideration not encountered in abiological applications of the azide-alkyne ligation, and two aspects of the standard procedure proved to be important in this regard. First, the use of ascorbate or *p*-hydro-

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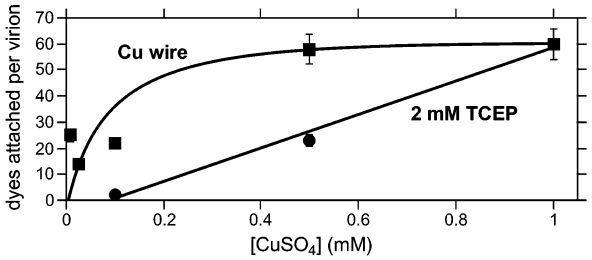

Figure 1. Reaction of **1** (2.0 mg/mL) and **4** (2.5 mM) in the presence of **6** (2 mM), as a function of reducing agent and Cu(II) concentration (pH 8.0, 4 °C, 16 h).

quinone reductants was found to induce substantial disassembly of the virus capsid. Second, while CPMV itself is stable to 20 mM Cu(II) and 10 mM Cu(I) in the presence of any combination of TCEP, alkyne, and azide, the formation of capsid-bound triazoles in the presence of copper(II) leads to virus decomposition.[8] Ligand **6** protects the virus from Cu-triazole-induced disassembly.

Some mechanistic considerations are relevant, in the context of those already published.[5a] We find that ligand **6** strongly inhibits Cu(II)-catalyzed oxidative coupling of terminal alkynes to diynes under otherwise standard conditions,[9] suggesting that it serves the important function of stabilizing the Cu(I) oxidation state in water. TCEP is a competent reducing agent for Cu(II) and reacts only very slowly with aliphatic azides, thus proving to be a useful additive, but it also binds to copper centers.[8] High TCEP/Cu ratios could therefore be inhibitory (Figure 1). The fact that the reaction of virus-azides with an excess of small-molecule alkyne gives somewhat better results than the converse suggests that the proposed[5a] Cu-acetylide intermediate may suffer an alternative decomposition pathway that becomes more important at low concentrations. However, the crucial point is that copper(I) activates alkynes toward azides (and other dipolar reagents[10]) but not toward anything else, so that selective ligations can be performed in complex biological environments.

The virus scaffold provides a demanding test of the ligation process in that a large number of attachments to each particle are required and are realized. The rates of Cu(I)-catalyzed azide-alkyne cycloadditions are shown here to be comparable to those of cysteine-maleimide reactions, heretofore the most rapid and reliable connections to virus capsids.[6,11] In addition to accelerating the bond-forming process, tris(triazolyl)amine **6** serves to protect proteins from the potential harmful effects of soluble copper species and may be a prototype for a new class of ligands.[7] We anticipate that the azide-alkyne ligation methodology should be applicable to a wide variety of biomolecules,[12] scaffolds,[5b] and cellular components, both in vitro and in vivo.

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Supporting Information Available: Experimental details; further information concerning studies of virus stability in the presence of the reaction components (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) (a) Kiick, K. L.; Saxon, E.; Tirrell, D. A.; Bertozzi, C. R. *Proc. Natl. Acad. Sci. U.S.A.* **2002**, *99*, 19−24. (b) Chin, J. W.; Santoro, S. W.; Martin, A. B.; King, D. S.; Wang, L.; Schultz, P. G. *J. Am. Chem. Soc.* **2002**, *124*, 9026−9027.

(2) (a) Lemieux, G. A.; Bertozzi, C. R. *TIBTECH* **1998**, *16*, 506−513. (b) Saxon, E.; Armstrong, J. I.; Bertozzi, C. R. *Org. Lett.* **2000**, *2*, 2141−2143. (c) Saxon, E.; Bertozzi, C. R. *Science* **2000**, *287*, 2007−2010.

(3) Huisgen, R. In *1,3-Dipolar Cycloaddition Chemistry*; Padwa, A., Ed.; Wiley: New York, 1984; Chapter 1, pp 1−176.

(4) Lewis, W. G.; Green, L. G.; Grynszpan, F.; Radic, Z.; Carlier, P. R.; Taylor, P.; Finn, M. G.; Sharpless, K. B. *Angew. Chem., Int. Ed.* **2002**, *41*, 1053−1057.

(5) (a) Rostovtsev, V. V.; Green, L. G.; Fokin, V. V.; Sharpless, K. B. *Angew. Chem., Int. Ed.* **2002**, *41*, 2596−2599. (b) Tornøe, C. W.; Christensen, C.; Meldal, M. *J. Org. Chem.* **2002**, *67*, 3057−3062.

(6) (a) Wang, Q.; Lin, T.; Tang, L.; Johnson, J. E.; Finn, M. G. *Angew. Chem., Int. Ed.* **2002**, *41*, 459−462. (b) Wang, Q.; Kaltgrad, E.; Lin, T.; Johnson, J. E.; Finn, M. G. *Chem. Biol.* **2002**, *9*, 805−811. (c) Wang, Q.; Lin, T.; Johnson, J. E.; Finn, M. G. *Chem. Biol.* **2002**, *9*, 813−819.

(7) We were alerted to the beneficial effects of tris(triazolyl)amine ligands by the observation of accelerating reaction rates (autocatalysis) during their preparation by the copper-catalyzed cycloaddition. Compound **6** was designed based on this observation and is a particularly powerful facilitator of this process. A full account of the development, coordination chemistry, and applications of multidentate triazoles and related ligands will be presented elsewhere.

(8) See Supporting Information.

(9) (a) Campbell, I. D.; Eglinton, G. *Org. Synth.* **1965**, *45*, 39−42. (b) Schenning, A. P. H. J.; Arndt, J.-D.; Ito, M.; Stoddart, A.; Schreiber, M.; Siemsen, P.; Martin, R. E.; Boudon, C.; Gisselbrecht, J.-P.; Gross, M.; Gramlich, V.; Diederich, F. *Helv. Chim. Acta* **2001**, *84*, 296−334.

(10) Hilgraf, R.; Rostovtsev, V. V.; Fokin, V. V.; Sharpless, K. B., unpublished results.

(11) The Diels−Alder reaction has been employed similarly [Yousaf, M. N.; Chan, E. W. L.; Mrksich, M. *Angew. Chem., Int. Ed.* **2000**, 39, 1943−1946. Pozsgay, V.; Vieira, N. E.; Yergey, A. *Org. Lett.* **2002**, *4*, 3191−3194], but azides and alkynes are both easier to prepare and more stable than dienes and activated dienophiles.

(12) Fazio, F.; Bryan, M. C.; Blixt, O.; Paulson, J. C.; Wong, C.-H. *J. Am. Chem. Soc.* **2002**, *124*, 14397−14402.

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