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Three-dimensional protein assemblies directed by orthogonal non-covalent interactions†

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In this report, an orthogonal non-covalent interaction strategy based on specific recognition between sugar and protein, and host–guest interaction, was employed to construct artificial three dimensional (3D) protein assemblies in the laboratory.

Protein self-assemblies inspire board research interest, due to their crucial role in nature,[1] as well as their potential applications as biocompatible and biodegradable materials.[2] Biologists demonstrated that self-assembled protein structures, *e.g.* filaments,[3a] microtubules,[3b] virus capsids,[3c] *etc.* are essential for various functions, including material and energy transportation, gene encapsulation *etc.* Meanwhile, these biomaterials have a precise and uniform nanostructure, which renders them significant advantages over other organic structures made from polymers or small molecules.

In the past decades, creative strategies have been developed to construct artificial protein nanostructures,[4] including controlling protein symmetry, developing fusion proteins[5] or genetically modifying the protein surface with functional groups.[4e] Different kinds of non-covalent interactions, *i.e.* metal coordination,[6] host–guest interactions,[7] hydrophobic interactions[8] *etc.*, have been used to obtain protein nanostructures, including nanowires, nano-rings, helical tubes, three-dimensional structures *etc.* For example, Liu and co-workers modified dimeric glutathione *S*-transferase with tripeptide Phe-Gly-Gly (FGG) on its symmetrical sides and then constructed a protein nanowire structure by connecting FGG with cucurbit[8]uril (CB[8]).[7a]

To our knowledge, in nature, proteins often form 1D, 2D or 3D nanostructures *via* multiple interactions orthogonally, *i.e.* the different interactions work independently.[9] Biodiversity contributes to the smart world significantly in its own way.[10] Thus it is desirable for chemists to achieve orthogonal self-assembly of proteins in the laboratory for biomimetic studies. In the past decades, various supramolecular structures, starting mostly from different synthetic building blocks, have been constructed *via* orthogonal self-assembly.[11] However, as far as we know, few reports demonstrated the formation of regular protein assemblies in the nm or μm scale *via* orthogonal interactions. A beautiful example was given by Ward and Hayashi, in which apomyoglobin and streptavidin were linked alternatively *via* dual molecular recognitions.[8d] Recently we demonstrated that by using a synthetic small molecular ligand composed of both sugar and rhodamine B (RhB), proteins self-assembled into 3D crystals with interpenetrating networks[12] or helical microtubes,[13] driven by protein–sugar interaction and π–π stacking of RhB. It was found that in this self-assembly process, the protein–sugar interaction took place first followed by the π–π stacking. To make the two interactions occur independently, *i.e.* to achieve orthogonal protein self-assembly, a new ligand is designed in the current research, in which π–π stacking of RhB was replaced by the molecular recognition based on cucurbit[8]uril (CB[8]). Then a new kind of artificial protein assembly was constructed *via* orthogonal non-covalent interactions, *i.e.* the recognition between sugar–protein and the host–guest interaction based on CB[8].

To perform orthogonal self-assembly of proteins, Concanavalin A (ConA), a homotetrameric protein exhibiting D2 symmetry, was selected as a model (concentration calculated as its monomer in this work). On each of its monomer, there is a sugar-binding site, favoring α-mannopyranoside (Man), which is one of the orthogonal interactions. For the other one, we chose the inclusion complexation based on CB[8]. Due to its high binding ability to guest molecules, many kinds of supramolecular polymers have been constructed.[14] Thus ligands containing Man and guest species to CB[8] were designed. As shown in Fig. 1, ligands **NapMan** and **AzoMan** having naphthyl or azobenzene groups as guests, respectively, were synthesized. As reported in the literature, one CB[8] could bind two naphthalene molecules at the same time

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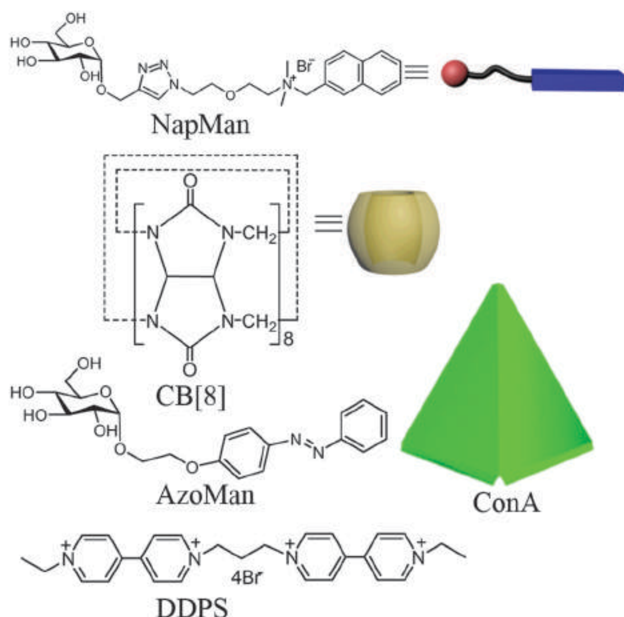

Fig. 1 Chemical structures and cartoon representation of **NapMan**, **AzoMan**, **DDPS**, CB[8] and ConA.

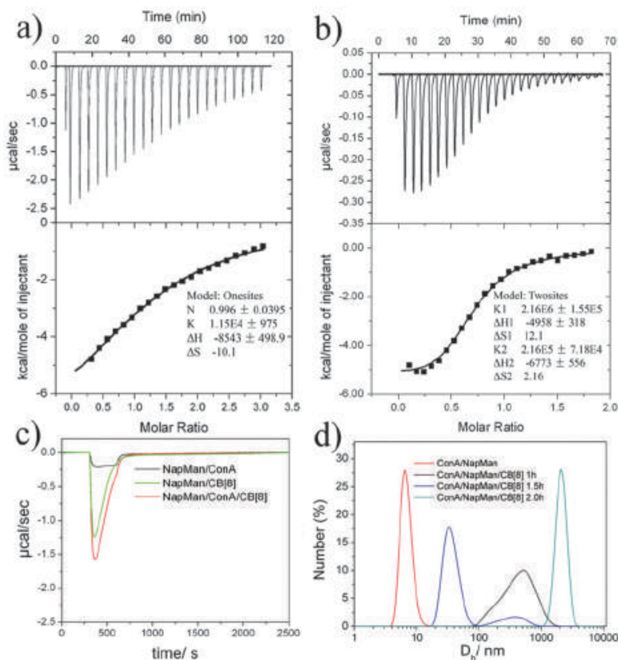

Fig. 2 ITC results of raw and integrated data for titration of (a) **NapMan** (1.5 mM) to Con A (0.1 mM) (calculated as monomer) and (b) mixture of ConA and **NapMan** (0.6 mM) to CB[8] (0.02 mM) in aqueous solution at 20 °C. (c) SIM-ITC data of 0.59 mM **NapMan** titrating into 0.05 mM ConA, 0.025 mM CB[8], and the mixture of 0.05 mM ConA and 0.025 mM CB[8]. (d) DLS results of ConA/**NapMan** solution after addition of CB[8] at different time intervals.

with a binding constant of approximately 10^{11}–10^{12} M^{-2}[15] or with one azobenzene and one dipyridyl salt (**DDPS**) forming a 1 : 1 : 1 supramolecular complex.[16] The two pairs of interactions were quite successful in making different kinds of supramolecular systems.[17]

In order to determine the ConA/Man recognition and the host–guest interactions based on CB[8] quantitatively and particularly to explore whether one interaction can be realized without the interference of the other, a series of isothermal titration calorimetry (ITC) measurements were performed as follows. First, the binding constant of **NapMan** with ConA was measured as 1.15×10^4 M^{-1} (Fig. 2a), while fitting the ITC result of **NapMan** with CB[8] with a two-site binding model gave high binding constants of $K_1 = 1.09 \times 10^6$ M^{-1}, $K_2 = 8.18 \times 10^5$ M^{-1} and a 2 : 1 stoichiometry of **NapMan** and CB[8] (Fig. S1, ESI†). The interaction was also supported by ^{1}H NMR (Fig. S2a, ESI†) and UV-vis spectroscopy (Fig. S2b and c, ESI†), and the latter further proved the 2 : 1 binding ratio of **NapMan** and CB[8]. Then the binding constants were measured again in the ternary system ConA/**NapMan**/CB[8]. As shown in Fig. 2b, an equivalent mixture of ConA and **NapMan** (0.6 mM) in aqueous solution, in which Man/ConA binding already existed, was titrated to the solution of CB[8] (0.02 mM). A two-site binding model gave binding constants of $K_1 = 4.56 \times 10^6$ M^{-1}, $K_2 = 3.51 \times 10^5$ M^{-1}, which were close to the constants of **NapMan** and CB[8] without ConA. Meanwhile, a mixture of CB[8] (0.5 mM) and **NapMan** (1 mM), was titrated to a solution of Con A (0.05 mM) with the binding constant measured as 1.4×10^4 M^{-1} (Fig. S3, ESI†), which was also consistent with that of **NapMan** and ConA without CB[8]. Moreover, the results obtained from single mode ITC (SIM-ITC) proved that the interactions took place simultaneously. As shown in Fig. 2c, the heat quantity (3.65 μcal) of **NapMan** titrating to the mixture of ConA/CB[8] was almost equal to the sum of the heat released from equivalent **NapMan** titrating to ConA (0.93 μcal) and to CB[8] (2.79 μcal) separately. Similarly, when **AzoMan** was used as a ligand, the consistency of binding constants in the four component system ConA/**AzoMan**/CB[8]/**DDPS** to the corresponding ones in literature also supported the possibility of orthogonal self-assembly (Fig. S4 and S5, ESI†).

To perform the orthogonal protein self-assembly, an equimolar **NapMan** and ConA was mixed with 0.5 equiv. CB[8] in 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer (20 mM) ([NaCl] = 20 mM, [$CaCl_2$] = 5 mM, [$MnCl_2$] = 5 mM), and the final concentration of **NapMan** and ConA was 0.15 mM. After the three components were mixed together, an assembled structure formed with its size increasing along with time until precipitates were formed after 3 h, as monitored by dynamic light scattering (DLS) (Fig. 2d). Similar results for the self-assembly of the four component system ConA/**AzoMan**/CB[8]/**DDPS** were also obtained from DLS (Fig. S6, ESI†). After the assembly was formed, addition of 10 equiv. mannose or 10 equiv. adamantane hydrochloride (Ada) to the ConA/**AzoMan**/CB[8]/**DDPS** solution induced disassociation of the assembly because of supramolecular competition (Fig. S7, ESI†). Moreover, the size distribution of the assembly can be controlled by addition of free mannose. When 5 equiv. mannose was added while ConA/**AzoMan**/CB[8]/**DDPS** were mixed together, the assembly size remained stable around several hundred nanometers without precipitation within at least 12 h. However, when

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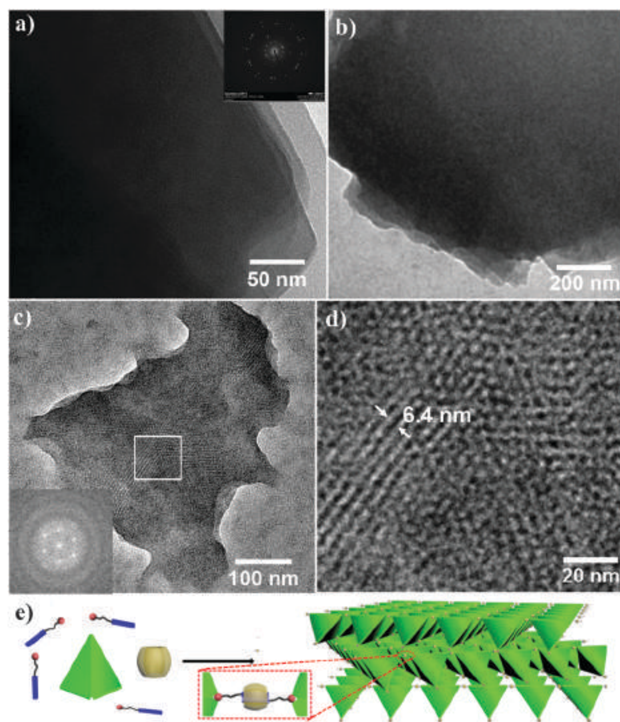

Fig. 3 (a) HR-TEM image of ConA/**AzoMan**/**DDPS**/CB[8] (diffraction pattern inserted). (b) TEM and (c) HR-TEM images of the ConA/**NapMan**/CB[8] assembly. The fast Fourier transform result was inserted in (c). (d) The zoomed-in image in the white box in (c). (e) Possible mechanism of the protein assembly formation.

10 equiv. mannose was used in the same experiment, no assembly was observed within 12 h (Fig. S8, ESI†).

More importantly, TEM was employed to observe the morphology of the assembly with negative staining (2% uranyl acetate). As shown in Fig. 3, the images clearly showed that both ConA/**AzoMan**/CB[8]/**DDPS** (Fig. 3a) and ConA/**NapMan**/CB[8] (Fig. 3b) assemblies were stacked layers. On the edge of the former structure (Fig. 3a), more than five layers were clearly observed. Each layer has a rather regular shape, although different layers could not stack into regular macroscopic objects. The diffraction pattern (inset of Fig. 3a) showed their crystalline state of protein assembly. Similarly, the diffraction pattern of the **NapMan**/CB[8]/ConA system was also clearly observed (inset of Fig. 3c). Furthermore, under a high resolution TEM (Fig. 3c and d), aligned regular patterns inside the layers of proteins were clearly observed. The size of the repeating unit was about 6.4 nm in the ConA/**NapMan**/CB[8] assembly. According to all the above results, a possible mechanism of orthogonal protein self-assembly is shown in Fig. 3e, *i.e.* ConA interacted with **NapMan** *via* the specific ConA/Man interaction, while CB[8] induced the dimerization of **NapMan** *via* CB[8]/Nap interaction. These components packed regularly into the protein layers, which finally stacked into macroscopic precipitates.

Finally, light responsiveness of the ConA/**AzoMan**/**DDPS**/CB[8] assembly was explored. It is known that under UV light, *trans*-azobenzene isomerizes into its *cis*-isomer and loses its binding ability to CB[8], while under visible light the *cis*-isomer can be transformed back to the *trans* one.[17] We found that this feature could persist in the protein assembly containing **AzoMan**. As shown in Fig. S9 (ESI†), after UV irradiation (λ = 365 nm) for about 30 min, the absorption intensity of ConA/**AzoMan**/**DDPS**/CB[8] at about 345 nm almost disappeared, while the absorption around 428 nm became higher (red line in Fig. S9, ESI†), showing the successful conversion of **AzoMan** from a *trans*- to a *cis*-isomer. Then the sample was placed under visible light and UV-vis spectroscopy was used to trace the isomerism of **AzoMan** from *cis* to *trans* in the assembly. It was found that within 230 min, the absorption at 345 nm was steadily recovered to the absorption intensity before UV irradiation (black line in Fig. S9, ESI†), while that at 428 nm went down to almost no absorption. The UV-vis absorption spectra clearly indicated that the isomerism of azobenzene had been reserved in the assembly. However, we did not observe obvious disappearance of the assembly under UV irradiation, which means that dissociation of the protein assembly did not occur completely. This phenomenon was unexpected. We speculate that the regular pattern inside the assembly might be too tight to be disrupted by the isomerism of azobenzene.

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