Stable Nanoparticles Prepared by Heating Electrostatic Complexes of Whey Protein Isolate-Dextran Conjugate and Chondroitin Sulfate

Qingyuan Dai,[†,‡] Xiuling Zhu,[‡] Shabbar Abbas,[†] Eric Karangwa,[†] Xiaoming Zhang,[*,†] Shuqin Xia,[†] Biao Feng,[†] and Chengsheng Jia[†]

[†] State Key Laboratory of Food Science and Technology, School of Food Science and Technology, Jiangnan University, Lihu Road 1800, Wuxi, Jiangsu 214122, People's Republic of China

[‡] College of Biological and Chemical Engineering, Anhui Polytechnic University, Beijing Middle Road, Wuhu, Anhui 241000, People's Republic of China

[*] (X. Zhang) E-mail: xmzhang@jiangnan.edu.cn. Phone: +86 510 85197217; Fax: +86 510 85884496.

Supporting Information

MATERIALS AND METHODS

pH and Salt Stability. The stability of biopolymer nanoparticles against pH and salt was determined using spectrophotometry and dynamic light scattering. WPI/polysaccharide suspensions were prepared by heating WPI/polysaccharide mixtures at pH 5.2 and 85 °C for 15 min. The suspensions were firstly added with NaCl stock solution (3 M NaCl) to reach a final concentration of 200 mM NaCl, and then adjusted to the desired pH values (1.0 to 8.0) using hydrochloric acid (2.0, 1.0, 0.1, and 0.01 M) or sodium hydroxide solution (0.1 and 0.01 M).

Free Amino Groups Measurement. The free amino groups of WPI, WPI/dextran mixture, and WPI-dextran conjugate were determined by the o-phthalaldehyde (OPA) method with some modifications [1]. OPA reagent was prepared by dissolving 40 mg of OPA in 1 mL of methanol and mixing with 2.5 mL of 20% (w/w) SDS, 25 mL of 100 mM sodium tetraborate, and then adding 100 μL of β-mercaptoethanol. The solution was diluted to make the volume up to 50 mL with deionized water. 200 μL of sample solution was added directly to 4 mL of OPA reagent. The mixed solution was incubated in a water bath at 35 °C for 2 min and the absorbance was measured at 340 nm. Lysine was used as a standard. The degree of glycosylation (DG) was calculated as follows:

$$DG(\%) = \frac{A_0 - A_t}{A} \times 100$$

Where A_0, A_t, and A were the levels of free amino groups in WPI/dextran mixture, WPI-dextran conjugate, and WPI, respectively. All samples were analyzed in

triplicate.

Fourier Transform Infrared Spectra (FT-IR) Measurements. Infrared spectra of WPI, dextran, WPI/dextran mixture, and WPI-dextran conjugate were carried out with a Fourier transform infrared spectrometer (Nicolet iS10, Thermo Fisher Scientific Inc, Waltham, USA) using KBr pellets technique. The WPI/dextran mixtures and WPI-dextran conjugates were diluted 4-fold with deionized water and centrifuged at 10,000*g* for 30 min. The supernatant solutions were then lyophilized. Pellets were prepared by pressing the mixture of samples and potassium bromide (KBr) with the mass ratio of 1:100. All spectra were collected in the wavenumber range from 4000 to 400 cm^{-1} using resolution of 2 cm^{-1}.

RESULTS AND DISCUSSION

pH Effect on Turbidity of WPI/Polysaccharide Suspensions with Salt. It is well-known that high salt concentration can destabilize polyelectrolyte complexes due to the electrostatic screening effect. As observed in Figure S1A, all suspensions in the presence of 200 mM NaCl had higher turbidity at a relatively wide range of pH values. For WPI/dextran and WPI/dextran/ChS, the turbidity of their suspensions significantly ($p < 0.05$) increased in the pH range from 4.0 to 6.0, which was due to the electrostatic screening effect weakening the electrostatic repulsion to the formation of large aggregates [2, 3]. However, in our case, the high turbidity of WPI/ChS suspensions occurred in the pH range from 3.0 to 5.0. Although the electrostatic screening effect indeed existed at lower or higher pH ranges, the suspensions containing salt had relatively low and constant turbidity. It was mainly due to the fact

that the electrostatic repulsion interactions were still strong enough to overcome the electrostatic screening effect, and to prevent the large aggregation at the pH values far away from the p*I* of WPI. These results are in agreement with previous studies [3-6].

pH Effect on Particle Diameter of WPI/Polysaccharide Suspensions with Salt. In the presence of salt, the large aggregates in the WPI/ChS suspensions occurred at the pH range of 3.0-6.0 (Figure S1B). The steric hindrance of ChS did not significantly improve the stability of WPI/ChS suspensions against salt. It has been reported that the pectin-coated heat-treated β-lg particulates did not significantly improve their pH stability in the presence of salt, which was due to the electrostatic screening effect and insufficient steric hindrance of pectin [6]. The particle diameters of WPI/dextran and WPI/dextran/ChS suspensions containing salt were relatively larger than those of their suspensions without salt in the pH range of 3.0-7.0. The results indicated that the introduction of neutral dextran did not improve the salt stability due to the lack of electrostatic interaction and the absence of steric hindrance.

Change in Free Amino Group Content. The degree of glycosylation (DG) of WPI-dextran conjugate was determined by an OPA assay from the loss of free amino groups of WPI in Figure S2. The glycosylation reaction between reducing end carbonyl groups of polysaccharide and free amino groups of protein would decrease the amount of free amino group in protein. Therefore, the amount of reacted amino groups of proteins is proportional to DGs of protein-polysaccharide conjugates [7-9]. The results demonstrated that WPI was successfully grafted with dextran after heat treatment, according to the remaining lower amino group content. Our findings are in

accordance with previous studies [8, 10, 11]. The analysis indicated that DGs of WPI-dextran conjugate 1 (incubated at 60 °C for 24 h), conjugate 2 (incubated at 60 °C for 48 h), and conjugate 3 (incubated at 60 °C for 72 h) were 5.2, 9.7, and 12.2%, respectively.

FT-IR Spectra of WPI, Dextran, WPI/Dextran Mixture, and WPI-Dextran Conjugate. FT-IR spectroscopy is particularly useful technique to characterize the protein primary structure. For protein, the amide I and II bands are the two most prominent vibrational bands [12]. The absorption peak of protein at 1600-1700 cm^{-1} (amide I) is attributed to the stretching vibrations of C=O (approximately 80%) and C-N groups (10-20%) [13]. The absorption at 1480-1575 cm^{-1} (amide II) is associated to the bending vibrations of N-H groups (40-60%) and the stretching vibrations of C-N groups (18-40%) [12]. For carbohydrate, there is a series of overlapping peaks located in the region of 950-1180 cm^{-1}, which mainly arise from the stretching vibrations of C-C and C-O groups and the bending vibrations of C-H groups. These peaks are often referred to as the "saccharide" bands and are the most intense bands in the mid-infrared spectrum, whereas these absorptions are weak in the IR spectra of most proteins [14]. The infrared spectra of WPI, dextran, WPI/dextran mixture, and WPI-dextran conjugate are shown in Figure S3.

It was found that the amide I and II bands of pure WPI (Figure S3a) centered approximately between 1646 and 1544 cm^{-1}, respectively. In the mixture of WPI and dextran, these characteristic peaks in the amide I and II bands (Figure S3c) also appeared. But the intensities of the FT-IR spectra of WPI/dextran mixture obviously

decreased with increasing heat-treatment time. Therefore, the results demonstrated that WPI was successfully glycosylated with dextran via Maillard reaction. Our findings are in accordance with previous studies [14]. Besides, the amide III band, which is known to be very complex and mainly results from C-N stretching and N-H deformation, is located at 1200-1300 cm^{-1} in the protein [14]. The spectral features of the amide III band for the WPI-dextran conjugate decreased in intensity with increasing reaction time. Consequently, the results also indicated the alteration of protein structure. Similar results were previously reported [14]. The results of FT-IR spectra of WPI-dextran conjugate are in agreement with their degree of glycosylation.

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Figure Captions

Figure S1. Effect of pH and 200 mM NaCl on turbidity(**A**), hydrodynamic diameter (D_h) (**B**), and polydispersity index (PDI) (**C**) of WPI/polysaccharide suspensions. Suspensions were prepared by heating WPI/polysaccharide mixtures at pH 5.2 and 85 °C for 15 min.

Figure S2. Free amino groups content. WPI, whey protein isolate; mixture, WPI/dextran mixture; conjugate 1, WPI/dextran mixture incubated at 60°C for 24h; conjugate 2, WPI/dextran mixture incubated at 60°C for 48 h; conjugate 3, WPI/dextran mixture incubated at 60°C for 72 h.

Figure S3. FT-IR spectra of WPI (a), dextran (b), WPI/dextran mixture (c), WPI-dextran conjugate 1 (d), WPI-dextran conjugate 2 (e), and WPI-dextran conjugate 3 (f).

Figure S1.

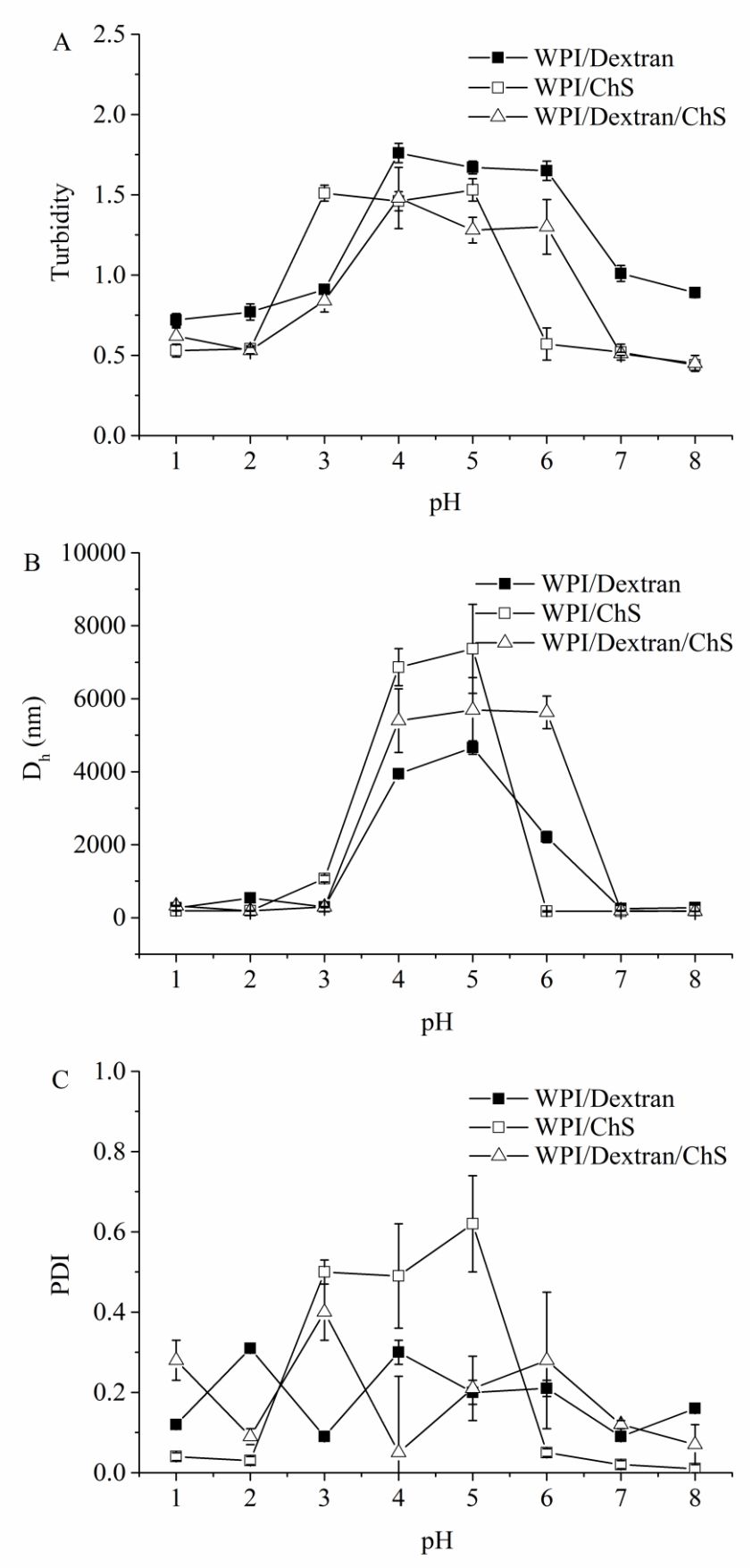

A
Turbidity
pH
WPI/Dextran
WPI/ChS
WPI/Dextran/ChS
B
D_h (nm)
pH
WPI/Dextran
WPI/ChS
WPI/Dextran/ChS
C
PDI
pH
WPI/Dextran
WPI/ChS
WPI/Dextran/ChS

Figure S2.

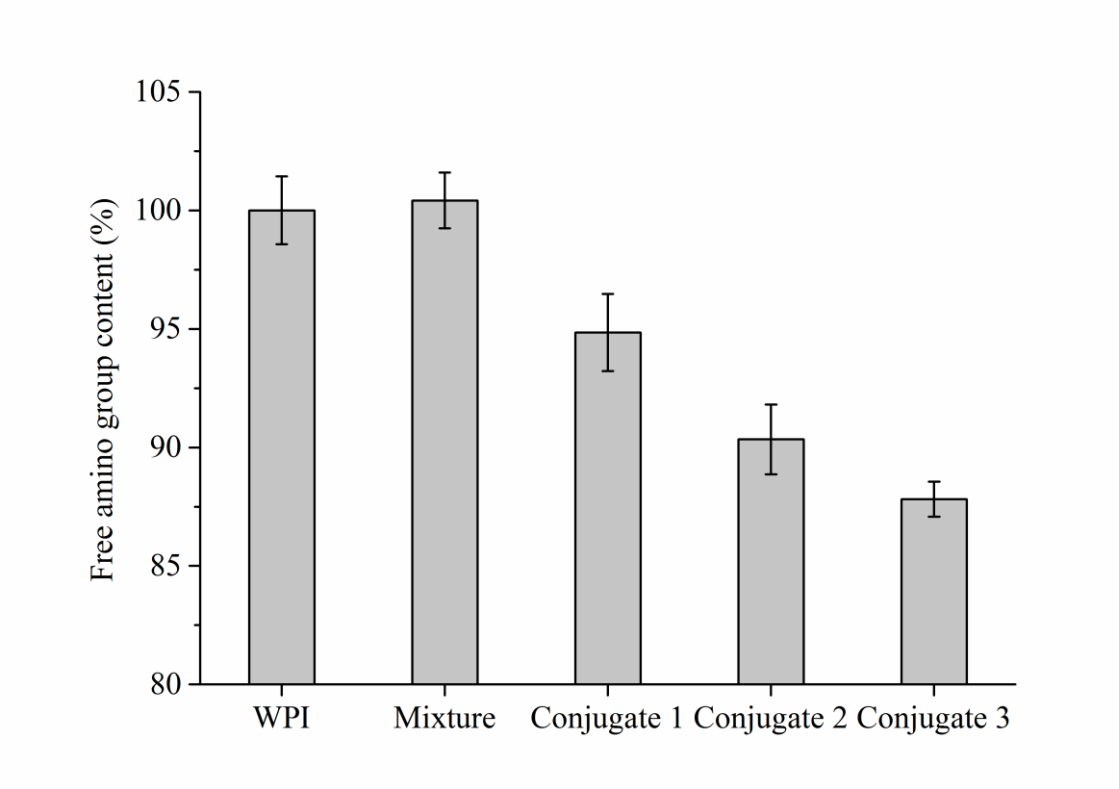
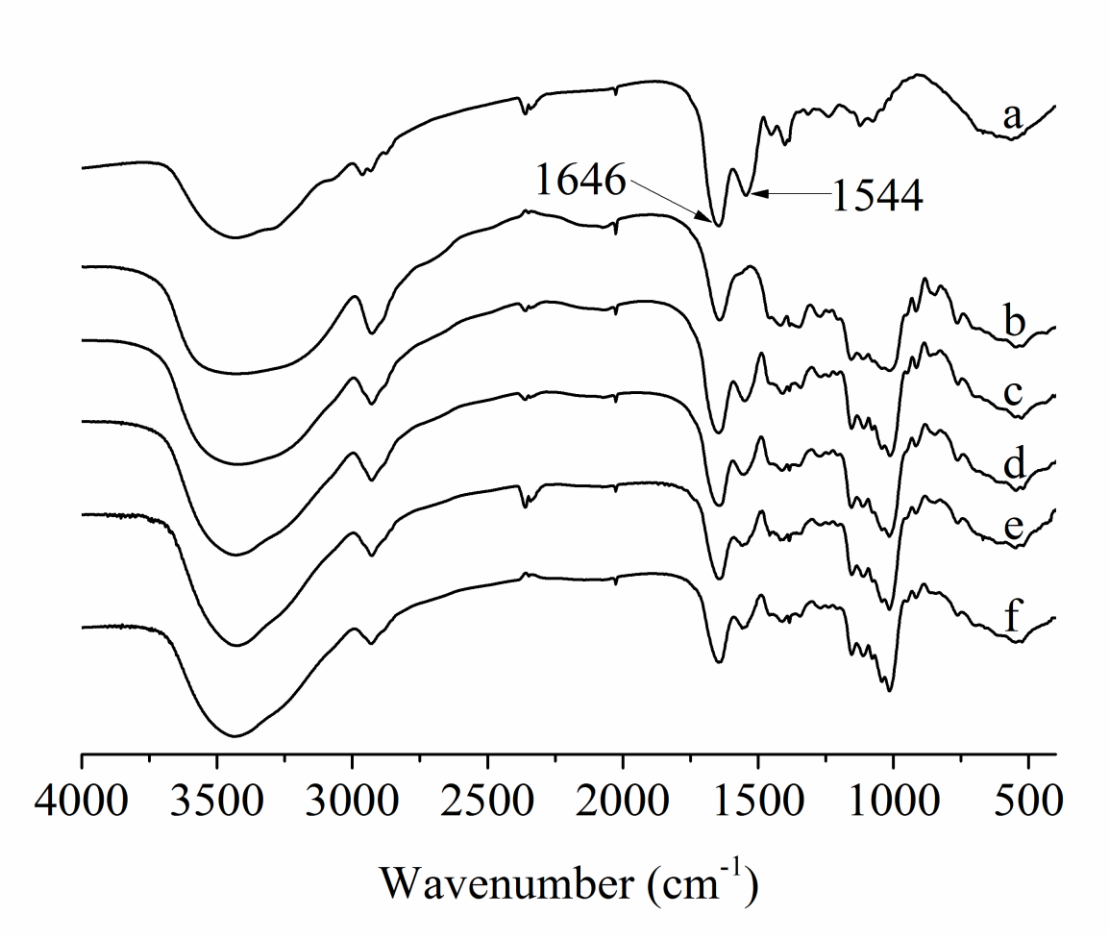

105
100
95
90
85
80
Free amino group content (%)
WPI
Mixture
Conjugate 1
Conjugate 2
Conjugate 3

Figure S3.


a
1646
1544
b
c
d
e
f
4000
3500
3000
2500
2000
1500
1000
500
Wavenumber (cm-1)