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Synthesis of a Bridging Ligand with a Non-denatured Protein Pendant: Toward Protein Encapsulation in a Coordination Cage

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Toward protein encapsulation by a synthetic host, we synthesized an ubiquitin-dangled ligand, a potential precursor of a nanoscale coordination cage. The key step is the addition of a C-terminal Cys76 SH group, which was introduced by Gly76Cys mutation, to a maleimide acceptor on the ligand. The C-terminal mutation and the SH addition to the ligand did not damage the ubiquitin moiety at all, neither structurally nor conformationally.

Encapsulation of proteins in synthetic hosts may enable the control of protein functions. With the expectation of enhanced stability and enzymatic activities, some proteins have been accommodated in synthetic host materials such as solid supports,[1] polymer matrices,[2] and reverse micelles.[3] In these structurally nonuniform hosts, however, the protein functions are dispersed. Furthermore, functional control and elaboration of the encapsulated proteins are difficult because they can no longer be analyzed by common spectroscopic or crystallographic methods. We envisioned the encapsulation of proteins in a structurally well-defined host, where the protein functions are not dispersed but distinctly controlled and can be observed using spectroscopic and crystallographic methods. This chemical approach has a great advantage over biological strategies with natural cages such as viruses which present difficulties in preparation and structural modification.[4]

Recently, rigid and bent ligands **1** have been shown to self-assemble into nanoscale spherical cages **2** upon complexation with Pd(II) ions (Figure 1).[5] Noting that the diameters of the spherical cages are comparable to those of proteins (up to 10 nm), we had an ambition to encapsulate proteins in the discrete cage host. The first step toward the protein encapsulation inside the cage is the development of a mild synthesis of a protein-functionalized ligand without denaturization of the protein's native folding. As our first target, we choose ubiquitin, a relatively small globular protein (76 residues, 8.6 kDa approximately 3–4 nm in diameter) which plays an important role in proteasomal degradation.[6] Here we report that terminal mutation (Gly76 to Cys76) of ubiquitin followed by coupling with a maleimide-functionalized bent ligand provides a mild and high-yielding method for attaching a protein on a ligand without denaturization.

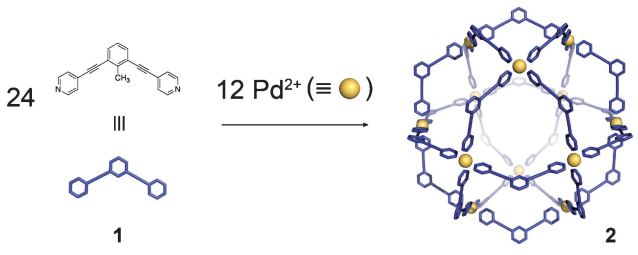
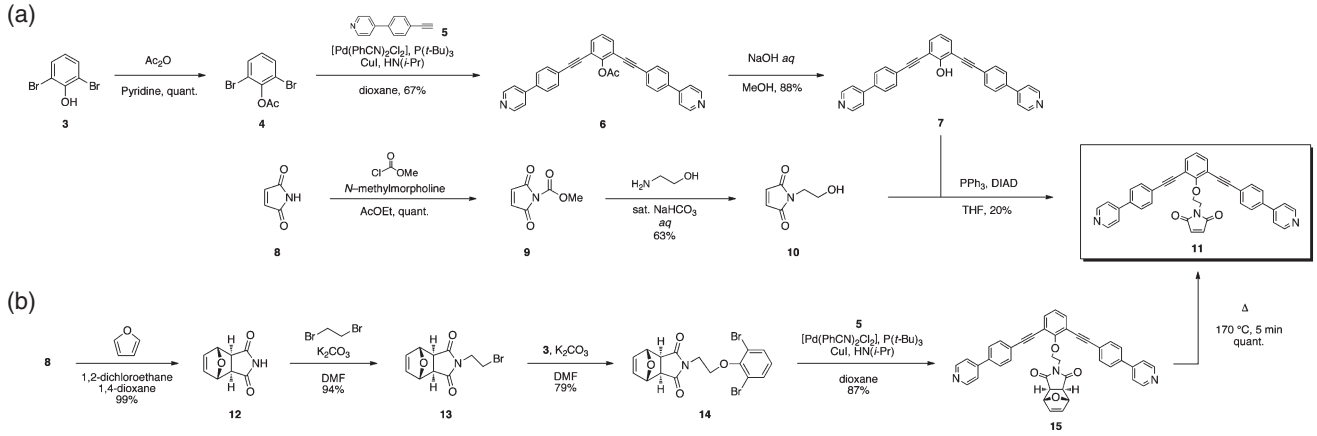


Figure 1. Schematic representation of self-assembly of a coordination sphere.

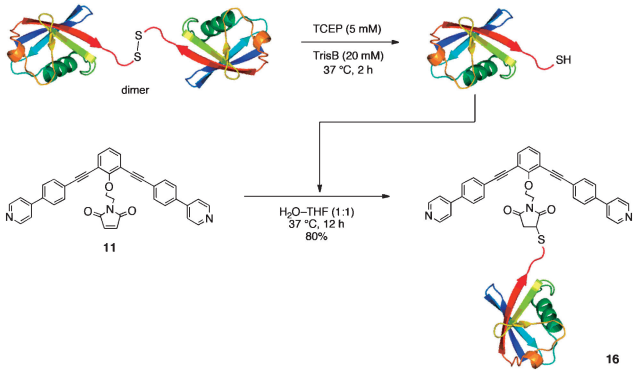
In our strategy for the protein encapsulation, one of the ligands involved in the cage **2** should be replaced by a protein-functionalized ligand. The key requirements in the synthesis of the protein-functionalized ligand are (1) selective coupling at a specific residue of ubiquitin with the ligand and (2) mild conditions that do not denature the ubiquitin native structure. The existing functional groups in ubiquitin (for example, NH_2 of Lys and COOH of Asp or Glu) are unavailable for the coupling with the ligands because two or more of these residues exist in the ubiquitin sequence and no selectivity among these residues is expected. In addition, chemical derivation of internal residues may lead to denaturization. We therefore designed the introduction of a Cys residue (SH group) by a mutation technique. Since the ubiquitin C-terminal sticks out from the folding structure and is conformationally flexible, mutation at this site is expected to retain the native structure. Thus we examined the mutation of C-terminal Gly76 into Cys76 (Gly76Cys mutation).[7]

The Gly76Cys-mutated ubiquitin was constructed by standard polymerase chain reaction (PCR) and genetic engineering techniques. The recombinant ubiquitin mutant was expressed and purified as described previously.[6] Among the number of methods available to couple a protein with an organic functional group, we chose thiol–maleimide coupling[8,9] since a covalent bond is irreversibly formed under very mild coupling conditions.

The maleimide part is covalently coupled to the concave of ligand **1**. We established the synthesis of ligand **11** via two synthetic routes. Initially, ligand **11** was prepared by route a (Scheme 1a). Commercially available **3** was acetylated (**3** → **4**;



Scheme 1. Two synthetic routes to maleimide-coupled ligand **11**.



Scheme 2. Introduction of ubiquitin to the synthetic ligand through thiol–maleimide coupling.

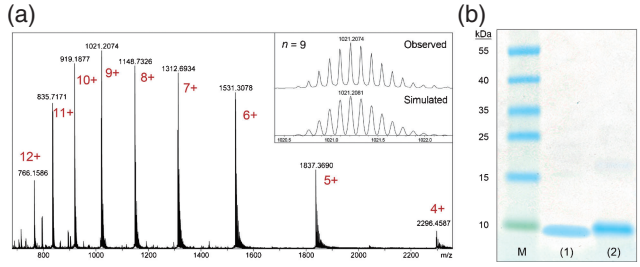


Figure 2. (a) A series of multi-protonated **16** (4+ to 12+) observed in the ESI-MS. (b) Purification of Gly76Cys ubiquitin (1) and Gly76Cys ubiquitin conjugated ligand **16** (2). M indicates the protein molecular weight standards.

≈100%) and coupled with 4-(4-pyridyl)phenylacetylene (**5**) by a Sonogashira reaction to give **6** (67%). The acetyl deprotection (**6** → **7**, 88%), *N*-(hydroxyethyl)maleimide (**10**), prepared from maleimide (**8**) in two steps (via **9**), was introduced by a Mitsunobu reaction to give maleimide-coupled ligand **11** in 20% yield. The overall yield was 7.4%.

To improve the overall yield, we also developed an alternative route (route b in Scheme 1b), in which the low-yielding Mitsunobu reaction is avoided and the protected maleimide is introduced first. Thus maleimide (**8**) was subjected to a Diels–Alder reaction with furan (**8** → **12**, 99%) and subsequently treated with 1,2-dibromoethane to give **13** (94%). Coupling of **13** with **3** (79%) followed by the Sonogashira coupling (**14** → **15**, 87%) and thermal deprotection (retro-Diels–Alder) gave **11** (≈100%). The overall yield was impressively improved to 64%.

Having synthesized the ligand and ubiquitin parts with appropriate reaction sites, we finally examined the coupling of these two components (Scheme 2). The Gly76Cys-mutated ubiquitin was actually obtained as a SS-bonded dimer and the disulfide bond was reductively cleaved before use with tris-(2-carboxyethyl)phosphine (TCEP, 5 mM) in Tris-HCl buffer (pH 7.5, 20 mM) at 37 °C for 2 h. The reduced monomer of the Gly76Cys ubiquitin was mixed with ligand **11** under neutral conditions in H_2O–THF (1:1) solution. We were relieved to find that the key coupling reaction proceeded quite smoothly: LC-MS analysis showed the quantitative formation of the coupled product and the protein conjugated ligand **16** was isolated in 80% yield after HPLC purification.

Characterization of **16** was carried out by ESI-MS, ^{1}H NMR spectroscopy, and SDS-PAGE. In the ESI-MS analysis, the molecular weight of **16** (9182.5830) was determined from a series of multi-protonated **16** (4+ to 12+) (Figure 2a). Ultra-high-resolution MS revealed m/z 1021.2074 for the 9+ species (calcd. 1021.2081; error < 1 ppm). The ^{1}H NMR spectrum of **16** showed the loss of olefinic protons (see Figure S1[10]). In SDS-PAGE, ligand **16** was clearly observed at a slightly heavier region than Gly76Cys ubiquitin (Figure 2b).

To confirm whether the folded structure of ubiquitin is retained after chemical coupling with ligand **11**, a ^{1}H–^{15}N HSQC spectrum was measured to observe the distribution of ubiquitin amide NH correlations on the 2D map. For the production of the fully ^{15}N-labeled protein, cells were grown in M9 media containing [^{15}N]NH_4Cl (1 g L^{-1}). The ^{15}N-labeled ubiquitin mutant was purified in the same manner as the unlabeled counterpart. As shown in Figure 3, the NH correlations of ligand **16** are quite similarly mapped as those of fully ^{15}N-labeled Gly76Cys ubiquitin, confirming the retention of the globular folded structure.

In summary, we have succeeded in preparing ubiquitin-functionalized, bispyridyl bridging ligand **16** in a high yield. It is remarkable that the C-terminal mutation strategy and the

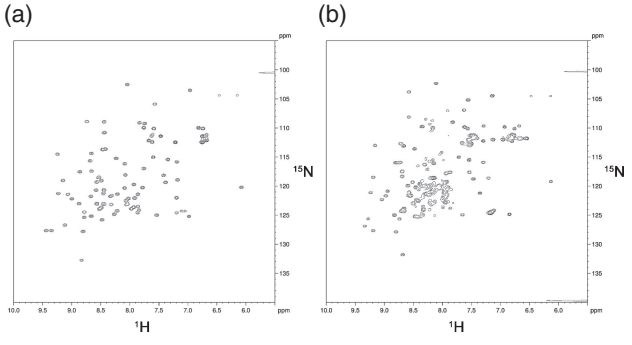


Figure 3. ^{1}H–^{15}N HSQC spectra of (a) ^{15}N-labeled Gly76Cys ubiquitin (500 MHz, $H_2O/D_2O = 90/10$, 300 K) and (b) ligand **16** (500 MHz, $H_2O/D_2O/CD_3CN/TFA = 81/9/10/0.1$ (pH 2.0), 300 K). The slight spectral changes were mainly due to the different solvent conditions in NMR measurements.

extremely mild conditions in the final coupling process do not damage the ubiquitin moiety, neither structurally nor conformationally. Self-assembly of a spherical coordination cage around ubiquitin is currently on-going.

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References and Notes

1 a) L. M. Ellerby, C. R. Nishida, F. Nishida, S. A. Yamanaka, B. Dunn, J. S. Valentine, J. I. Zink, *Science* **1992**, *255*, 1113. b) C. Lei, Y. Shin, J. Liu, E. J. Ackerman, *J. Am. Chem. Soc.* **2002**, *124*, 11242.

2 a) G. Zhu, S. R. Mallery, S. P. Schwendeman, *Nat. Biotechnol.* **2000**, *18*, 52. b) N. Kameta, M. Masuda, H. Minamikawa, N. V. Goutev, J. A. Rim, J. H. Jung, T. Shimizu, *Adv. Mater.* **2005**, *17*, 2732. c) M. Yan, J. Ge, Z. Liu, P. Ouyang, *J. Am. Chem. Soc.* **2006**, *128*, 11008. d) J. Kim, J. W. Grate, *Nano Lett.* **2003**, *3*, 1219. e) D. V. Volodkin, N. I. Larionova, G. B. Sukhorukov, *Biomacromolecules* **2004**, *5*, 1962. f) P. Walde, S. Ichikawa, *Biomol. Eng.* **2001**, *18*, 143.

3 a) G. A. Ayala, S. Kamat, E. J. Beckman, A. J. Russell, *Biotechnol. Bioeng.* **1992**, *39*, 806. b) C. R. Babu, P. F. Flynn, A. J. Wand, *J. Am. Chem. Soc.* **2001**, *123*, 2691.

4 a) F. P. Seebeck, K. J. Woycechowsky, W. Zhuang, J. P. Rabe, D. Hilvert, *J. Am. Chem. Soc.* **2006**, *128*, 4516. b) M. Comellas-Aragonès, H. Engelkamp, V. I. Claessen, N. A. J. M. Sommerdijk, A. E. Rowan, P. C. M. Christianen, J. C. Maan, B. J. M. Verduin, J. J. L. M. Cornelissen, R. J. M. Nolte, *Nat. Nanotechnol.* **2007**, *2*, 635. c) I. J. Minten, L. J. A. Hendriks, R. J. M. Nolte, J. J. L. M. Cornelissen, *J. Am. Chem. Soc.* **2009**, *131*, 17771. d) B. Wörsdörfer, K. J. Woycechowsky, D. Hilvert, *Science* **2011**, *331*, 589.

5 a) M. Tominaga, K. Suzuki, M. Kawano, T. Kusukawa, T. Ozeki, S. Sakamoto, K. Yamaguchi, M. Fujita, *Angew. Chem., Int. Ed.* **2004**, *43*, 5621. b) S. Sato, J. Iida, K. Suzuki, M. Kawano, T. Ozeki, M. Fujita, *Science* **2006**, *313*, 1273. c) K. Suzuki, S. Sato, M. Fujita, *Nat. Chem.* **2010**, *2*, 25.

6 a) S. Vijay-Kumar, C. E. Bugg, W. J. Cook, *J. Mol. Biol.* **1987**, *194*, 531. b) A. Hershko, A. Ciechanover, *Annu. Rev. Biochem.* **1998**, *67*, 425.

7 T. Hirano, O. Serve, M. Yagi-Utsumi, E. Takemoto, T. Hiromoto, T. Satoh, T. Mizushima, K. Kato, *J. Biol. Chem.* **2011**, *286*, 37496.

8 a) U. K. Nässander, P. A. Steerenberg, H. Poppe, G. Storm, L. G. Poels, W. H. De Jong, D. J. A. Crommelin, *Cancer Res.* **1992**, *52*, 646. b) D. Kirpotin, J. W. Park, K. Hong, S. Zalipsky, W.-L. Li, P. Carter, C. C. Benz, D. Papahadjopoulos, *Biochemistry* **1997**, *36*, 66. c) K. Maruyama, N. Takahashi, T. Tagawa, K. Nagaike, M. Iwatsuru, *FEBS Lett.* **1997**, *413*, 177. d) F. J. Martin, D. Papahadjopoulos, *J. Biol. Chem.* **1982**, *257*, 286.

9 G. T. Hermanson, *Bioconjugate Techniques*, 2nd ed., Academic Press, San Diego, **2008**.

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