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ARTICLE TYPE

Tunable self-assembly of nanogels into superstructures with controlled organization

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The tunable pH-dependent lysozyme/xanthan gum nanogels were fabricated in alkali-coupled thermal treatment conditions. The nanogels could be self-assembled into superstructures with different controlled sizes as the pH value 10 of the system was regulated.

Multi-scale control of materials is always a challenging task in many biomedical and nanotechnology fields.¹⁻² Self-assembly based on non-covalent interactions provides a powerful strategy for structured organization at a microcosmic level.³ Over the past

- ¹⁵ decade, various self-assembled nanoparticles, regardless of organic, inorganic or hybrid materials, have been widely prepared. The discrete nanoparticles with favourable size and distribution tend to gather in a more stable system driven by Entropy.⁴ While superstructures with inorganic nanoparticles
- ²⁰ usually fabricated for especial optoelectronic or other practical applications.⁵⁻⁶ The common methods were cross-linking, solvent evaporation and template-patterning after the initial nanoparticles were synthesized.⁷ However, completely avoiding of organic reagents and chemical process, assembly into well-defined
- ²⁵ superstructures with accurate control is still a difficult challenge. Especially for biopolymers, their complicated charges and conformation properties made the self-assembly behavior complex and difficult to control. Therefore, there is scarce report about biopolymers self-assembly into superstructures.
- ³⁰ Most biopolymers commonly carry the free hydroxyl or/and amino groups along their backbone, resulting in different charged and soluble properties.⁸⁻⁹ Their nanoparticles self-assembly behavior would exhibit great differences in various conditions. Taken chitosan for example, a reversible Pickering emulsion
- ³⁵ system could be established base on its dissolution behavior in acidic and alkaline solution.¹⁰⁻¹¹ For most natural proteins, deprotonation and protonation of amines when the pH above or below of pI play crucial roles in their self-assembly behavior and structure stability.¹²⁻¹³ It is widely regard that steric effect
- ⁴⁰ maintains the stability of nanoparticles solutions. Moderate changes in the pH value, resulting in the zeta potential changes for nanoparticles, may promote and controlled fabricate superstructures by self-assembly of nanoparticles.

Our group interestingly found lysozyme (Ly) and xanthan ⁴⁵ gum (XG) have intensive interactions which was very sensitive to pH changes. Especially for their soluble complexes that suffered thermal treatment exhibited controlled self-assembly phenomenon. In this study, a series of assemblies with controlled organization and sizes were established by self-assembly of pH-⁵⁰ tunable lysozyme/xanthan gum nanogels (Ly/XG NPs). The tunable repeatability of Ly/XG NPs solution induced by pH adjusting was also investigated.

To prepare Ly/XG pH-tunable solution, XG and Ly solutions (1.0 mg/ml) were prepared in purified water with stirring for 6h 55 and 2h at 25°C, respectively. An equal amount of XG solution was added into the Ly solution before pre-alkalization (about pH 9.5). The final mixture pH value was adjusted to 12.5 and followed by stirring for another 15min. Subsequently, the mixtures were incubated at 80°C for 15min and cooled to room 60 temperature spontaneously. The pH of disposed solution was regulated to desired values. The average size, zeta potential, transmittance and TEM of the series solutions were characterized. The average size and zeta potential were measured by a commercial laser light scattering instrument (Malvern ZEN3690, 65 Malvern Instruments) at 25°C. The transmittances of each XG/Ly solutions were measured with a previous modified method using 722E visible-infrared spectrometer at 600 nm against a blank of pure water.14 The TEM of XG/Ly NPs was obtained using a

JEOL transmission electron microscope (H-7650, Hitachi, Japan).



Fig. 1 Transmittance of Ly/XG solutions as the function of pH values (A) and cycles (B)



Fig. 2 Distribution (A) and size (B) of Ly/XG nanoparticles as a function of pH values

Figure 1A showed that at high pH (>9, stage I), the s transmittance of Ly/XG mixture were more than 90% and the solution was transparent (as showed in the Fig.1A), indicating XG and Ly were consolute in the solution. At the moderate pH condition (6-9, stage□), the transmittance of the mixture sharply decreased with the pH decreasing. The transmittance at pH 9 was

- ¹⁰ about 90%, and the solutions obviously became micro blue opalescent when at pH was 8.5 (middle inset in Fig.1A). But the transmittance decreased to 30% when pH was 6.0. It was regard that the structure of Ly could be damaged and exposed more hydrophobic region during the high thermal treatment. The
 ¹⁵ characters of isoelectric point still exist but have been changed. As the Fig.1A shown, the pI of Ly transferred into the range between 9.0 and 10 after alkali coupled thermal treatment in the presence of xanthan gum. When pH higher than 9.0, Ly was
- deprotonated and the both biopolymers were both negative. ²⁰ While in range of 9.0 and 6.0, Ly was protonated, conferring cationic behavior. Ly and XG therefore undergo interassociations leading to the formation of gel nanoparticles driven by the electrostatic and hydrophobic interaction at the □ stage. It was noteworthy that the transmittance has maximum reduction ²⁵ rate when pH was about 8.5, indicating that the Ly/XG NPs system was unstable in current environment. While as the pH further decreased (<6.0, stage □) the structure of NPs swelled and
- they self-assembled into superstructures (although have been confirmed as NPs with larger size later) with loose structure for ³⁰ internal repulsion from Ly.

The transmittance conversion between pH 11 and 7 (Fig.1B) exhibited the formed Ly/XG NPs has excellent tunable self-assembly and dissociation properties. The tunable behavior was pH-dependent. It illustrated the Ly/XG NPs processed favourable ³⁵ repeatability when suffered pH conversion. The size of the Ly/XG NPs (pH 7.0) was slightly increased only after the turntable 5 cycles, indicating high stability and favorable switchable behavior (Fig.S1 and Fig.S2).



Fig. 3 TEM of Ly/XG nanoparticles as the system pH was 9 (A), 8.5 (B), 8(C), 7(D) and 6 (e, f)



Scheme 1 Illustration of Ly/XG self-assembly behaviour at the different pH condition

In the acidization, Ly/XG NPs was formed during the stage II and the size of the NPs was increased with the pH deceasing $_5$ (Fig.2A). As the Fig.1 shown, we believed the NPs with good distribution when pH ranged from 6.0 to 9.0. We artificially divided the stage II into two small groups. During the \Box_1 stage, the size of NPs sharply increased from 89.7nm to 366.8nm, while reached a platform (\Box_2) about 370nm in the pH range of 7 and 8.

- ¹⁰ As the pH further decreased, the size of Ly/XG complex became bigger on the base of □₂ and then exhibited a steady level (□). As we know, Ly was deprotonated at the pI condition. At pH about 9, the disordered coil of XG molecules for denaturation would wrap the Ly up for strong hydrophobic driving and shared
- ¹⁵ favorable distribution and size. While Ly protonatied in the Ly/XG complex (□₁), the NPs swelled for the repulsion between Ly molecules and exhibited an increasing trend in the hydrate particle size. The increasing behavior would be balanced with the inter-attraction between Ly and XG and displayed a platform.
 ²⁰ The NPs could be self-assembly in superstructures by aggregation
- for larger size to maintain the stability of the system as the pH below 6.0 (III) (Scheme 1).

Although different sizes were shown in the pH range (5.0-9.0), the Ly/XG complex systems were all stable without phase ²⁵ separation. To confirm our hypothesis, the zeta potential was measured (Fig.S3). In the pH range of 7.0-9.0, the zeta potential was about -27mV which could make the NPs considerable stable. The zeta potential follow increased to about -40 mV for sustaining the increasing size with pH reduction. The ³⁰ polydispersity (PDI) at different pH values also revealed the aggregation behaviour of Ly/XG NPs for increased values with

pH decreasing (Fig.S4). To further verify our hypothesis, the morphology of Ly/XG solutions with different pH was characterized by TEM and the ³⁵ results were displayed in Fig.3. It was obvious that the trend of Ly/XG complex sizes with different pH was completely consistent with the results shown in Fig.2. It is worth mentioning that Ly/XG NPs were self-assembled into superstructures when the pH was 6.0, which also explained why the size increased in

- ⁴⁰ the III stage. The higher resolution image (Fig.3f) clearly shows the discrete cell for the superstructures was also small Ly/XG NPs. Unfortunately, the Ly/XG NPs aggregated without any regular rules so that the superstructures distribution was not very well.
- ⁴⁵ The Ly/XG mixtures suffered thermal treatment (80°C, 15min) with other pH values (12 and 12.2) also exhibited pH-response

(Fig.S5). However, it was obvious that it the solution did not show good responsive behavior and hard to control as compared with the mixtures that disposed at pH 12.5. Even so, the Ly/XG 50 mixtures heated at pH 12.2 trended to have responsive behavior that was close to that of the Ly/XG mixtures disposed at pH 12.5. The comparative results illustrated appropriate thermal treatment in certain condition would make the Ly/XG mixtures show selfassembly behavior. The phenomenon revealed the aggregation 55 behavior has large relations with Ly and XG structures, especially with the Ly higher structure. Additionally, FTIR was further to explore the potential chemical changes during the disposed process (Fig.S6). The results obviously demonstrated the structures of Ly and XG undergone great changes during the 60 alkali-coupled heat-treatment. For Ly, the most distinctive peaks for proteins are amide I and II bands approximately between 1650 cm⁻¹ and 1540 cm⁻¹, respectively.¹⁵ It clearly demonstrated the intensity of the two absorptions decreased sharply as it suffered alkali-coupled thermal treatment. Additionally, the band intensity 65 of 1441 cm⁻¹, which assigned to asymmetric and symmetric stretching vibrations of COO, increased significantly as the disposed pH increasing. The variation in the spectrum further confirmed the protonation/deprotonation behaviour of COOH and NH₂ that was closely resulted from pH change.¹⁶ The XG ⁷⁰ spectrum about 1441 cm⁻¹ shared the same trend with Ly for pyruvate that produced the negative charges.¹⁷ The other obvious phenomenon was the 1725 cm⁻¹ attributed to the acetyl group in the side chain of XG disappeared during the alkali treatment¹⁸, indicating a stronger hydrophobicity for XG. So, moderate

⁷⁵ changes in conformation and charges for Ly and XG were crucial to self-assembly behaviour, indicating electrostatic and hydrophobic interaction were the dominant driving powers.

Conclusions

⁸⁰ Tunable Ly/XG nanogels were fabricated through regulating the pH of Ly/XG mixtures which has been exposed moderate alkalicoupled thermal treatment. The switched behavior could be transferred between cosolubilization and nanogels when the pH of solutions changed between 7 and 11. The pH-response of the ⁸⁵ samples exhibited favorable repeatability. Co-solution, nanogels and superstructures based on the Ly/XG NPs could be obtained at different pH stages as it was tunable when adjusted. The self-assembly phenomenon in this paper illustrated superstructures based nanoparticles could be controlled via regulation in the proper conditions for biopolymers.

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