Barium Ion Induced Assembly of an Artificial Protein Nanocage, TIP60

Protein nanocages can encapsulate various molecules within their inner space and are expected to be useful for drug delivery. Typically, protein nanocages are produced through the assembly of multiple molecules, and thus efficient encapsulation of drug molecules requires a technique for disassembling and reassembling the nanocages. However, the process often reduces the yield of the nanocages. Here we have developed a system for inducing the formation of protein nanocages by metal ions using TIP60, an artificial protein nanocage composed of 60 molecules.

Protein nanocages have potential applications in medical and biotechnological fields. Studies on encapsulation of drugs in their inner space have been widely conducted [1, 2]. The protein nanocages are composed of multiple protein molecules and generally obtained as caged structure after the purification. For efficient drug encapsulation in inner space, disassembly and reassembly of the nanocage is necessary. Since this process often needs harsh treatment such as acidification, the yield of drug-incorporated nanocages is not sufficiently high. We assumed that this problem could be solved by designing protein molecules that cannot form nanocages in the preparation stage but can become caged structure upon external stimulation.

In this study, we designed a protein that responds to metal ions to form a nanocage based on the protein nanocage TIP60 (Fig. 1(a)), which has a soccer ball-shaped structure and is composed of 60 molecules of artificially designed fusion protein [3, 4]. TIP60 was synthesized by a typical *E. coli* protein expression system and was obtained as a caged structure even immediately after purification, suggesting that the fusion proteins assemble into the cage in the cell. Therefore, we attempted to design a fusion protein that does not assemble into a nanocage.

The three-dimensional structure of TIP60 was determined by our group previously (Fig. 1(a)). Based on the tertiary structure, we assumed that K67 is a key residue maintaining the 60-mer structure. K67 is located at the interface of fusion proteins and forms intermolecular hydrogen bonds. We thus constructed a K67E mutant. Electrophoresis of the K67E mutant showed that no 60-mer was produced after purification (Fig. 1(b)). The predicted structure of the K67E mutant suggested that the mutant has a number of oxygen atoms at the interface of fusion proteins, which are known to coordinate with alkaline earth elements such as Ca ions. We hypothesized that the mutant would form a 60-mer structure in the presence of alkaline earth elements. As a result, the band corresponding to the 60-mer nanocage was observed in the presence of Ca, Sr, and Ba ions, with Ba ions giving the highest yield among the three metal ions at a concentration of 1 mM (Fig. 1(b)) [5].

The structure of metal ion (Ba)-induced TIP60 (mTIP60) was analyzed using small-angle X-ray scattering (SAXS) analysis (Fig. 2(a)). The SAXS data showed that mTIP60 had a spherical shape that almost closely matched that of the original TIP60. The radius of gyration (R_g) from Guinier analysis and the maximum particle dimension (D_{max}) from the pair-distance distribution function $P(r)$ were estimated to be 9.4 nm and 21.8 nm, respectively. Interestingly, mTIP60 had a more right-shifted peak of $P(r)$ than that of the original TIP60, suggesting that the inner space did not contain any other molecule in the case of mTIP60 (Fig. 2(b)). The unassembled fusion proteins (in the absence of Ba ions) showed a left-shifted $P(r)$ typically observed in an elongated shaped molecule. The structural estimation by a dummy atom model generated by DAMMIF and DAMMIN suggested that the unassembled protein had a dimeric form (Fig. 2(c)). We then analyzed the structure of mTIP60 by cryogenic electron microscopy (cryo-EM) in KEK-SBRC (Fig. 2(d)), indicating that the overall structure was the almost same as that of the original TIP60. Additionally, we found a potential map attributable to Ba ions that was not observed in the original TIP60 at the interface of fusion proteins corresponding to the mutation position. These data strongly indicated that the mutated amino acid residue (K67E) was involved in Ba coordination, which stabilized the 60-mer structure. To further confirm that whether Ba ions stabilize the 60-mer structure, we performed an experiment to remove Ba ions using EDTA. The addition of EDTA led to the disassembly of mTIP60 into smaller oligomers, indicating that the presence of Ba ions was essential for maintaining the 60-mer structure [5].

Finally, we tested the capability of mTIP60 to encapsulate a different molecule in its inner space. We added single-stranded DNA (ssDNA) with Ba ions to the K67E mutant with two additional mutations, and successfully obtained a nanocage that encapsulated the ssDNA [5].

Taken together, our results demonstrate that metal-induced assembly of mTIP60 can be a fundamental technique for use as a drug carrier. By designing a protein that forms a nanocage in response to external stimulation, we were able to overcome the limitations of harsh treatments and achieve efficient drug encapsulation. This approach has the potential for a wide range of medical applications and can be further optimized for advanced drug delivery systems.

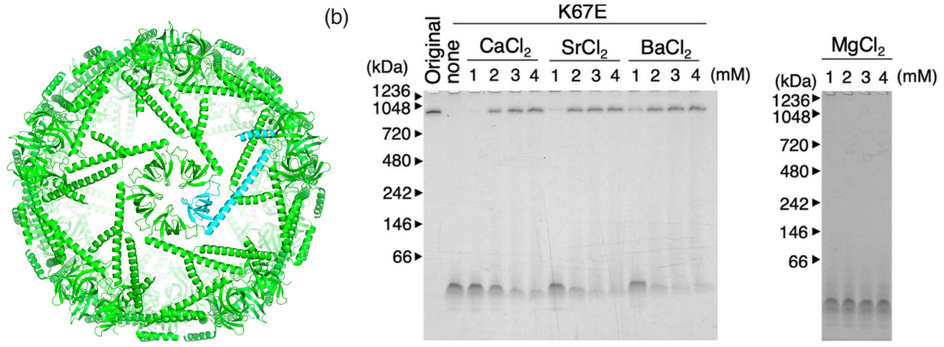

Figure 1: (a) The three-dimensional structure of the original TIP60 (PDB ID: 7EQ9). Single subunit was shown in cyan. **(b)** Electrophoresis results. The band appeared at molecular weight lower than 66 kDa molecular weight marker was unassembled K67E mutant. The band appeared at the position corresponding to that of original TIP60 was assembled 60-mer. This panel **(b)** is reproduced from reference [5]. Copyright 2023 American Chemical Society.

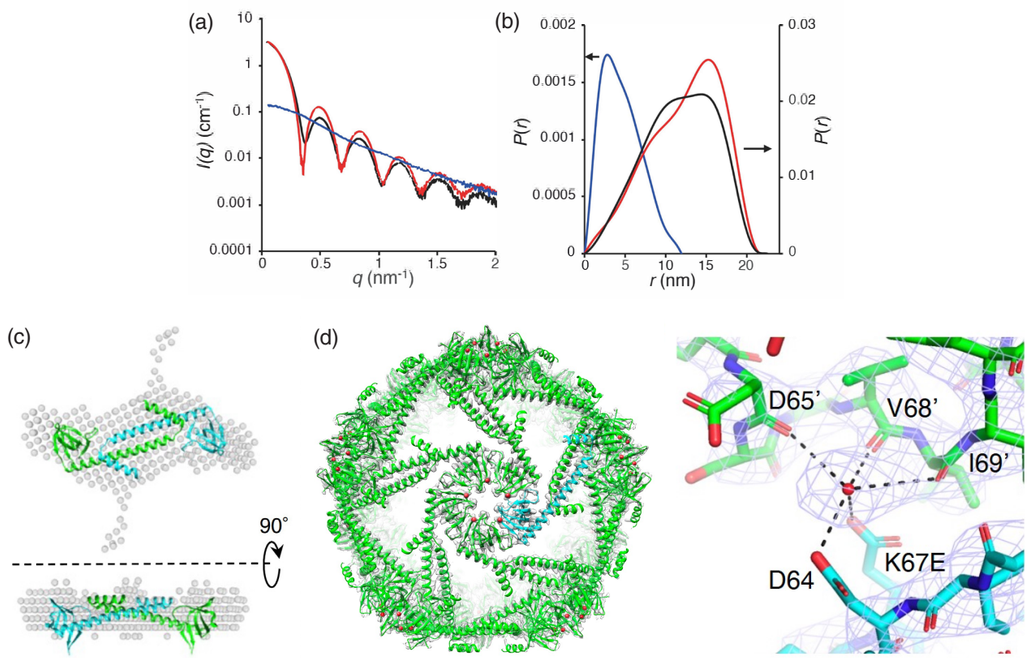

Figure 2: (a-c) Results of SAXS analysis. **(a)** Scattering curves of the original TIP60 (black), TIP60 (K67E) with EDTA (blue), and mTIP60-Ba (red). **(b)** Pair-distance distribution of the original TIP60 (black), mTIP60-Ba (red), and TIP60 (K67E) with EDTA (blue). **(c)** Dummy atom model of TIP60 (K67E) with EDTA (gray spheres). The cartoon model of the dimer extracted from the cryo-EM structure of the original TIP60 was superimposed using the program SUPCOMB. **(d)** Cryo-EM potential map and refined model of mTIP60-Ba (left panel) and close-up view of the cryo-EM structure and map of a Ba ion surrounded by oxygen atoms in amino acid residues (right panel) (PDB ID: 7XM1). This Figure is adapted and reproduced from reference [5]. Copyright 2023 American Chemical Society.

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