Supplementary Material (ESI) for Chemical Communications

Electronic Supplementary Information

Stimuli-responsive nanoparticles composed of naturally occurring amphiphilic proteins

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1. Materials

Bovine β-casein was purchased from Sigma-Aldrich (USA). 8-Anilino-1-naphthalene sulfonate (ANS) and 2,4,6-trinitrobenzenesulfonate (TNBS) were purchased from Tokyo Chemical Industry, Co., Ltd. (Tokyo, Japan). 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) was purchased from Peptide Research Institute (Osaka, Japan). All other chemicals were of the highest purity commercially available.

2. Preparation of casein nanoparticels (CNPs)

β-Casein was dissolved in 50 mM phosphate buffer (pH 7.0). Then, an appropriate amount of aqueous stock solution of EDC in the same buffer was added to obtain the concentration of 25 ~ 250 mM, and the reaction mixture was placed at 4, 20 or 40 °C for 24 h under gentle stirring. The final concentration of β-casein was 5 mg/mL in each case. The EDC stock solution was used immediately after the preparation. The chemical treatment at 40 °C for 24 h resulted in the formation of insoluble aggregates (Fig. S1A), and the further characterization was impossible. On the other hand, CNPs can be obtained by the chemical treatment at 4 or 20 °C.

The aqueous solution containing the cross-linked products after the incubation at 4 °C for 24 h was passed through a 0.2 μm Millipore membrane filter to remove the insoluble fraction. The filtrate was then applied to a size-exclusion column (PD10 sepharose column, GE Healthcare) and the first elution was pooled. The SDS-PAGE analysis of cross-linked products showed that both samples were substantially purified and could not migrate the polyacrylamide gels due to the significant increase in molecular weight (Fig. S1B).

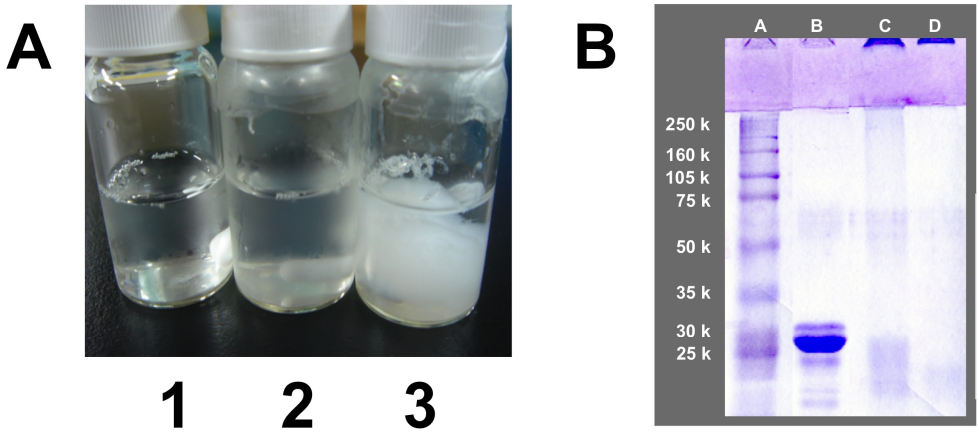

Fig. S1. (A) A photograph of the reaction mixture containing cross-linked products with 250 mM EDC after placing at 4, 20 or 40 °C (from 1 to 3) for 24 h under gentle stirring. (B) SDS-PAGE analysis of purified cross-linked products. Lane A: molecular weight markers; lane B: β-casein; lanes C and D: β-casein cross-linked with 50 mM (C) or 250 mM (D) EDC.

3. Characterization of CNPs

3-1. Determination of degree of cross-linking

The degree of cross-linking was determined by the quantification of residual primary amino groups in CNPs using TNBS according to the procedures of Ref. 20. The degree of cross-linking was calculated based on the calibration curve constructed with free β-casein.

3-2. Fluorescent measurements

An aqueous stock solution of ANS was added to the aqueous solution of CNPs (50 mM phosphate buffer, pH 7.0). The final concentrations of ANS and CNPs were 10 μM and 0.5 mg/ml, respectively. Fluorescent spectra were measured using luminescent spectrometer (LS55C, Perkin Elmer). The excitation wavelength was 350 nm, and the fluorescent intensity was measured at 480 nm. In the absence of β-casein, ANS fluorescence was negligible. The measurements were conducted at least three times for independently prepared samples and the average values and the standard deviations were determined.

3-3. Dynamic light scattering measurements

The particle size distribution and the ζ -potential of casein micelles and CNPs were measured by Zetasizer Nano ZS (MARVERN Instruments, U.K.) with a 532 nm laser. The scattering angle was set at 90°. All samples were prepared in 50 mM phosphate buffer (pH 7.0) and equilibrated for more than a half hour before the measurements. Data collection was conducted at 15 °C. The protein concentration was adjusted to 0.5 mg/mL. The measurements were

conducted at least three times for independently prepared samples and the average values and the standard deviations were determined. Definition of polydispersity index (PDI) in this manuscript was followed by the international standard (ISO 13321) as noted in the manufacturer's manual. Here, PDI can be correlated with the width of size distribution. The PDI value of non-cross-linked β-casein micelles was 0.33. By contrast, The PDI values of β-casein micelles cross-linked with 25 and 250 mM EDC were 0.14 and 0.16, respectively.

3-4. Characterization of CNPs by AFM measurements

AFM images were obtained on a NanoScope IIIa controller equipped with a MultiMode scanning probe system (Digital Instruments) operated in tapping mode under air atmosphere. Silicon cantilevers (Nanosensors, NCH-10V) were used throughout the study. Squares of natural mica were stuck to the AFM sample plates using epoxy glue and were used as substrates. AFM specimen preparations were made using divalent cations to bridge between the negatively charged mica substrate and the micellar backbone. Typically, a stock solution of β-casein or cross-linked β-casein samples was diluted to 1 ng/μL in 10 mM Tris buffer (pH 7.5) containing 1 mM $MgCl_2$, and a 5 μL portion of the solution was spotted directly onto freshly cleaved mica. The solution was incubated on the surface for 30 s, and the surface was gently rinsed with H_2O. Then, the sample was blown to dry with compressed N_2 and was further dried under vacuum for 90 min.

The high-resolution AFM images and the corresponding cross-section profiles of non-cross-linked or cross-linked samples were shown in Fig. S2. From the AFM image, it is apparent that the native casein molecules possess different sizes and shapes in their aggregate structures, and thus did not allow the particle analysis procedure to report reliable data. On the other hand, the particle analysis on the AFM images of cross-linked samples gave results typical to single-component distribution. Moreover, chemical cross-linking treatment with increased concentration of EDC doubled the specific height of the aggregate, which means, in a general sense, rigidity enhancement for the particle matter under examination.

3-5. Effect of the protein concentration on the turbidity change

The aqueous solution of CNPs was prepared in 50 mM phosphate buffer (pH 7.0). The concentrations of CNPs were 0.5, 1.0, 1.5 and 2.0 mg/mL. The turbidity change of CNPs solution at 600 nm upon the increase in solution temperature was followed using UV-vis spectrophotometer (V550, Jasco, Japan). The rate of temperature increase was set at 2 ºC/min. The results were shown in Fig. S3.

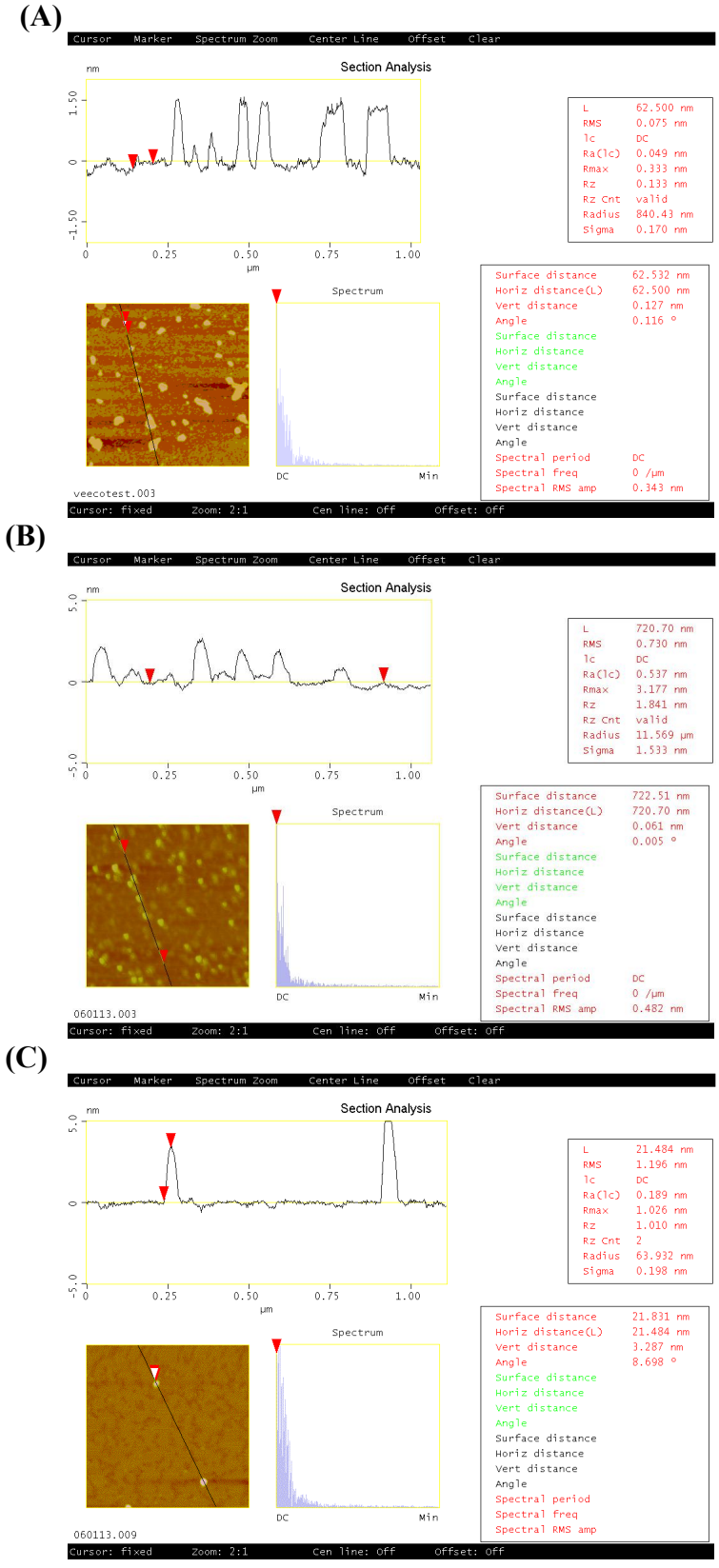

Fig. S2 The cross-section profiles of the high-resolution AFM images corresponding to non-cross-linked β-casein micelles (A) and cross-linked β-casein micelles prepared with 25 (B) and 250 (C) mM EDC.

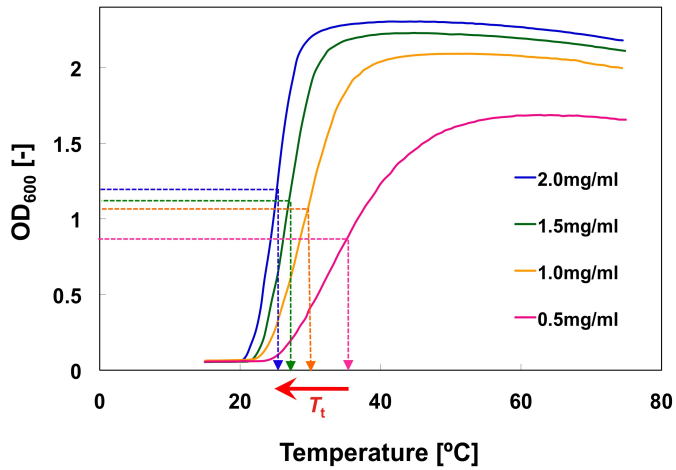

Fig. S3 The turbidity change of CNPs solution upon the increase in solution temperature.

3-6. Durability test of CNPs upon the repeated heating and cooling cycles

Durability of CNPs upon the repeated heating and cooling cycles were followed by the turbidity change of CNPs solution at 600 nm as described in **3-5**. First, the aqueous solution of CNPs was prepared in 50 mM phosphate buffer (pH 7.0) at 15 ºC ([CNPs] = 1.0 mg/mL). The solution was heated at 45 ºC for 10 min under stirring, then OD_{600} was measured. After cooling the solution at 4 ºC for 10 min, then OD_{600} was measured. This process was repeated in the first 4 cycles, and no significant change in turbidity was observed. The next 2 cycles, the solution was heated at 60 ºC for 10 min under stirring, then OD_{600} was measured. Again, clear solution was obtained after cooling at 4 ºC for 10 min. Finally, the solution was heated at 75 ºC for 10 min under stirring, then cooling at 4 ºC for 10 min after measuring the OD_{600} value. In the last 4 cycles, the OD_{600} was gradually increased after the cooling process, suggesting that some portion of CNPs remained in aggregated forms. The results were shown in Fig. S4.

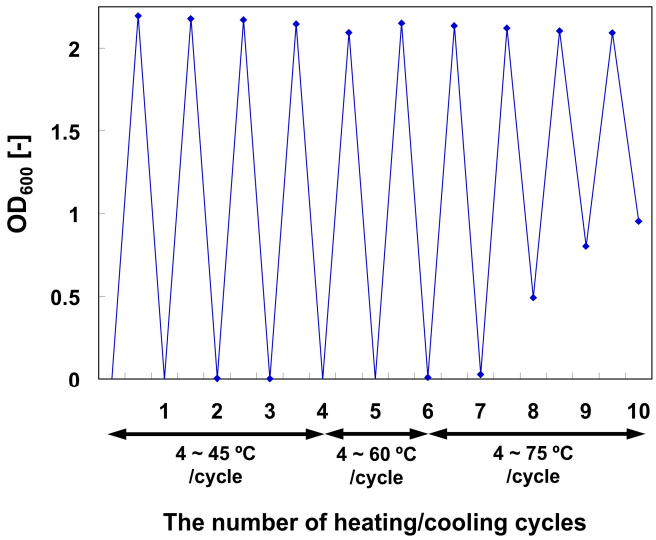

Fig. S4 The turbidity change of CNPs solution upon the repeated heating and cooling cycles.