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Near-Quantitative Aqueous Synthesis of Rotaxanes via Bioconjugation to Oligopeptides and Proteins

Carson J. Bruns,[†,‡] Hanwei Liu,[†] and Matthew B. Francis*[,†,∥]

[†]Department of Chemistry, [‡]Miller Institute, University of California, Berkeley, Berkeley, California 94720, United States
[∥]Materials Sciences Division, Lawrence Berkeley National Laboratory, Berkeley, California 94720, United States

Supporting Information

ABSTRACT: In spite of widespread interest in rotaxane-based molecular machines and materials, rotaxanes have not been attached covalently to proteins. We describe the near-quantitative aqueous synthesis of [2]rotaxanes based on neutral and charged aqueous hosts—cucurbit[7]uril (CB7) and cyclobis(paraquat-*p*-phenylene) ($CBPQT^{4+}$), respectively—using the thiol-ene addition of cysteine and maleimide as a stoppering protocol. After verifying the high efficiency of the reaction using glutathione (GSH) as an oligopeptide stopper, we have employed cytochrome C (CytC) as a protein stopper to produce the first well-characterized protein-rotaxane bioconjugates. We anticipate that this methodology will enable the preparation of novel materials that combine the unique properties of proteins and mechanical bonds.

Rotaxanes are a class of ring-threaded molecules that are of interest for a variety of potential applications enabled by the mechanical bond.[1] Switchable and cleavable rotaxanes have been leveraged in a variety of controlled-release applications[2] relevant to drug[3] and gene[4] delivery, as well as in artificial molecular machines,[5] such as molecular muscles,[6] sequence-specific molecular assemblers,[7] switchable catalysts,[8] and molecular electronic devices[9] capable of logic[10] and memory[11] operations. These applications often involve the attachment rotaxanes to surfaces[12] or macromolecules.[13] Rotaxanes have not been attached covalently to proteins, however, despite literature examples of protein-based,[14] protein-mounted,[15] and peptide-binding[16] pseudorotaxanes, as well as peptide-based,[17] peptide-binding,[18] and protein-binding[19] rotaxanes. Motivated by our interest in making protein-based biomaterials,[20] we aimed to develop a bioconjugation strategy for rotaxanes, which we anticipate will enable researchers to create materials that combine the advantageous properties of proteins and mechanical bonds. For example, we have shown[21] that cucurbituril-based rotaxanes can be deployed as stimulus-activated contrast agents for the sensitive detection of analytes by ^{129}Xe magnetic resonance techniques, and we would like to attach these rotaxane-based contrast agents to enzymes and antibodies in order to detect biochemical events with high specificity.

Major improvements in the efficiency of rotaxane synthesis have come from the advent of covalent,[22] noncovalent,[23] and active templates,[24] coupled with efficient reactions under both thermodynamic and kinetic control.[25] A prominent "click" reaction[26] is the Cu^{I}-catalyzed azide-alkyne cycloaddition (CuAAC), which has enabled the efficient synthesis of hundreds of mechanically bonded molecules,[27] with seminal examples based on active metal,[28] transition metal,[29] π-donor/π-acceptor,[30] and DNA[31] templates. We were surprised to find that the thiol-maleimide Michael addition,[32] a click reaction employed ubiquitously for bioconjugation[33] on account of its high efficiency in aqueous solvents, rapid reaction times, and shelf-stable precursors, has been almost ignored in rotaxane synthesis. Thiol-maleimide coupling has been employed on only two occasions[34] to obtain rotaxanes, both of which were performed in organic solvents in moderate (≤71%) yields. Water-soluble design motifs for constructing rotaxanes are needed to take advantage of this reaction's superior efficiency in aqueous solvents. Molecular hosts commonly employed in the aqueous synthesis of rotaxanes include (i) cyclodextrins,[35] (ii) cucurbiturils,[36] and (iii) charged cyclophanes, such as Stoddart's cyclobis(paraquat-*p*-phenylene) ($CBPQT^{4+}$),[37] cationic or anionic derivatives of Diederich's[38] tetraoxaparacyclophanes,[39] Hunter's tetralactam macrocycles,[40] and Ogoshi's[41] pillararenes.[42] We have selected cucurbit[7]uril (CB7) and $CBPQT^{4+}$ (Figure 1) as representative neutral and charged model systems, respectively, for evaluating the efficiency of the thiol-maleimide Michael addition as a bioconjugate stoppering protocol, employing glutathione (GSH) as a model oligopeptide on account of its single free thiol group originating from its central cysteine residue.

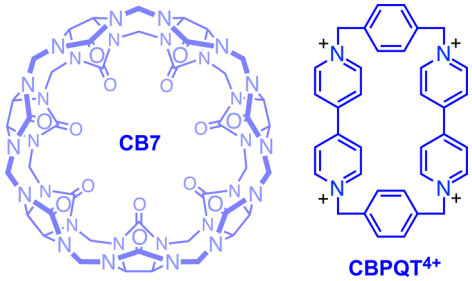

Figure 1. Structural formulas of $CBPQT^{4+}$ and CB7, model hosts for the aqueous bioconjugation of rotaxanes.

Near-quantitative aqueous syntheses of GSH-capped rotaxanes based on CB7 and $CBPQT^{4+}$ model systems are summarized in Figures 2 and 3, respectively. In each case, a pseudorotaxane self-assembles in aqueous solution from a maleimide-terminated precursor threading through the annulus

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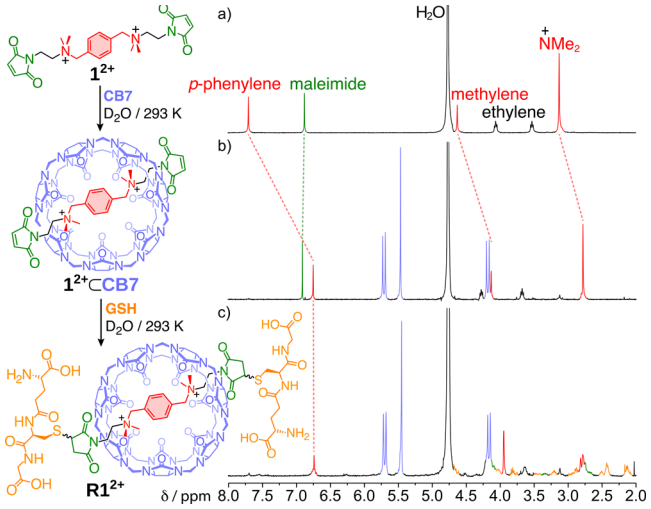

Figure 2. Near-quantitative stoppering of a CB7-based [2]rotaxane by maleimide-thiol addition to GSH, monitored *in situ* by ^{1}H NMR spectroscopy (D_2O, 400 MHz, 293 K). (a) ^{1}H NMR spectrum of maleimide-terminated xylylene-bisaminium precursor $\mathbf{1}^{2+}$. (b) ^{1}H NMR spectrum of pseudorotaxane $\mathbf{1}^{2+}\subset$CB7 (2 mM). (c) Crude ^{1}H NMR spectrum of [2]rotaxane $\mathbf{R1}^{2+}$ upon addition of 2.2 eq. GSH.

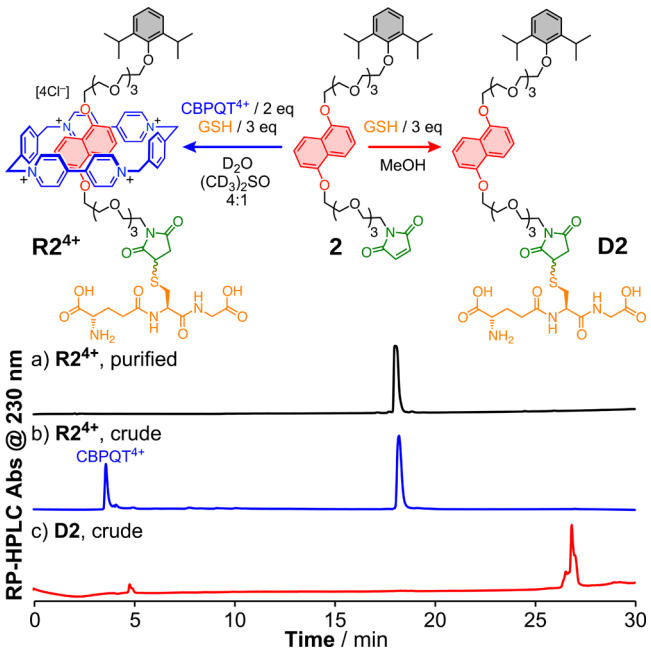

Figure 3. Stoppering of a DNP/CBPQT^{4+}-based [2]rotaxane $\mathbf{R2}^{4+}$ by bioconjugation to GSH. Analytical RP-HPLC (5–95% MeCN in H_2O, 0.1% TFA, 30 min, C18 column) absorbance traces (230 nm, AU) of (a) purified $\mathbf{R2}^{4+}$ and (b) crude $\mathbf{R2}^{4+}$ are compared with (c) DNP-based dumbbell **D2**, revealing that the reaction is near-quantitative because only excess CBPQT^{4+} (no **D2**) is detected in the crude sample of $\mathbf{R2}^{4+}$.

of a macrocyclic host. Bis-maleimide precursor $\mathbf{1}^{2+}$ is based on a quaternized *p*-xylylene-bisaminium recognition unit, which binds[43] CB7 with an association constant, K_a, on the order of 10^9 M^{-1} at room temperature in H_2O. The maleimide-terminated semidumbbell **2** is based on an oligoether-functionalized 1,5-dioxynaphthalene (DNP) recognition unit, which binds[44] CBPQT^{4+} with a K_a value on the order of 10^6 M^{-1} at room temperature in H_2O. The equilibrated pseudorotaxane solutions are exposed to a slight molar excess of GSH to obtain the corresponding [2]rotaxanes $\mathbf{R1}^{2+}$ in the case of CB7 and $\mathbf{R2}^{4+}$ in the case of CBPQT^{4+}. The synthesis of these compounds and their characterization by NMR spectroscopy and mass spectrometry are described in the Supporting Information (SI).

The aqueous stoppering reactions are complete within 10 min at room temperature at 2 mM concentration with respect to the maleimide precursors. Near-quantitative conversions for each reaction have been confirmed by *in situ* ^{1}H NMR spectroscopy (Figure 2, **Figure S4** in the SI) as well as analytical reversed-phase high-performance liquid chromatography (RP-HPLC; Figure 3 and **Figure S1** in the SI), assuming all products are detected by these methods. The *p*-xylylene resonances in the ^{1}H NMR spectrum of $\mathbf{1}^{2+}$ (Figure 2a) are shifted to lower frequencies upon addition of CB7 (Figure 2b) owing to the formation of pseudorotaxane $\mathbf{1}^{2+}\subset$CB7, and the maleimide signal disappears (Figure 2c) upon addition of GSH, which reacts to give the succinimide-thioether adduct of [2]rotaxane $\mathbf{R1}^{2+}$. Analogous behavior is observed (**Figure S4** in the SI) in the case of the donor-acceptor recognition motif, except semidumbbell **2** cannot be compared to pseudorotaxane $\mathbf{2}\subset$CBPQT^{4+} or [2]rotaxane $\mathbf{R2}^{4+}$ on account of its insolubility in the aqueous solution (20% v/v Me_2SO) chosen for the reaction—i.e., compound **2** dissolves only in the presence of ≥1 eq. CBPQT^{4+} as its chloride salt. For the sake of comparison, we prepared (Figure 3) GSH-capped DNP dumbbell **D2** in MeOH. Only two elution peaks appear in the analytical RP-HPLC trace (Figure 3b) of the crude rotaxane-stoppering reaction; unbound CBPQT^{4+} elutes at 4 min and $\mathbf{R2}^{4+}$ elutes at 18 min in a 5–95% H_2O-MeCN gradient (0.1% TFA) over 30 min on a C18 column. The high reaction efficiency is further confirmed by the fact that free dumbbell **D2**, which elutes (Figure 3c) at ca. 27 min, was not detected after stoppering the rotaxane. On a preparative scale, $\mathbf{R2}^{4+}$ was isolated in 88% yield after removal of excess CBPQT^{4+} and GSH by preparative RP-HPLC. We have not observed any evidence for the retro-Michael addition in any of our samples, even after days of standing in D_2O at mM concentrations.

Having verified the high efficiency of the maleimide-thiol addition for the aqueous synthesis of rotaxanes, we turned our attention to the covalent attachment of rotaxanes to proteins (Figure 4). We chose yeast *iso*-1-cytochrome *c* (CytC) as a model protein because (i) it contains a single solvent-accessible cysteine (residue 102), which is not bound in a disulfide bridge, (ii) it is a relatively small protein (12 708 Da, accounting for its heme center and acetylated *N*-terminus), which enables us to confirm the attachment of the relatively low molecular weight rotaxanes (<2 kDa) by gel electrophoresis, and (iii) its structure is known (PDB 1YCC).[45]

The bioconjugation of a *p*-xylylene/CB7 [2]rotaxane to CytC involves the addition of a CB7-threaded semirotaxane based on semidumbbell $\mathbf{1GS}^{2+}$ (derived from $\mathbf{1}^{2+}$ and GSH; see the SI) at 1 mM concentration in 20 mM HEPES buffer at pH 7.0. The protein was found to precipitate in phosphate buffer or in the presence of excessive CB7. In the case of the DNP/CBPQT^{4+} rotaxane bioconjugation, the semirotaxane $\mathbf{2}\subset$CBPQT^{4+} was added at 2 mM concentration with respect to **2** in 10 mM phosphate buffer at pH 7.5. Approximately 10% v/v Me_2SO was required to maintain the solubility of $\mathbf{2}\subset$CBPQT^{4+}. In both protocols, the concentration of CytC was 20 μM and the reaction time was approximately 30 min.

The excessive small-molecule reactants were separated from the protein and recovered by multiple rounds of spin concentration against a membrane with a 10 kDa cutoff molecular weight. The crude products were analyzed by

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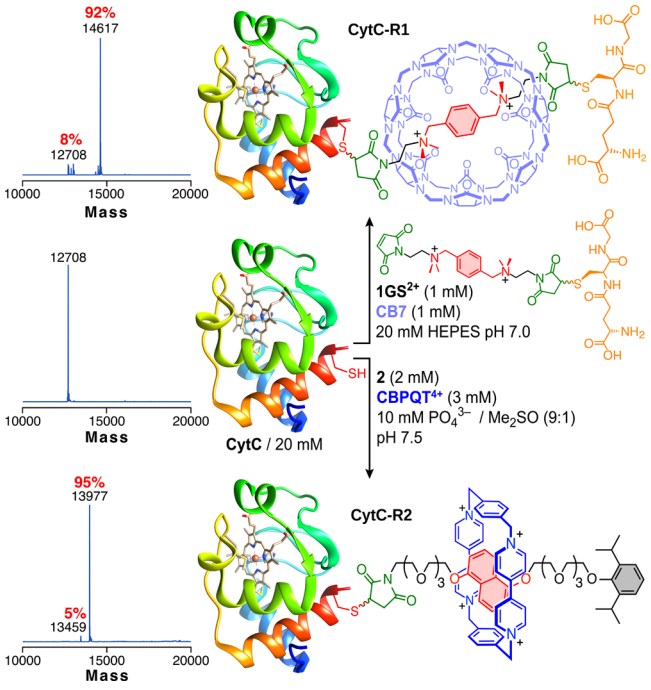

Figure 4. Aqueous synthesis of protein-mounted rotaxanes comprising CytC and the model systems based on CB7 and $CBPQT^{4+}$ hosts. Deconvoluted ESI-TOF-MS of the crude reaction products indicate that CytC is modified with >90% efficiency in each case.

electrospray ionization time-of-flight mass spectrometry (ESI-TOF-MS; Figure 4). CB7-Based protein-rotaxane **CytC-R1** is initially obtained alongside ca. 8% impurities comprising mainly CytC and doubly oxidized CytC ($[M + 32]^+$). Bioconjugates of the unthreaded semidumbbell $\mathbf{1GS}^{2+}$ were not detected. On the other hand, $CBPQT^{4+}$-based protein-rotaxane **CytC-R2** is accompanied by a 5% impurity comprising bioconjugate **CytC-2** possessing the unthreaded DNP semidumbbell, yet the CytC starting material was fully consumed. In both cases, the gradual ring-opening hydrolysis[32c] of the succinimide could be detected by ESI-MS in some of the population, and hydrolysis was more significant at higher pH.

To confirm further the formation of protein-stoppered rotaxanes, we compared CytC and the corresponding bioconjugates—both with and without a ring—by SDS-PAGE (Figure 5). In spite of their small differences (1−2 kDa) in molecular weight, CytC can be separated electrophoretically from the dumbbell- and rotaxane-mounted bioconjugates. Based

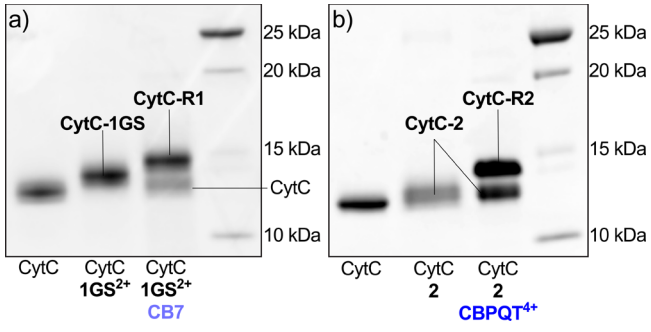

Figure 5. SDS-PAGE analysis of bioconjugation reactions for (a) **CytC-R1** and (b) **CytC-R2**, with samples comprising CytC and the CytC-dumbbell bioconjugates for comparison.

on the optical densities of the bands (Coomassie stain), **CytC-R1** represents (Figure 5a) up to 80% of the population after electrophoresis, whereas the remaining 20% is CytC. The protein-mounted dumbbell lacking CB7 (**CytC-1GS**) was not detected unless it was prepared separately. These results are in reasonable agreement with the ESI-TOF-MS data. In the second case (Figure 5b), the band corresponding to protein-rotaxane **CytC-R2** is accompanied by a second band corresponding to dumbbell-only bioconjugate **CytC-2** in an approximately 65:35 ratio, which is substantially lower than that (95:5) observed by ESI-TOF-MS. It is plausible that some of the $CBPQT^{4+}$ rings are released from the dumbbell during the SDS-PAGE protocol. The relatively harsh conditions of the denaturing process (100 °C in SDS-saturated 3:2 Tris-HCl buffer/glycerol for 10 min) and/or electrophoresis (150 V in MOPS buffer) may open the ring-strained $CBPQT^{4+}$ box because it is sensitive to reductants[46] and nucleophiles.[47] Recognizing that the thiol-maleimide addition is reversible under some conditions,[32b] it is also possible for intact $CBPQT^{4+}$ rings to be released in the event of a retro-addition during equilibration, because the molarity (~5 μM) of the sample is close to the K_d (~1 μM) of the pseudorotaxane. We cannot rule out the possibility, however, that the ESI-TOF-MS results are biased by inequivalent ionization potentials of **CytC-R2** and **CytC-2**. The ESI-TOF-MS and SDS-PAGE results were reproduced in samples aged in pure water for more than 24 h, suggesting that the retro-addition[32b] is not significant under ambient conditions.

In conclusion, we have developed an efficient stoppering protocol for the mild, rapid, near-quantitative aqueous syntheses of water-soluble rotaxanes, and applied this chemistry to produce the first examples of protein bioconjugates containing mechanical bonds. This new class of hybrid molecules may enable a variety of potential applications. For example, because rotaxanes are often employed as molecular switches and machines,[5−11] it may be possible to harness the controlled mechanical motion of a rotaxane ring in order to regulate the function of a protein, e.g., by changing its conformation or influencing the access of substrates to an active site. These possibilities are currently being investigated.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/jacs.6b10231.

Synthetic procedures and characterization data (PDF)

■ AUTHOR INFORMATION

Corresponding Author

*mbfrancis@berkeley.edu

ORCID

Matthew B. Francis: 0000-0003-2837-2538

Notes

The authors declare no competing financial interest.

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