Hierarchical self-assembly of native protein and its dynamic regulation directed by inducing ligand with oligosaccharide

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ABSTRACT

Proteins self-assemble into kinds of sophisticated architectures is ubiquitous in nature. Construction of these nanostructures in laboratory is helpful for understanding this physiological process and obtaining new functional materials. Although current state-of-the-art strategies have been developed, effective methods to regulate the process are still deficient. Herein, hierarchical self-assembly of Con A induced by a new designed oligosaccharide inducing ligand (RhB-triMan) was reported. The self-assembly of Con A can be regulated in the presence of transition metal ion Mn^{2+} due to the opening of spirolactone of RhB, and the assembly process can be further regulated by changing the molar ratio of ligands to proteins.

1. Introduction

As significant building blocks of organisms, proteins can self-assemble into a variety of sophisticated topological and functional architectures with different dimensions, which play pivotal roles in series of life processes [1–5]. Inspired by these precise and functional natural protein assemblies, various innovative strategies [6,7] have been proposed for the construction of artificial protein assemblies in the past few decades. With the development of supramolecular chemistry, kinds of weak interactions (e.g. host–guest interactions [8,9], π–π stacking interactions, metal [10–13] coordination and hydrophobic interactions etc.) have been employed to develop novel methods for fabricating artificial protein nanostructures due to their dynamic reversibility and hierarchical tunability. Benefiting from these advantages, a large number of well-designed artificial protein assemblies have been constructed, including 1D nanowire, 2D nanoring and network, 3D crystal structure etc. For example, Tczan and coworkers exploited the directionality and strength of metal coordination interactions to guide the formation of protein assemblies, generating 1D helical nanotubes and 2D crystalline sheets [10].

Recently, we have successfully prepared 3D crystalline frameworks through dual supramolecular interactions by using the small molecular inducing ligand, which consists of a saccharide moiety and a rhodamine B (RhB) moiety tethered by a flexible oligo(ethylene glycol) linker, in which saccharides could bind with lectins through molecular recognition and the RhBs are prone to dimerize via π–π stacking interaction [14]. The feasibility and practicality of this strategy have been well demonstrated by the further successful extension on construction of kinds of protein assemblies with morphologies from 1D to 2D and 3D structures via changing carbohydrate and protein pairs [15–19]. In addition, slight tuning of the linker length of the ligand may affect the protein-assembly morphologies, because appropriate linker length is required for tuning the carbohydrate-protein interactions and protein-protein interactions (PPIs) in ordered packing of proteins [16].

According to the pervious kinetics study about the ligand-induced protein self-assembly, the recognition between lectin and carbohydrate is the premise of the dimerization of RhB. Therefore, it’s envisioned that introduction of carbohydrate with stronger binding ability to protein could improve the efficiency and even change the direction of protein self-assembly. To further understand detail information of protein self-assembly directed by the inducing ligand, we herein report the self-assembly of protein Con A induced by a new ligand (RhB-triMan) without a flexible linker. The study shows that the molar ratio of RhB-triMan to Con A have large influence on the protein assembly kinetics and morphologies. The assembly processes revealed kinetic acceleration with the increase of ligand contents and the assembled morphologies can be mediated by stoichiometry ratio. Moreover, the protein self-assembly processes induced by the new ligand exhibited classical hierarchical self-assembly characteristics, i.e. the morphologies transformed from vesicles to short nanofibers with the time went by. Furthermore, computational studies have also been conducted for the in-depth understanding of the formation of each morphology, providing

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guidance on the design strategy to achieve tunable protein assemblies.

2. Results and discussion

2.1. Design of new small molecular inducing ligand RhB-triMan and noncovalent interactions

Concanavalin A (Con A) is a homo-tetramer protein exhibiting $D_2$ symmetry, as a tetrahedron with a side length of 7.9 nm and the distance from center to vertex is about 4.8 nm (Fig. 1a), which can specifically bind with various carbohydrates containing terminal $\alpha$-$D$-mannosyl and $\alpha$-$D$-glucosyl groups in the presence of two divalent ions of Ca$^{2+}$ and Mn$^{2+}$. It is worth noting that the triMan ($\alpha$-$D$-mannopyranosyl-(1 $\rightarrow$ 2)$\alpha$-$D$-mannopyranosyl-(1 $\rightarrow$ 2)$\alpha$-$D$-mannopyranose, Fig. 1b) binds to Con A much stronger than $\alpha$-mannopyranoside (Man) [20,21]. To investigate the effects of triMan on self-assembly process of protein, a new inducing ligand (RhB-triMan, Fig. 1c) was designed and prepared by coupling between triMan and RhB hydrazide [22] in the presence of catalytic amount of Y(OTf)$_3$ [23]. To illuminate the self-assembly mechanism of Con A induced by the new ligand RhB-triMan, the binding constant of RhB-triMan with ConA was firstly measured by isothermal titration calorimetry (ITC), and a value of $2.84 \times 10^5$ M was afforded, which is almost one order higher than the binding constant of triMan and ConA (ca. $2.45 \times 10^4$ M) (Fig. S1).

Considering that ring-opening process of the rhodamine spirolactam could be induced by some metal ions (such as Cu$^{2+}$, Zn$^{2+}$ etc) [22], whether the indispensable divalent ions of Ca$^{2+}$ and Mn$^{2+}$ for specific recognition of Con A and oligosaccharides could affect self-assembly process through the ring-opening process will be explored. In addition, according to the previous studies, the ligand-induced protein self-assembly proceeded via a cascade mode, which involved two different supramolecular interactions, the carbohydrate bind with protein immediately through molecular recognitions followed by dimerization of the RhB by $\pi$-$\pi$ stacking. Therefore, with a strong binding motif triMan, the ratio of the ligands to proteins on the influence of protein self-assembly could be carefully investigated.

2.2. Influences of transition-metal ion on the spirolactam ring-opening of the RhB-triMan

To verify the existence of spirolactam in RhB-triMan, Cu$^{2+}$ ions which have been reported for ring-opening of spirolactam were used to make pretest analysis. It’s shown that upon the addition of Cu$^{2+}$ to a solution of RhB-triMan in 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer (pH = 7.4, [HEPES] = 20 mM, [Na$^+$] = 20 mM), a significant color change and an obvious enhancement of ultraviolet–visible (UV–vis) spectrum peak in 450–600 nm range were simultaneously observed (Fig. S2). However, when Ca$^{2+}$ was added to the RhB-triMan solution, there was no obvious color change and characteristic peak of ring-opened RhB in UV–vis absorption spectra (Fig. S3), whether to increase Ca$^{2+}$ concentration or extend observation time and even raise temperature. After treatment of RhB-triMan solution with MnCl$_2$, no difference was observed during the first few hours compared with the case of Ca$^{2+}$ involved. However, the color of the solution changed from the original colorless to pink after standing at room temperature overnight and the color further deepened as the time prolonged. Such phenomena were well illustrated by UV–vis and photoluminescence (PL) spectra (Fig. 2). According to the above experimental results and the $^1$H NMR spectra (Fig. S4), we speculate that the Mn$^{2+}$ rather than Ca$^{2+}$ could induce
the spirolactam ring-opening of the RhB-triMan. Thus, a possible structure of the spirolactam ring-opened RhB-triMan was proposed (Fig. 1c).

### 2.3. Preparation and characterization of the protein assemblies

Con A and RhB-triMan were dissolved in HEPES buffer (pH = 7.4, [HEPES] = 20 mM, [Na\(^+\)] = 20 mM, [Ca\(^{2+}\)] = 5 mM, and [Mn\(^{2+}\)] = 5 mM) with the same concentration of 200 μM (calculated as binding sites) and incubated at 4 °C for several days. During the incubation, the self-assembly process was monitored by dynamic light scattering (DLS) and transmission electron microscopy (TEM). No obvious assembly phenomenon was observed before the incubation time reached about 18 h. At this moment, some vesicles were observed from TEM images (Fig. 3d) accompanied with increase of the incubating time. 20 h later, fused and some ruptured vesicles were observed (Fig. 3e) when the assembled time was further extended. It was interesting to note that the number of such short nanofibers greatly increased. According to these results, a possible mechanism for evolution of self-assembly morphology was proposed in Fig. 3g. The protein vesicles were firstly formed followed by fusion to some large composite vesicles. With the increase of the size of the composite vesicles, some of them gradually ruptured and fused due to the instability of these structures. Finally, the ruptured vesicles fused further and curled to short nanofibers. This dynamic evolution process of the self-assembled morphologies showed classical hierarchical feature.

### 2.4. Study on the hierarchical self-assembly process

Why the self-assembly of RhB-triMan induced Con A undergoing such hierarchical processes? According to the previous research, the rate-determining step of ligand-induced protein self-assembly process is the dimerization of rhodamine B moiety, because the recognition between carbohydrate and lectin could occur immediately. Whereas in this case, Mn\(^{2+}\) induced RhB-triMan spirolactam ring-opening could be an even more significant time-dependent process than the dimerization of RhB. When the two components were equivalently mixed together, the tri-Man moiety bind with Con A immediately, but the rhodamine B moiety retarded further self-assembly process because the rhodamine spirolactam is harmful for π-π stacking. Therefore, no assembly was observed during a considerable period of time. However, with increase of the processing time, the Mn\(^{2+}\) continuously promotes the spirolactam opening of RhB-triMan to restore the original conjugated structure of rhodamine B, thus the dimerization of rhodamine B moiety recovered gradually and the number of assemblies increased at the same time. In the meantime, the dimerization of RhB was also verified by UV–vis absorption spectra (Fig. S6a) and circular dichroism (CD) spectra (Fig. S6b). As shown in Fig. S6d, the peaks appeared at around 525 nm and 580 nm in the CD spectra were signals of RhB dimerization with different aggregation states, which corresponding to the signal of UV–vis spectra. In addition, the control experiments supported the role of the dimerization of RhB moiety, the DLS result showed there was no assembly formed even standing after 1 week when the triMan mixed with Con A (Fig. S7) and the self-assembled vesicles dissociated immediately in the presence of excess β-cyclodextrin (β-CD, 1 mM) due to destruction of π-π stacking of rhodamine (Fig. S8).

To further investigate the kinetics of the RhB-triMan induced Con A self-assembly processes, the molar ratio of ligand RhB-triMan to

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**Fig. 2.** Ring-opening process of the rhodamine spirolactam induced by 5 mM Mn\(^{2+}\). (a) Ultraviolet–visible (UV–vis) absorption spectrum and (b) Photoluminescence (PL) spectra at different incubation time. (c) Photographs of color changing with time.
When the ratio was increased to 2, two peaks in the DLS result indicated formation of self-assembled structures in different sizes after mixing the ligand (400 $\mu$M) and Con A (200 $\mu$M monomer) for 2 h (Fig. 4a). Correspondingly, coexistence of vesicles and short nanofibers were observed in the TEM images, and it can be seen clearly that some vesicles were fused with each other to form the fibers (Fig. 4b). After 10 h incubation, the solution was characterized by DLS again, disappearance of the peak at $\sim$100 nm of DLS (Fig. 4a) implied the almost complete transformation of vesicles to nanofibers. TEM images further showed that there were only some long nanofibers with helical structure and almost no vesicles could be found (Fig. 4c). 24 h later, much longer nanofibers than those at initial state were observed in TEM images (Fig. 4d).

Keeping the concentration of Con A at 200 $\mu$M, when the molar ratio of ligand (600 $\mu$M) to Con A was further increased to 3, DLS monitoring indicated the self-assembly process started in a very short time (Fig. 5a), this process was much faster than the case when the molar ratio of ligands to proteins was 2. Meanwhile, according to TEM images (Fig. 5c), long and tangled fibers could be observed during the self-assembly process. In addition, the AFM images (Fig. 5d) showed that entanglement between long fibers became more and more obvious with increase of incubation time. Intriguingly, observable amount of aggregate precipitation could be immediately formed, after mixing the ligand (800 $\mu$M) and protein (200 $\mu$M monomer) with a molar ratio of 4. According to the above-mentioned results, the self-assembled morphologies largely depend on the molar ratio of ligands to proteins, which makes it possible to control the self-assembled morphologies and the self-assembly kinetics by changing the stoichiometry of the molar ratio of ligand to protein at the same time.

2.5. Computational studies of the self-assembly process

Further, computational studies have also been conducted to understand the hierarchical self-assembly process, providing guidance on the design strategy to achieve tunable protein self-assemblies. For the Con A tetramer with four binding sites, there are five kinds of binding states of Con A and RhB-triMan, including free Con A and the conjugates of Con A attached with different numbers of RhB-triMan (Con A/ $n$RhB-triMan, $n = 0$–4). Assuming that each binding site has the
same ability to bind with triMan, the probability distribution function \( F_n \) for Con A-nRhB-triMan conjugates to the concentration ([C]) of Con A and molar ratio \( (R) \) of ligand to Con A was constructed, combining with the binding constant \( (k) \) of Con A and RhB-triMan measured from ITC, the deduction process was shown in Supporting Information. Based on the calculated results of the probability distribution function, the distribution diagrams were obtained (Fig. S10), and the fractions of each conjugates at a given concentration or molar ratio of ligands to proteins could be easily afforded from the diagrams. During the studies of the effect of changing molar ratio of RhB-triMan to Con A on self-assembly, the final concentration of Con A was kept at 100 μM all the time. According to the calculation results of probability distribution, when the final concentration of Con A was 100 μM and the RhB-triMan and Con A were with equal molar ratio, the probabilities of Con A binding with one or two RhB-triMan was ~32% and 36% (Fig. 6) respectively. When the molar ratio of ligands to proteins was increased to 2 and even to 3, the number of RhB-triMan of Con A binding also increased, but the probability of forming Con A/2RhB-triMan conjugate still remained at a very high value (Fig. 6 and Table S1), therefore, the assemblies tend to form a fibrous structure at this concentration. Meanwhile, when the molar ratio of ligands to proteins was 3, we found that the probability of forming Con A/3RhB-triMan conjugate also increases, this could explain the observation of the bifurcated structures. However, when the molar ratio of ligands to proteins continued to increase to 4, the probability of Con A binding with 3 or 4 RhB-triMan account for the major proportion (41%, 39%) and it was easy to form cross-linking networks and led to formation of precipitation. The precipitate are mainly some irregular sheets, some of them has neat arrangement, and the lattice spacing of the sheets with neat arrangement is about 5 nm, the TEM image is provided in Fig. S11. Therefore, the assembly kinetics could be easily controlled by the molar ratio of ligands to proteins, the whole process could be summarize as below Fig. 6.

3. Conclusion

In conclusion, hierarchical self-assembly of a natural tetrameric protein was induced by a new ligand comprising of triMan and RhB with spirolactam structure in the presence of Mn²⁺. Experimental and computational studies shown that the transformation from vesicles to nanofibers are largely affected by the mole ratio of ligands to proteins, increase of the ratio may significantly accelerate the morphology evolution. These results will facilitate the understanding of the self-assembly mechanisms of protein in nature and provide a new way to realize the regulation and control of protein self-assembly.

4. Experimental

4.1. Materials and methods

Rhodamine B (98%) and YOTf (98%) were purchased from TCI Shanghai; Con A (95%) was purchased from Sigma-Aldrich; CuSO₄ (98%), 4-(2-hydroxyethyl)-1-piperazinethanesulfonic acid (HEPES) were purchased from J&K Chemical; NaCl (98%), CaCl₂ (98%) and MnCl₂ (98%) were purchased from Sinopharm Chemical Reagent Co., Ltd.

4.2. Instruments and measurements

Nuclear magnetic resonance (NMR) spectra were obtained at a
Fig. 5. Self-assembly of RhB-triMan/ConA under a ratio (L:P) of 3. (a) Variation of particle size measured by DLS; (b) Diagram of multi-fibers. (c) TEM images. (d) AFM image.

Fig. 6. Summary of the ratio of ligands to proteins on the influence of protein self-assembly.
400 MHz Bruker AVANCE III HD spectrometer. Matrix Assisted Laser Desorption Ionization-Time of Flight (MALDI-TOF/TOF-MS) Spectra were performed using AB SCIEX (USA) 5800 instrument with the reflection mode. Isothermal titration calorimetry (ITC) experiments were conducted on a Micro-Cal VP-ITC system at 20.00 ± 0.01 °C. Dynamic light scattering (DLS) was taken by 3D modulated laser light scattering from LS Instruments. Photoluminescence (PL) spectra were recorded on a QM 40 spectrometer of PTI. UV–vis spectroscopy (UV–Vis) spectra were taken by a Lambda750 spectrophotometer of Perkin-Elmer with a 1 mm cuvette. Circular dichroism (CD) spectra was taken by a Chirascan instrument (Applied Photophysics) with a 1 mm cuvette. Transmission electron microscopy (TEM) images were taken with Tecnai G2 instrument (200 kV). The TEM sample prepared as followed: the copper grid was put on a glass slide to give a hydrophilic treatment. 3–5 µL of sample solution was dropped on the copper grid and standing for 1 min. The excess solution was removed with filter paper followed by negative staining with 1 wt% uranyl acetate aqueous for 30 s. Atomic Force Microscope (AFM) experiments was performed in tapping mode with RTESP probe in Bruker Multimode VIII SPM equipped with a J scanner.

4.3. Synthesis of RhB-triMan

To a solution of RhB hydraylide (0.20 g, 0.44 mmol) in anhydrous MeOH (10 mL) was added trMan (186 mg, 0.37 mmol) and Y(OTf)₃ (59 mg, 0.11 mmol) under argon atmosphere. After stirring at room temperature for 72 h, the solvent was removed under reduced pressure, and the crude product was purified through silica gel column chromatography (isopropanol/ ammonium hydroxide, 3/1) to give RhB-triMan (0.26 g, 0.28 mmol) as a white solid in 63% yield. 1H NMR (400 MHz, MeOD) δ 7.91–7.82 (m, 1H), 7.61–7.43 (m, 2H), 7.12–6.95 (m, 1H), 6.57–6.24 (m, 6H), 5.28–5.24 (m, 1H), 5.00–4.93 (m, 1H), 4.07–4.01 (m, 1H), 3.99–3.89 (m, 2H), 3.88–3.44 (m, 12H), 3.44–3.33 (m, 8H), 1.23–1.12 (m, 12H); MALDI-TOF/TOF-MS of RhB-triMan: calcd for [C₉₆H₁₄₆O₇7N₁₃]⁺ = 943.42, found 943.58. (1H NMR spectrum and MALDI-TOF of RhB-triMan see Figs. S12 and S13).

CRediT authorship contribution statement

Haijun Chen: Conceptualization, Methodology, Validation, Investigation, Writing - original draft. Guang Yang: Conceptualization, Methodology. Ensong Zhang: Formal analysis, Methodology. QiQi Ge: Resources. Rongying Liu: Methodology, Resources. Libin Wu: Resources. Yingfei Feng: Methodology, Resources, Writing - review & editing. Guosong Chen: Conceptualization, Methodology, Supervision, Writing - review & editing, Project administration, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.eurpolymj.2020.109871.