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

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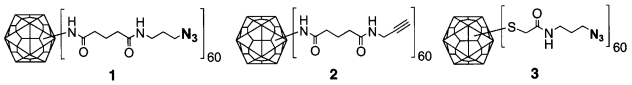
Azides and alkynes are highly energetic functional groups with particularly narrow distributions of reactivity. Thanks to their weak acid−base properties, they are nearly inert toward biological molecules and toward the reaction conditions found inside living cells. At the same time, azide and alkyne 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

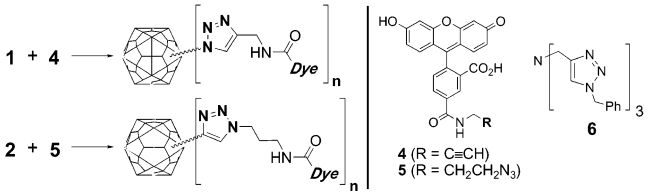


Table 1. [3 + 2] Cycloaddition Reactions of Virus-Azides/Alkynes **1**−**3** with Dye-Alkyne **4** and Dye-Alkyne **5**[e]

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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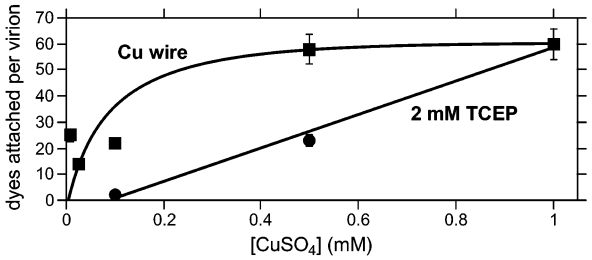

Figure 1. Reaction of **1** (2.0 mg/mL) and **4** (2.5 mM) in the presence of **6** (2 mM), as a function of reducing agent and Cu(II) concentration (pH 8.0, 4 °C, 16 h).

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.

Acknowledgment. We thank The David and Lucille Packard Foundation (#2001-17733), the NIH (EB00432, N01-C0-27181), and The Skaggs Institute for Chemical Biology for support of this work. R.H. thanks DAAD, Swiss National Science Foundation, and Novartis Foundation for financial support. We are also grateful to Mr. Peng Wu for the preparation of dye-azide and dye-alkyne reagents, and to Profs. John E. Johnson and Tianwei Lin for sharing their expertise with CPMV.

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.

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(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.

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(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.

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Supporting Information

A. Materials and Methods.

1. General.

All reagents chemicals were used as received. The viruses were prepared using the previously described methods; the cysteine-containing mutant virus employed is the one previously designated VEF*Cys*B.[1,2] Unless otherwise noted, "buffer" refers to 0.1 M potassium phosphate buffer, pH 7.0. The virus was stored in buffer at a concentration of about 10 mg/mL. Size exclusion columns for purification of virus from reaction mixtures were prepared by pre-swelling Bio-Gel® P-100 Gel (from BioRad, 23 g) in buffer (400 mL) and loading the gel into Bio-Spin® disposable chromatography columns (from BioRad). For 80 μL of virus solution (1 mg/mL), approximately 1 mL of the prepared gel is required. Sucrose gradient ultracentrifugation separation of virus samples was performed on a 30 mL gradient [made of 20% (w/w) sucrose solution in buffer, frozen at -20°C and defrosted just before use] with centrifugation at 38,000 rpm for 3 hours with Beckman SW41 Ti rotor, giving rise to well-separated bands. Virus concentrations were measured by absorbance at 260 nm; virus at 0.1 mg/mL gives a standard absorbance of 0.8. The average molecular weight of the CPMV virion is 5.6×10^6.

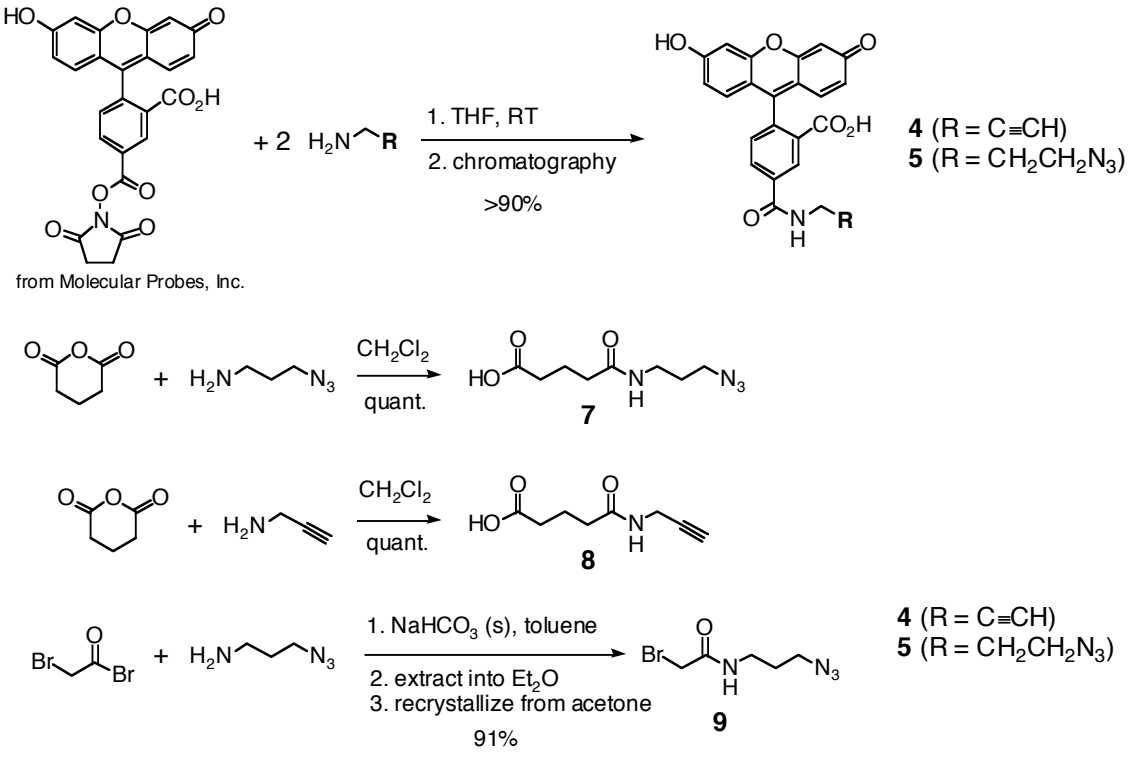
2. Electron Microscopy.

TEM analyses were performed by depositing 20 μL aliquots of each sample onto 100-mesh carbon-coated copper grids for 2 minutes. The grids were then stained with 20 μL of 2% uranyl acetate and viewed with Philips CM100 electron microscope. ^{1}H and ^{13}C NMR spectra were measured on a Varian-300 or Brucker AMX-500 spectrometer as indicated. FPLC analyses were performed with AKTA Explorer (Amersham Pharmacia Biotech) equipment, using Superose™-6 size-exclusion or Hitrap-Q ion-exchange columns. In the former case, 0.05 M potassium phosphate buffer (pH 7.0) was used as eluent; intact virions show retention times of approximately 25 minutes at an elution rate of 0.4 mL/min, whereas broken particles and individual subunit proteins elute only after 50-60 minutes.

3. Reagents

(a) Compounds **4**, **5**, and **7**–**9** were prepared in high yield by the condensation reactions shown in Scheme S1.

Scheme S1.



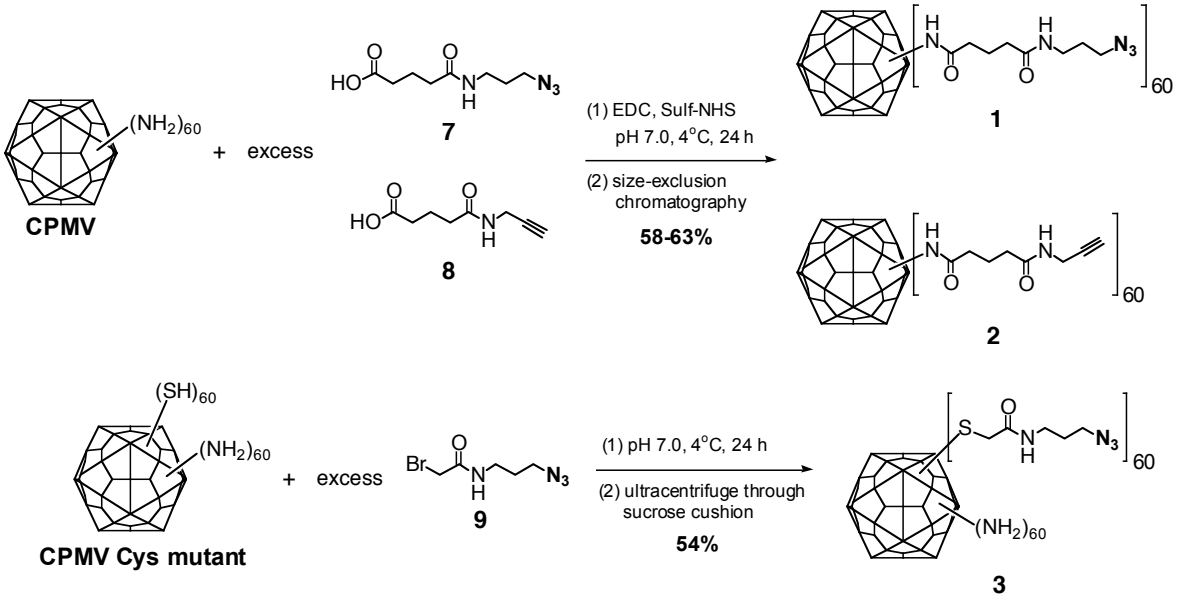
(b) Tris(triazolyl)amine **6**. Tripropargylamine (0.98 g; 7.5 mmol) in acetonitrile (10 mL) was treated sequentially with benzylazide (197.0 μL; 26.3 mmol), 2,6-lutidine (874 μL; 7.50 mmol) and $Cu(MeCN)_4PF_6$ (0.141 g; 0.378 mmol, 1.7 mol% with respect to total alkyne units). Upon addition of the copper salt, the reaction warmed and was cooled in an ice bath. After stirring at room temperature for two days, a white crystalline solid precipitated from the reaction. Acetonitrile (4 mL) was added to the entire reaction mixture, which was then heated and allowed to cool to room temperature to induce precipitation of the desired compound. Filtration and washing with cold acetonitrile afforded fine, white, needle-like crystals (1.71 g; 43%). A second crop was obtained by evaporation of the solvent and recrystallization of the residue from hot acetonitrile (0.57 g; 14%). Mp 138-139 °C. ^{1}H NMR ($CDCl_3$): δ 3.70 (s, 6 H, N-CH_2-triazole), 5.49 (s, 6 H, $PhCH_2$), 7.26 (m, 6 H), 7.34 (m, 9 H), 7.67 (s, 3H, triazole). ^{13}C NMR ($CDCl_3$): δ 134.7, 129.1, 128.7, 128.0, 123.8, 54.1, 47.0. Electrospray mass spectrum: (m/z) 531 (MH+). Anal. Calcd for $C_{30}H_{30}N_{10}$: C, 67.90; H, 5.70, N, 26.40. Found: C, 67.71; H, 5.73; N, 26.32.

(c) CPMV alkyne and azide conjugates.

Wild-type CPMV was decorated with 60 azides or alkynes by the trapping of the sulfo-N-hydroxysuccinimide esters of **8** and **9**, generated *in situ*, with the uniquely reactivity lysine (K38) on the exterior surface of the virus coat protein (capsid),[1] giving virions **1** and **2** (Scheme S2). These reactions are directly analogous to our previously reported use of isolated NHS esters and the loading of 60 functional groups on the virion was substantiated by established methods.[1] Experimental details appear below. While carboxylate residues on

the exterior surface of CPMV may be activated by excess carbodiimide/N-hydroxysuccinimide in this procedure, the resulting NHS ester residues should be harmlessly hydrolyzed since there are no available amines at pH 7 to trap them. In addition, a CPMV mutant containing cysteine inserted in the solvent-exposed E-F loop of the large protein subunit[2] was alkylated by bromoacetamide **10** to give structure **3**, again bearing 60 azide groups per virus particle.

Scheme S2.



WT-CPMV-alkyne 1. Wild-type CPMV (20 mg) was added to a mixture of **7** (42.8 mg, 0.2 mmol, 1000-fold excess relative to the viral asymmetric unit), 1-ethyl-3-(3-dimethlaminopropyl)-carbodiimide hydrochloride (EDC, 191.7 mg, 1 mmol) and *N*-hydroxysulfosuccinimide (sulfo-NHS, 86.8 mg, 0.4 mmol) in 4 mL buffer, and the reaction mixture was incubated at 4 ºC for 48 h. After dialysis in buffer (4L) to eliminate most of the excess small-molecule reagents, the product was purified by ultracentrifugation at 42,000 rpm over a 2 cm 40% (w/w) sucrose cushion, and the resulting transparent pellets were redissolved in 2 mL buffer. The concentration of virus was determined by OD_{260} as 5.8 mg/mL; yield 58%. The intact nature of the derivatized viral particles was determined by analytical size-exclusion FPLC.

WT-CPMV-azide 2. Performed as above using wild-type CPMV and **8**, giving a yield of 63% of recovered derivatized virus particles.

Mutant CPMV-azide 3. VEF*Cys*B (17 mg) and **9** (17.6 mg, 0.1 mmol) in an 80:20 mixture of buffer and DMSO (total 8 mL) were allowed to stand at 4 ºC for 48 h. Initial separation of virus from reagent was accomplished by the addition of another 8 mL of buffer to precipitate the organic reagent, and centrifugation of the mixture at 16,000 *g* for 10 min. The supernatant was further purified by ultracentrifugation at 42,000 rpm over a 2 cm 40% (w/w)

sucrose cushion, and the resulting transparent pellets were redissolved in 0.5 mL buffer. The concentration of virus was determined by OD_{260} as 18.5 mg/mL; yield 54%.

4. "Click reactions" on derivatized viruses

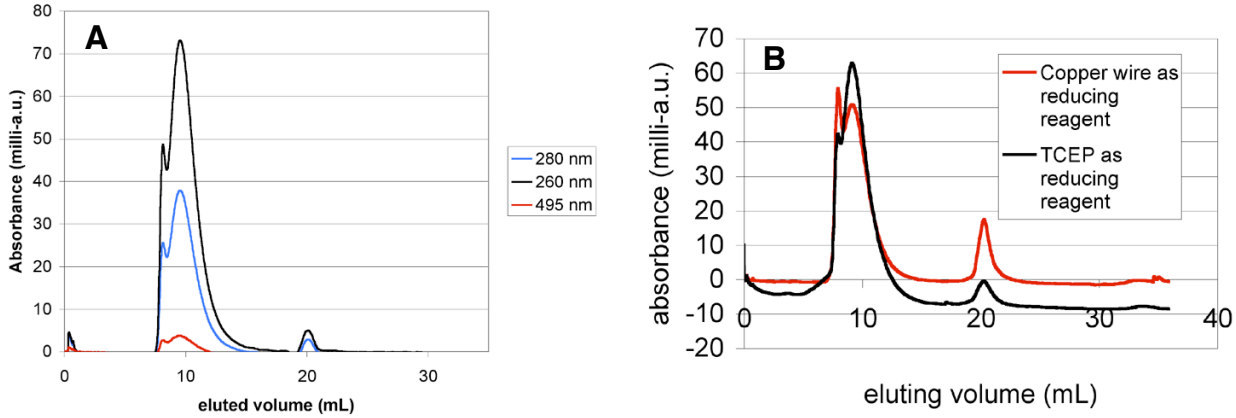
(a) Procedure A, TCEP as reducing reagent. Virus conjugates **1** or **3** (200 μg) and dye-alkyne **4** (0.25 mmol) were added to a solution of tris(triazolyl)amine **6** (2 mM) and TCEP (2 mM) in a mixture of 0.1 M potassium phosphate buffer (pH 8.0, 95 μL) and *tert*-butanol (5 μL) at 4°C. $CuSO_4$ was then added to a concentration of 1 mM. Following incubation at 4 °C for 16 h, the mixture was purified by passage through a P-100 size exclusion column (centrifugation at 800 *g* for 6 min). This filtration was repeated with fresh columns until all the excess reagents were removed (typically twice). The recovery of derivatized viruses was usually about 90% with 60 copies of dyes loaded per virion (determined as described below); all such samples were composed of >95% intact particles (determined by analytical size-exclusion FPLC and TEM). Virus concentrations were determined by measuring the absorbance at 260 nm, and dye concentrations were obtained by measurement of absorbance at 495 nm, with molar absorbtivity calibrated for each use by mixing known quantities of dye with CPMV (1 mg/mL).

(b) Procedure B, copper wire as reducing reagent. Performed analogously to procedure A, using a short length of copper wire instead of TCEP as the reducing agent. These reactions required approximately 24 h to reach completion, and the resulting samples contained a larger percentage of aggregated virus particles (40-50%), detected both by size-exclusion FPLC and TEM.

B. Characterization and Virus Stability

1. Representative FPLC and TEM data

Figure S1. Size-exclusion FPLC of products (Superose-6 column). (A) Product of coupling of **1** with **4**, using procedure A, after purification. The sharp shoulder in front of the main peak is assigned to virus particles aggregated by virtue of Cu-triazole coordination (see Fig. S3). Note the detection of dye at 495 nm. (B) Comparison of the reaction of **1** with **4** using the two procedures described above. The reaction mediated by copper wire shows both a larger shoulder and a larger peak at 20 mL, the latter indicative of disassembled viral protein.



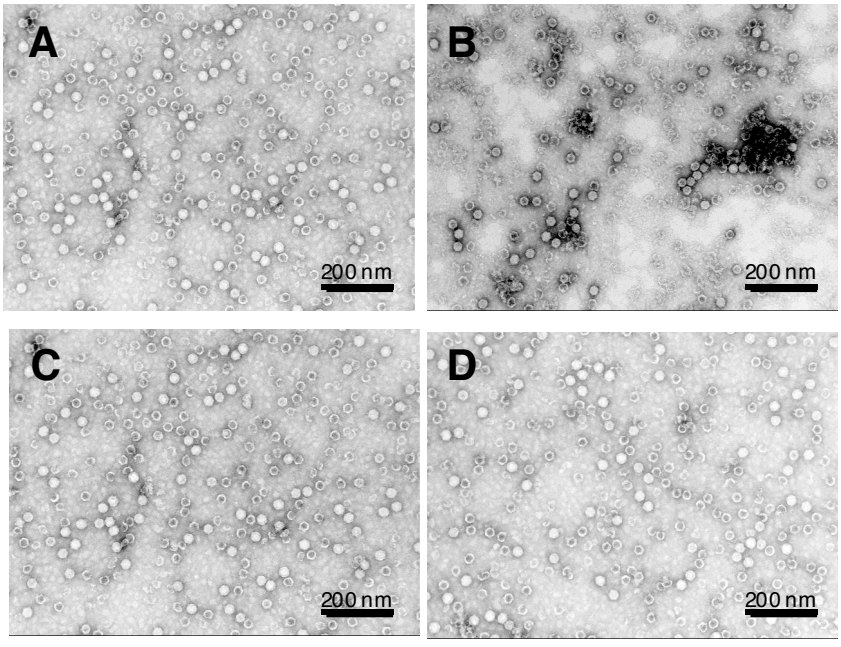


Figure S2. TEM of purified products at 0.2 mg/mL.
(A) **1** + **4**, procedure A;
(B) **1** + **4**, procedure B;
(C and D) samples A and B, respectively, incubated with 2.5 mM EDTA for 1 h.

Note the greater amount of aggregation in sample B, and the disruption of aggregation by addition of EDTA.

2. Stability of CPMV-Triazole Conjugates

The use of ascorbate (as originally described) or *p*-hydroquinone as reducing agents for Cu(II) gave poorly reproducible results in virus-based coupling reactions. To investigate this phenomenon, a preliminary exploration of the stability of CPMV in the presence of a variety of species was undertaken, with the results shown in Table S1. Ascorbate and hydroquinone were both shown to induce decomposition of the capsid (entries 1 and 2), accounting for the poor recovery of virus-triazole adducts in the presence of these reductants. The CPMV scaffold alone is stable to 20 mM Cu(II) and 10 mM Cu(I) in the presence of any combination of TCEP, alkyne, and azide (entries 3 and 4). However, the corresponding incubation of CPMV-alkyne or CPMV-azide adducts with complementary reagents in the presence of Cu(I) leads to virus decomposition (entries 5 and 6). Under these conditions, triazole adducts on the virus are being formed, and Cu(II) is being generated in stoichiometric amounts.

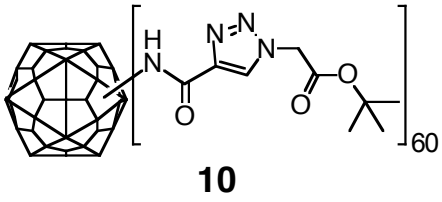
A preformed triazole was attached to the virus by procedures analogous to those described above to give **10** (Figure S3). In the presence of excess azide, alkyne, and TCEP, **10** was completely stable (Table S1, entry 7). The addition of Cu(I) or Cu(II) salts to **10** induced rapid breakup of the virus capsid with precipitation of the resulting protein (entries 8 and 9). Under these conditions (in air), it is presumed that a Cu(II)–triazole adduct is the culprit. Figure S3 shows size-exclusion FPLC data supporting these observations.

Table S1.

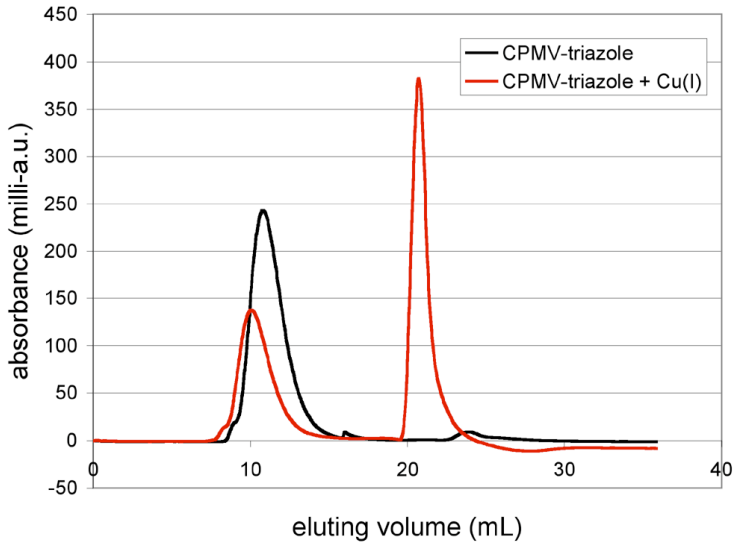
Entry	Virus	$CuSO_4$ (mM)	CuI (mM)	**4** [a,c] 2 mM	**5** [b,c] 2 mM	reducing agent [d]	TCEP [c] 10 mM	Stable (Y/N)
1	CPMV	–	–	–	–	Asc	–	N
2	CPMV	–	–	–	–	HQ	–	N
3	CPMV	20	–	+/–	+/–	–	+/–	Y
4	CPMV	–	10	+/–	+/–	–	+/–	Y
5	**1**	–	10	+	–	–	–	N
6	**2**	–	10	–	+	–	–	N
7	**10**	–	–	+	+	–	+	Y
8	**10**	20	–	–	–	–	–	N
9	**10**	–	10	–	–	–	–	N

(a) 2 mM; results are the same with other water-soluble alkynes such as propargylamine. (b) 2 mM; results were the same with other water-soluble azides such as 2,3-dihydroxy-1-azidopropane. (c) +/– denotes the same results in the presence or absence of the indicated reagent. (d) Asc = ascorbate (1 mM); HQ = hydroquinone (1 mM).

Figure S3. Demonstration of decomposition in the presence of virus-bound triazole plus copper. "CPMV-triazole" refers to species **10** (see below), which gave the FPLC trace shown, characteristic of intact virions. This species was stable for days in solution at room temperature or 4°C. In contrast, the exposure of a solution of **10** to 1 mM $CuSO_4$, 1 mM CuI, or copper wire plus 0.2 mM $CuSO_4$ led to rapid aggregation and decomposition; shown here is the result with $CuSO_4$ after several hours. Note the displacement of the first peak to shorter retention volume, indicating aggregation, and the large peak corresponding to disassembled CPMV protein at ca. 20 mL.



10



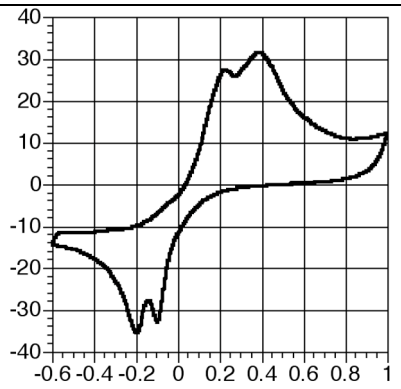
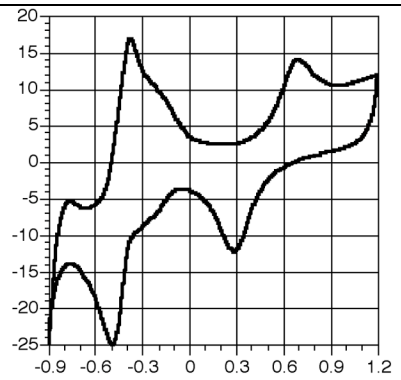
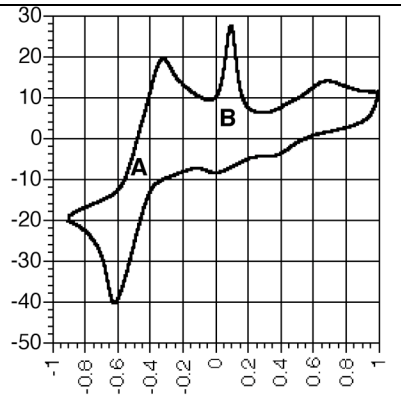
3. Electrochemistry.

Preliminary investigations of the copper/TCEP/tris(triazolyl)amine system were undertaken in 90:10 water:*tert*-butanol containing 0.2 M $LiClO_4$, as close as possible to the protein ligation reaction conditions. Other than the presence of electrolyte, the only major difference was the omission of buffer; pH in electrochemistry experiments was measured and controlled by the addition of NaOH solution to mixtures made acidic by TCEP. The results shown in Table S2 were unchanged in the pH range from 4 to 7; voltammograms shown were acquired at pH 7. The important observations are as follows:

(i) TCEP is approximately 400 mV more reducing than Cu(I) under these conditions, and is thus thermodynamically competent to reduce Cu(II) to Cu(I).
(ii) When the TCEP:Cu ratio is 2:1 or higher, a second electroactive species in addition to free TCEP is observed at $E_{1/2}$ = +488 mV, approximately 430 mV more positive than the Cu(I/II) couple in the absence of TCEP. This species is tentatively assigned as a Cu•TCEP complex of uncertain stoichiometry.
(iii) When equimolar amounts of TCEP and Cu are present, an irreversible wave assigned to solvated Cu ion is observed. In this situation, the electrochemical oxidation of Cu(I) to Cu(II) is proposed to be followed immediately by chemical reduction back to Cu(I) by TCEP, accounting for the irreversible nature of the wave. Increasing the scan rate to 1 V/s allows a small portion of the Cu return wave to be visible, but scanning at higher rates could not be performed. These data suggest that the Cu•TCEP binding constant is not very large, but that the coordination of TCEP to Cu significantly stabilizes the Cu(I) oxidation state.
(iv) Tris(triazolyl)amine **6** is not electroactive in the available electrochemical window.

(v) The addition of **6** to any of the mixtures above does not change the observed cyclic voltammetry response, which suggests one of three situations: (a) the binding of **6** to Cu under these conditions is weak (unlikely in light of other observations described throughout), (b) the resulting complex is not electroactive, or (c) the redox potential of the Cu•**6** complex is outside the available electrochemical potential range. An unidentified solid precipitated from these solutions after 20-30 minutes. Further studies are underway with more soluble multidentate triazole ligands.

Table S2. Cyclic voltammetry results [working electrode = Pt disk; auxiliary = Pt wire; reference = Ag/AgCl; solvent and electrolyte specified in text; scan rate = 200 mV/s; *x*-axis = potential (V vs. Ag/AgCl); *y*-axis = current (μA)].

4.0 mM $CuSO_4$ Two species are present, perhaps representing different populations of ligands (aquo, sulfate, *t*-BuOH) on the metal; at 0.25 mM a single wave is visible with $E_{1/2}$ = +49 mV.	E_{ox} = 0.389, 0.228 V E_{red} = –0.196, -0.098 V $E_{1/2}$ = +97 mV, +65 mV	
$CuSO_4$ (0.5 mM) + TCEP (1 mM) Wave 1 is coincident with the only observed in cyclic voltammetry of TCEP alone.	<u>wave 1</u> (TCEP) E_{ox} = –0.378 E_{red} = –0.495 $E_{1/2}$ = –437 mV <u>wave 2</u> (Cu•TCEP) E_{ox} = +0.692 E_{red} = +0.284 $E_{1/2}$ = +488 mV	
$CuSO_4$ (1 mM) + TCEP (1 mM) [voltammogram is unchanged in the presence of 0.5 mM **6**]	peak B = +97 mV	

4. Reactivity of TCEP with azide.

Reaction mixtures containing 1-2 mM azidoacetic acid and 10-20 mM TCEP (in the presence and absence of 1 mM $CuSO_4$) in D_2O at pH 8.0 were monitored by NMR. No reaction was observed after 48 hours at 4°C (the conditions under which virus ligation reactions were performed), but complete reduction of the azide (and the formation of one equivalent of phosphine oxide) was observed after 24 hours at room temperature. Note that these tests were performed at higher concentrations than reactions involving virus-derived reagents, and azidoacetic acid should be substantially more reactive toward phosphines than aliphatic azides of the type employed for virus conjugation. Thus, the rate of azide decomposition by TCEP observed here is almost certainly much greater than that experienced in the virus ligations of interest, and is therefore not competitive with the azide-alkyne cycloaddition process.

References

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(2) Wang, Q.; Lin, T.; Johnson, J. E.; Finn, M. G. *Chem. Biol.* **2002**, *9*, 813-819.