Soy Protein/Soy Polysaccharide Complex Nanogels: Folic Acid Loading, Protection, and Controlled Delivery

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ABSTRACT: In this study, we developed a facile approach to produce nanogels via self-assembly of folic acid, soy protein, and soy polysaccharide. High-pressure homogenization was introduced to break down the original aggregates of soy protein, which benefits the binding of soy protein with soy polysaccharide and folic acid at pH 4.0. After a heat treatment that causes the soy protein denaturation and gelation, folic acid-loaded soy protein/soy polysaccharide complex nanogels were fabricated. The nanogels have a polysaccharide surface that makes the nanogels dispersible in acidic conditions where folic acid is insoluble and soy protein forms precipitates after heating. More importantly, the protein and polysaccharide can inhibit the reactions between dissolved oxygen and folic acid during UV irradiation. After the preparation and storage of the nanogels in the presence of heat, oxygen, and light in acidic conditions, most of the folic acid molecules in the nanogels remain in their natural structure and can be released rapidly at neutral pH, that is, in the intestine. Because most food and beverages are acidic, the nanogels are a suitable delivery system of folic acid in food and beverages.

INTRODUCTION

Driven by increasing demands for preventing or delaying the onset of chronic diseases, extensive studies have been carried out to devise novel encapsulation materials and methods to incorporate functional ingredients into food. Considering instability under conditions encountered in food processing and storage, such as temperature, oxygen, and light, protection systems are needed for the functional ingredients before consumption. In recent years, the applications of food nanotechnology have grown very fast. By nanotechnology, we can shift the focus from macroscopic properties to the nanoscale and manipulate or self-assemble molecule by molecule to design and develop novel functional food with improved water solubility, thermal stability, oral bioavailability, sensory attributes, and physiological performance.

Folate constitutes a group of water-soluble B vitamins which are present in many chemically related derivatives. Folate is vital for all living creatures. Our bodies cannot synthesize it, and we depend entirely on our diets to provide this vitamin. Folate deficiency may be linked to diseases such as Alzheimer’s disease, neural tube defects, pregnancy complications, and several types of cancer. Folic acid (FA), a synthetic form of folate, is the oxidized and most stable form of folate, which is reduced to biologically active tetrahydrofolate in vivo. However, FA is sensitive to UV radiation that breaks the C9–N10 bond to yield inactive pteridine and (p-aminobenzoyl)-L-glutamic acid (Scheme 1). It has been reported that the photodegradation of FA can be reduced in the presence of HSA (human serum albumin) and β-lactoglobulin in neutral solutions in which FA is soluble. Considering that most food and beverages are acidic in which FA is insoluble, more studies are needed for the loading, protection, and delivery of FA.

Hydrogel nanoparticles (nanogels) formed by self-assembly of natural polymers such as protein and polysaccharide have been expected to be suitable systems for encapsulation,
protein, and delivery of bioactive compounds in functional food because of safety and nutrition.\textsuperscript{3,14,15} Previously, we fabricated nanogels by heating an ovalbumin and chitosan mixed solution at the pH where the protein and polysaccharide carry opposite charges.\textsuperscript{16} Heat treatment causes denaturation and gelation of ovalbumin; thus, parts of the chitosan chains are frozen in the core, while the other parts of the chitosan chains extend out as the shell of the nanogel. The nanogels do not dissociate over a wide range of pH conditions even when the protein and polysaccharide carry the same charge due to the cross-linking of the protein. Up to now, various polysaccharides and globular proteins have been used for fabricating stable nanogels via heat treatment by several groups.\textsuperscript{17–20} Several influential factors, including pH, ionic strength, ratio of protein to polysaccharide, polysaccharide type, and heating time and temperature, have been investigated. On the other hand, the effects of the interactions between protein and polysaccharide on the parameters influencing the formation of complexes and coacervates, the characterization of the kinetics of phase separation and multiscale structure of the complexes and coacervates, and the investigation of the functional properties of complexes and coacervates in food applications have been studied.\textsuperscript{21–23} However, very few protein/polysaccharide nanogels have been reported as carriers for encapsulation, protection, and delivery of bioactive compounds. Furthermore, the complex nanogels produced from plant proteins have not been reported.

Plant proteins have attracted much attention in the food industry because of increased consumer concerns over the safety of animal-derived products and/or their dietary preferences and food choices.\textsuperscript{24} However, proteins of plant origin do not function effectively at acidic pH environments because the proteins precipitate at pH values close to their isoelectric point.\textsuperscript{25,26} Soy protein is an abundant byproduct of the soybean oil industry. It has good functional properties for food processing due to its high nutritional value and emulsifying and gelling properties.\textsuperscript{27} The isoelectric point of soy protein is about pH 4.8.\textsuperscript{28} The poor solubility of soy protein at pH around 4.8 limits the applications of soy protein in the food and beverage industries because most food and beverages are acidic.

Soy-soluble polysaccharide, which is extracted from the byproduct of the isolation of soy protein, is a negatively charged polysaccharide possessing high water solubility, low bulk viscosity, and high temperature stability.\textsuperscript{29} It is composed of a main rhamnogalacturonan backbone branched with β-1,4-galactan, α-1,3- or α-1,5-arabinan chains, and homogalacturonan, which contains galacturonic acid (about 20% of the total sugar).\textsuperscript{30,31} The polysaccharide also contains a small amount of hydrophobic protein which contributes to its interfacial activity.\textsuperscript{32} The polysaccharide has been used as an emulsifier in acidic emulsion-based beverages and a source of dietary fiber in fortified food.\textsuperscript{33} Recently, we used soy protein and soy polysaccharide to fabricate a stable oil-in-water emulsion.\textsuperscript{34} The emulsion can encapsulate hydrophobic bioactive compounds inside oil droplets and act as a delivery system for hydrophobic bioactive compounds.

In this study, for the purpose of loading, protection, and delivery of amphiphilic bioactive compounds, such as FA, we developed a simple, green, and effective approach to produce nanogels from soy protein and soy polysaccharide. We used high-pressure homogenization to break down the original aggregates of soy protein, which benefits the binding of the soy protein with soy polysaccharide and FA at pH 4. After a heat treatment that induced the soy protein gelation, we obtained stable FA-loaded nanogels with a soy polysaccharide surface. The structure, stability, and FA protection and release properties of the nanogels were investigated. The results demonstrate the nanogels are a suitable FA delivery system.

### MATERIALS AND METHODS

**Materials.** Acid-soluble soy protein (Soyasour 4000K, moisture 3.5%, crude protein/dry 95.8%) and soybean-soluble polysaccharide (Soyafibe-S-CA100, crude protein 6.3%, moisture 5.6%, ash 7.4%) were from Fuji Oil Co. Ltd. FA (98%), NaNO\(_3\) (99%), and pectinase from Aspergillus aculeatus (aqueous solution, ≥3800 units/mL) were from Sigma-Aldrich. NaCl, NaOH, and HCl were analytical grade and from Sinopharm Chemical Reagent Co. All materials were used without further purification. Deionized water was used in all experiments.

**Preparation of Soy Protein/Soy Polysaccharide Nanogels.** Acid-soluble soy protein was dissolved in water with an apparent concentration of 23 mg/mL. After 3 h of stirring, NaNO\(_3\) with a final concentration of 0.02% was added to inhibit microbial growth. The solution was adjusted to pH 3.75–4 and stirred overnight at 37 °C. The undissolved component was removed by centrifugation at 8000 rpm for 30 min. The apparent protein concentration in the supernatant was 20 mg/mL determined by the solid weight after lyophilization. Soy polysaccharide was dissolved in water. After overnight stirring and the addition of NaNO\(_3\), a soy polysaccharide stock solution with an apparent concentration of 50 mg/mL and a NaNO\(_3\) concentration of 0.02% was obtained.

For the purpose of simplification, in this study, the protein concentration was directly calculated according to the powder weight of the acid-soluble soy protein, which does not include the protein bound covalently to the polysaccharide. The polysaccharide concentration was directly calculated according to the powder weight of the soybean-soluble polysaccharide, which includes the protein bound covalently to the polysaccharide.

The polysaccharide stock solution was diluted with water. Then the protein stock solution was added to the diluted polysaccharide solution. The pH of the mixture was adjusted to 4.0. The final protein concentration in the mixed solution was 5 mg/mL, the final polysaccharide concentration was 15 mg/mL, and the pH was 4.0 if there is no specific indication in this paper. After 3 h of stirring, the mixture was homogenized using a high-pressure homogenizer (AH100D, ATS Engineering Inc.) at 600 bar for 2 min, immediately followed by a heat treatment at 90 °C for 1 h. The resultant protein/polysaccharide nanogel solution was adjusted to different pH values with 0.5 mol/L NaOH or HCl solution, and then 3 mol/L NaCl solution was added to reach a 0.2 mol/L final NaCl concentration. The nanogel solutions containing designed pH values and NaCl concentrations were stored at 4 °C to investigate the stability.

**Preparation of FA-Loaded Soy Protein/Soy Polysaccharide (FA/Protein/Polysaccharide) Nanogels.** FA/protein/polysaccharide nanogels were prepared by mixing FA with the protein and polysaccharide at pH 7.4, followed by pH adjustment, high-pressure homogenization, and heat treatment. Following are the details of the preparation: 7.88 g of pH 7.4 soy polysaccharide solution with a 50 mg/mL concentration was added to 5 mL of water, followed by addition of 6.25 mL of 1 mmol/L of pH 7.4 FA solution and 6.25 mL of pH 7.4 soy protein solution with a 20 mg/mL concentration. In the mixture, the final concentration of protein was 5 mg/mL, that of polysaccharide 15 mg/mL, and that of FA 0.25 mmol/L, and the volume was 25 mL. The pH of the mixture was adjusted to 4.0. After 3 h of stirring, the mixture was homogenized at 600 bar for 2 min and then was immediately heated at 90 °C for 1 h to produce FA/protein/polysaccharide nanogels.

**Release of FA from FA/Protein/Polysaccharide Nanogels.** A 5 mL FA/protein/polysaccharide nanogel solution was dialyzed (cutoff molecular weight 3500) against 50 mL of release buffer (phosphate-buffered saline (PBS), 10 mmol/L of pH 7.4 phosphate
buffer containing 0.15 mol/L NaCl at 37 °C. Periodically, 2 mL of the release buffer was taken out and the same volume of the fresh buffer was added. The soy protein/soy polysaccharide nanogel solution without FA was dialyzed at the same conditions, and the 280 nm absorption in the release buffer was regarded as the background. The FA concentration was calculated by the 280 nm absorption in the release buffer after deduction of the background. The FA working curve was obtained by standard FA solutions prepared by dissolving FA in 10 mmol/L of pH 7.4 phosphate buffer with different FA concentrations.

UV Irradiation. A sample of 3 mL was placed in a glass bottle and then was irradiated by a UV radiometer (peak λ = 365 nm, 500 W, Beijing Changtuo Technology Co. Ltd.) at a distance of 30 cm for 1 h to investigate the photodegradation of the FA. For all the solutions undergoing UV irradiation, no NaN₃ was added.

ξ Potential Measurements. ξ potentials were measured at 25 °C on a ZetaSizer Nano ZS90 (Malvern Instruments). The electrophoresis mobility ζ₆ was measured, and the ξ potential was calculated by the Dispersion Technology Software provided by Malvern according to the Henry equation: ζ₆ = (2εₑεᵣ/3η)(f/ka), where εₑ, εᵣ, and f(ka) are the dielectric permittivity of the solvent, the viscosity of the solution, and Henry's function. For the protein and the polysaccharide solutions, the ξ potential samples were prepared by adjusting the stock solutions to the desired pH values and then separately diluting the protein solutions to 1 mg/mL and the polysaccharide solutions to 5 mg/mL with the solution containing the same pH value and 5 mmol/L NaCl. The ξ potential samples of the protein/polysaccharide complex nanogels were prepared similarly in which the protein concentration was 0.2 mg/mL.

Atomic Force Microscopy (AFM) Measurements. AFM images were acquired in tapping mode on a Digital Instruments Nanoscope IV (Veeco Instruments). The AFM sample was prepared by drying the diluted solution on a freshly cleaved mica surface naturally at room temperature for 2 days.

Dynamic Light Scattering (DLS) Measurements. DLS measurements were carried out on a Malvern Autosizer 4700 (Malvern Instruments). The measurements were performed at 25 °C and a fixed scattering angle of 90°. The apparent z-average hydrodynamic diameter (Dₐ) and polydispersity index (PDI) were obtained by CONTIN mode analysis. The DLS sample was prepared by diluting the nanogel solution to a protein concentration of 0.1 mg/mL with the same pH aqueous solution. All the nanogel solutions reported in this paper were fabricated and analyzed in at least two batches to assess the reproducibility; the average value and deviation were reported.

Fluorescence Measurements. FA fluorescence emission spectra were recorded on a fluorescence spectrophotometer (FLS-920, Edinburg Instruments) at 25 °C and an excitation wavelength of 348 nm. For the FA loaded in the nanogels, the sample was prepared by releasing the loaded FA from the nanogels at pH 7.4, followed by isolating the released FA via ultrafiltration (cut-off molecular weight 3000; MicroconYM-3, Millipore) and then diluting the isolated FA in the ultratrate 10 or 20 times to avoid self-quenching. For the free FA dissolved at pH 7.4 directly, the FA solution was diluted to 0.025 mmol/L for fluorescence measurement.

UV Absorption Measurements. The absorption at 280 nm was recorded on a spectrophotometer (Shimadzu UV-2550) to calculate the FA concentration in the ultratrate after deduction of the background. The same ultratrate was performed for the soy protein/soy polysaccharide nanogel solution without FA, and the 280 nm absorption in the ultratrate was regarded as the background.

Transmission Electron Microscopy (TEM) Measurements. TEM observations were conducted on a Philips CM120 electron microscope. The TEM sample was prepared by depositing diluted nanogel solution onto a carbon-coated copper grid. The sample was dried naturally at room temperature for at least 3 days.

Polysaccharide Hydrolysis. The soy polysaccharide on the nanogel surface was hydrolyzed by pectinase. For monitoring the size change by real time DLS measurement, the hydrolysis was performed at 25 °C and pH 4.0 to reduce the hydrolysis rate. During the DLS measurement, 10 μL of 0.5% pectinase solution was added to a polystyrene cuvette containing diluted nanogel solution prepared by diluting 60 μL of the original nanogel solution with 3 mL of pH 4.0 aqueous solution. The nanogel size was measured every 6 min.

RESULTS AND DISCUSSION

Preparation and Characterization of Soy Protein/Soy Polysaccharide Nanogels. The soy protein was characterized by SDS—polyacrylamide gel electrophoresis (Figure S1 in the Supporting Information). The result indicates the soy protein is mainly composed of glycinin (about 20 and 35 kDa bands) and β-conglycinin (about 50 kDa band). Figure 1 shows the protein is positively charged and negatively charged when the solution pH is lower and higher than 5.3, respectively. The solubility of the protein is poor in aqueous solutions in the pH range of 4—7. At pH 3.75—4, when the original soy protein concentration is 23 mg/mL, about 90% of the protein remains in the supernatant after centrifugation at 8000 rpm for 30 min. However, the DLS result reveals that the supernatant contains dispersible protein aggregates whose Dₐ is 179 nm. The AFM image (Figure 2A) confirms the protein aggregates exist in the supernatant; their average diameter and height are 179 and 38 nm, respectively. In our previous study, heating the soy protein solution of 5 mg/mL concentration at pH 3.25 and 80 °C for 1 h caused partial denaturation of the protein; the α-helix content decreased, but the β-sheet and random coil contents increased. In this study, when the 5 mg/mL soy protein solution in the pH range of 3.5—4.0 is heated at 90 °C for 1 h, the protein aggregates to larger particles which precipitate soon after storage; when the heated solution is changed to pH 5.0, precipitation happens immediately.

Soy polysaccharide is soluble in the pH range of 2.0—8.0. There are some aggregates with a Dₐ of 464 nm in the 15 mg/mL polysaccharide solution at pH 4.0, which may result from the 6.3% hydrophobic protein, but the scattering intensity is not strong. No further aggregation happens when the polysaccharide solution is heated at 90 °C for 1 h. Figure 1 shows the polysaccharide is negatively charged in the pH range of 3.0—8.0. Therefore, electrostatic attractions exist between the protein and polysaccharide in the pH range of 3.0—5.3. According to the literature, the polysaccharide contains only some galacturonic acid (about 20% of the total sugar), which implies the neutral side chains of the polysaccharide can make the protein/polysaccharide complex dispersible in aqueous
solution. In addition, the polysaccharide contains about 6.3% hydrophobic protein, which can increase the interactions between the protein and polysaccharide. Furthermore, the hydrophobic protein in the polysaccharide can take part in the gelation with the soy protein. Therefore, it is possible that a part of the polysaccharide chains can be fixed in the core of the soy protein/polysaccharide complex nanogel, while the other part of the polysaccharide chains can extend out as the shell of the nanogel.

Table S1 in the Supporting Information shows the protein/polysaccharide complexes formed at pH 4.0 are not stable. Heating the protein/polysaccharide complex solution at 90 °C for 1 h cannot produce stable nanogels. This phenomenon is different from the result of ovalbumin and chitosan complex solution in which nanogels with a chitosan shell are fabricated after heat treatment. It is possible that the self-aggregation of the soy protein prevents the binding of the soy protein with the soy polysaccharide; as a result, the polysaccharide cannot prohibit the protein from precipitation after the heat treatment. To solve this problem, we used high-pressure homogenization to destroy the aggregates of the soy protein. For the protein particles, after a homogenization at 600 bar for 2 min, the average diameter is 156 nm and the average height is 34 nm, shown in the AFM image (Figure 2B), while the \( D_n \) is 161 nm, which are smaller than the sizes of the protein particles before homogenization mentioned above. For the soy protein/polysaccharide complex, only homogenization at pH 4.0 without heat treatment can produce narrow dispersible nanogels, but the nanogels are not stable when the solution is changed to pH 5.0 and higher (Table S1). Possibly, the homogenization condition of 600 bar for 2 min could not produce enough cross-links. Therefore, we adopted a heat treatment at 90 °C for 1 h immediately after the homogenization to induce the soy protein denaturation and then to fix the structure of the soy protein/polysaccharide complex nanogels. The produced nanogels having a \( D_n \) of 155 nm are stable; the AFM image in Figure 2C shows the nanogels are dispersible.

The data in the Supporting Information show that the preparation conditions influence the stability of the nanogels. Table S2 (Supporting Information) shows a suitable homogenization pH range is 3.5–4.0. In this pH range, the soy protein and soy polysaccharide carry opposite charges and the protein is dispersible before heating, which benefit the formation of the protein/polysaccharide complex. Table S3 (Supporting Information) reveals that increasing the homogenization pressure can reduce the nanogel size, but the resultant nanogels aggregate when the medium is changed to pH 5.0 and contains 0.2 mol/L NaCl. The data in Table S4 (Supporting Information) demonstrate that a higher heating temperature and longer heating time result in more stable nanogels because of stronger gelation of the protein. Table S5 (Supporting Information) shows the nanogels produced from a WR (weight ratio of soy protein to soy polysaccharide) of 1:2 to 1:5 are stable. Increasing the soy protein concentration from 5 to 10 mg/mL causes a small increase of the nanogel sizes (Tables S4 and S5). When the protein concentration is further increased to 15 mg/mL, the nanogels are not stable during storage.

The protein/polysaccharide nanogel solution, produced at pH 4.0 with a protein concentration of 5 mg/mL, a polysaccharide concentration of 15 mg/mL, homogenization at 600 bar for 2 min, and a heat treatment at 90 °C for 1 h, was adjusted to pH 2.0–8.0, and NaCl was added or not to investigate the stability. The nanogel solutions are homogeneous in appearance, and their sizes do not change significantly after storage in media of pH 2.0–8.0 without the addition of NaCl (Table 1). The 5 mg/mL protein solution is not soluble at pH 5.0. The nanogels are stable at pH 5.0 after 3 months of storage, indicating the nanogels have a polysaccharide surface that can protect the soy protein from precipitation at pH 5.0. Changing the medium pH from 4.0 to 8.0 results in an increase of the nanogel size because the polysaccharide chains carry

**Table 1. DLS Results of the Nanogels Prepared from Soy Protein/Soy Polysaccharide Complex after 3 Months of Storage in Different pH Media with and without 0.2 mol/L NaCl**

<table>
<thead>
<tr>
<th>pH</th>
<th>fresh (( D_1 ) (nm))</th>
<th>( \text{PDI} )</th>
<th>after 3 months (( D_2 ) (nm))</th>
<th>( \text{PDI} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH 3.0</td>
<td>167 ± 0.2</td>
<td>0.20 ± 0.02</td>
<td>198 ± 2</td>
<td>0.20 ± 0.02</td>
</tr>
<tr>
<td>pH 4.0</td>
<td>159 ± 0.3</td>
<td>0.14 ± 0.03</td>
<td>173 ± 2</td>
<td>0.18 ± 0.01</td>
</tr>
<tr>
<td>pH 5.0</td>
<td>160 ± 0.1</td>
<td>0.20 ± 0.01</td>
<td>183 ± 1</td>
<td>0.19 ± 0.03</td>
</tr>
<tr>
<td>pH 6.0</td>
<td>184 ± 2.5</td>
<td>0.22 ± 0.01</td>
<td>208 ± 2</td>
<td>0.27 ± 0.05</td>
</tr>
<tr>
<td>pH 7.0</td>
<td>190 ± 0.3</td>
<td>0.22 ± 0.01</td>
<td>205 ± 2</td>
<td>0.31 ± 0.04</td>
</tr>
<tr>
<td>pH 8.0</td>
<td>198 ± 0.2</td>
<td>0.34 ± 0.02</td>
<td>214 ± 3</td>
<td>0.36 ± 0.03</td>
</tr>
<tr>
<td>pH 9.0</td>
<td>202 ± 0.2</td>
<td>0.20 ± 0.02</td>
<td>209 ± 2</td>
<td>0.34 ± 0.03</td>
</tr>
<tr>
<td>pH 10.0</td>
<td>204 ± 0.3</td>
<td>0.18 ± 0.02</td>
<td>202 ± 2</td>
<td>0.34 ± 0.03</td>
</tr>
</tbody>
</table>

The nanogels were produced at pH 4.0 with a protein concentration of 5 mg/mL, a polysaccharide concentration of 15 mg/mL, homogenization at 600 bar for 2 min, and a heat treatment at 90 °C for 1 h.
more negative charges at higher pH as shown in Figure 1, which make the polysaccharide chains on the nanogel surface more extended. At pH 8.0, both the protein and polysaccharide carry negative charges (Figure 1). Because the gelation during the heat treatment has fixed the structure of the complex nanogels, the nanogels do not dissociate when electrostatic repulsion exists between the protein and polysaccharide. In the presence of 0.2 mol/L NaCl, the sizes increase to more than 300 nm for the nanogels stored in pH 4.0 and 5.0 media, but the nanogels stored in pH 2.0, 3.0, 6.0, 7.0, and 8.0 media do not change their sizes much after 3 months. In addition, the nanogels can be stored as lyophilized powder. After lyophilization and rehydration, the nanogel sizes are almost the same as the sizes of freshly prepared ones.

As we know, nanogels swollen in water are much larger than the dried ones. To confirm the produced nanogels have a hydrogel structure, we measured the swelling ratio, which is the ratio of the average volume measured by DLS to the average volume obtained by the AFM image ($V_{DLS}/V_{AFM}$). The $D_h$ (z-average hydrodynamic diameter) of the nanogels is 155 ± 5 nm (Table 1), and the nanogels measured by DLS are supposed to be spherical in shape. For the AFM image measured in tapping mode, the vertical data measured are close to the real values and the lateral dimensions measured are larger than the real values because of the broadening effect of the tip.42 The average radius ($r$) of the nanogels shown in Figure 2C is 59 ± 24 nm after subtraction of 7 ± 2 nm of the tip radius, and the average height ($h$) is 13 ± 6 nm. The height is much smaller than the diameter, indicating the nanogels are soft and collapsed on the mica surface. The nanogels in the AFM image are supposed to be a part of the sphere, so the average volume is estimated using the equation

$$V_{AFM} = n h^3(r^2 + h^2)/2h = h^3/3.$$  

The estimated swelling ratio ($V_{DLS}/V_{AFM}$) is 26.6, confirming that the nanogels have a low-density structure and can contain a large amount of water.

Figure 1 shows the nanogels have $\zeta$ potentials similar to those of the polysaccharide in the pH range of 3–8, indicating the nanogels have a polysaccharide surface. The enlarged AFM image of the nanogels (Figure 2D) shows the core/shell structure visually, in which the average thickness of the shell is 16 ± 3 nm, further confirming the nanogels have a polysaccharide surface. As a result, the nanogels are stable in the pH range of 3–8 after long-term storage.

Pyrene was used as a fluorescence probe to characterize the hydrophobicity of the nanogels. Pyrene has a much lower solubility in water than in hydrocarbons. It migrates from the hydrophilic into the hydrophobic patches of the protein. The digital photograph in Figure S3 of the Supporting Information shows precipitates after the protein and polysaccharide and FA mixed solution being changed from pH 7.4 to pH 5.0. Further changing the pH to 4.0, the precipitates disappear and the solution contains dispersible yellow particles. On the other side, the yellow FA precipitates appear soon after the polysaccharide and FA mixed solution being changed from pH 7.4 to pH 5.0. These phenomena can be explained by the facts that (1) the soy protein is insoluble at pH 5.0 and dispersible at pH 4.0, (2) the FA can bind with the protein, forming dispersible FA/protein complex particles at pH 4.0, and (3) the light yellow solution indicates a part of the FA can bind with the polysaccharide, whereas the other part of the FA precipitates at pH 4.0. The results shown in Figure S3 confirm that the interactions between the protein and FA increase when their mixed solution is changed from pH 7.4 to pH 4.0.

The Free FA in the mixtures was isolated by ultrafiltration after equilibrium at pH 7.4 overnight. The protein concentration was 5 mg/mL and the polysaccharide concentration was 15 mg/mL in the nanogel solution.

Table 2. Free FA Concentrations in Different Mixtures of pH 7.4

<table>
<thead>
<tr>
<th>sample</th>
<th>FA concn in feed (mmol/L)</th>
<th>FA concn in ultrafiltrate (mmol/L)</th>
<th>recovered FA in ultrafiltrate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FA + 5 mg/mL protein</td>
<td>0.250</td>
<td>0.215 ± 0.005</td>
<td>86 ± 2</td>
</tr>
<tr>
<td></td>
<td>0.500</td>
<td>0.435 ± 0.004</td>
<td>87 ± 1</td>
</tr>
<tr>
<td>FA + 15 mg/mL polysaccharide</td>
<td>0.250</td>
<td>0.241 ± 0.007</td>
<td>96 ± 3</td>
</tr>
<tr>
<td></td>
<td>0.500</td>
<td>0.506 ± 0.008</td>
<td>101 ± 2</td>
</tr>
<tr>
<td>FA + protein/polysaccharide nanogels</td>
<td>0.250</td>
<td>0.225 ± 0.005</td>
<td>90 ± 2</td>
</tr>
<tr>
<td></td>
<td>0.500</td>
<td>0.447 ± 0.006</td>
<td>89 ± 1</td>
</tr>
<tr>
<td>FA</td>
<td>0.500</td>
<td>0.485 ± 0.008</td>
<td>97 ± 2</td>
</tr>
</tbody>
</table>

**Table 2. Free FA Concentrations in Different Mixtures of pH 7.4**

and nanogels was changed from pH 7.4 to pH 4.0, FA precipitated. This result indicates the nanogels cannot prohibit the FA from precipitation by means of direct mixing.

The deprotonation and protonation make FA hydrophilic at pH 7.4 and hydrophobic at pH 4.0, which benefits the hydrophobic interactions between FA and the hydrophobic patches of the protein. The free FA in the mixtures was isolated by ultrafiltration after equilibrium at pH 7.4 overnight. The protein concentration was 5 mg/mL and the polysaccharide concentration was 15 mg/mL in the nanogel solution.

Preparation and Characterization of FA-Loaded Protein/Polysaccharide (FA/Protein/Polysaccharide) Nanogels. FA has pH-dependent solubility which is more than 1 mmol/L at pH 6 but decreases to less than 2.3 μmol/L at pH 4 due to the protonation of the carboxylic groups.13 The binding of FA with separate soy protein, soy polysaccharide, and the protein/polysaccharide nanogels at pH 7.4 was investigated first. After equilibrium overnight, the free FA in the mixture was separated by ultrafiltration. At the conditions of 5 mg/mL soy protein, 15 mg/mL soy polysaccharide, and 0.25 mmol/L FA, the separate protein and nanogels can bind only about 10% of the FA, and the polysaccharide almost cannot bind FA as shown in Table 2. When the mixed solution of FA and polysaccharide solution being changed from pH 7.4 to pH 5.0. Further changing the pH to 4.0, the precipitates disappear and the solution contains dispersible yellow particles. On the other side, the yellow FA precipitates appear soon after the polysaccharide and FA mixed solution being changed from pH 7.4 to pH 4.0. These phenomena can be explained by the facts that (1) the soy protein is insoluble at pH 5.0 and dispersible at pH 4.0, (2) the FA can bind with the protein, forming dispersible FA/protein complex particles at pH 4.0, and (3) the light yellow solution indicates a part of the FA can bind with the polysaccharide, whereas the other part of the FA precipitates at pH 4.0. The results shown in Figure S3 confirm that the interactions between the protein and FA increase when their mixed solution is changed from pH 7.4 to pH 4.0.

The FA/protein complex particles formed at pH 4.0 are not stable and precipitate gradually during storage. Therefore, we mixed FA with soy protein and soy polysaccharide at pH 7.4 and then adjusted the mixture to pH 4.0, followed by homogenization and heat treatment to produce FA-loaded nanogels. After production at pH 4.0, the nanogel solution was changed to pH 2.0, 3.0, 3.5, and 5.0 to investigate the stability. In the pH range of 3.0–5.0, the FA/protein/polysaccharide nanogels have $D_h$ values (Table 3) similar to those of the nanogels without FA (Table 1). Furthermore, the FA/protein/polysaccharide nanogel solutions are homogeneous (Figure S3 of the Supporting Information) and do not change their sizes significantly (Table 3) after 6 months of storage in the pH range of 3.0–5.0. These results verify that the FA is loaded inside the nanogels and the nanogels are a suitable carrier of FA.
in acidic conditions. The data in Table 3 demonstrate that, in the presence of soy protein and soy polysaccharide, the binding of the protein with the polysaccharide and the binding of the protein with FA can simultaneously suppress the precipitation of the protein after heat treatment and suppress the precipitation of FA at pH 4.0. Although the protein and polysaccharide bind only about 10% of the FA at pH 7.4, the FA/protein/polysaccharide nanogels load the FA completely at pH 4.0.

Figure S3 (Supporting Information) shows the precipitates in the mixture of the protein and FA at pH 5.0. The long-term stability of FA/protein/polysaccharide nanogels in media of pH 3.0−5.0 suggests the nanogels have a polysaccharide surface. To further confirm the structure of the nanogels, polysaccharide hydrolysis was performed using pectinase.32 The mixed solution of diluted FA/protein/polysaccharide nanogels and pectinase was incubated at pH 4.0 and 25 °C to digest the soy polysaccharide; meanwhile DLS measurement was performed in real time. Before the addition of pectinase, the size was 172 nm (Figure 3). After the addition of pectinase, the size decreased gradually to 140 nm and then increased, and the scattering intensity also decreased and then increased, while the PDI did not change significantly. It is worth noting that the scattering intensity is proportional to both the number of scatterers and the square of the scatterer’s mass.46 During the hydrolysis process, the polysaccharide on the nanogel surface was digested, which caused the nanogel mass to decrease and thus the nanogel size and the scattering intensity to decrease. However, the nanogels without a polysaccharide surface aggregated during the process, which resulted in an increase of the nanogel mass and therefore increases of the nanogel size and the scattering intensity. From the decrease of the $D_h$ value before and after the digestion, we can estimate that the polysaccharide layer of the nanogel surface is about 16 nm, which is the same as the polysaccharide layer of the protein/polysaccharide nanogels shown in AFM image (Figure 2D).

The TEM image in Figure 4 shows FA/protein/polysaccharide nanogels have a spherical shape and are dispersible.

![Figure 4. TEM image of FA/protein/polysaccharide nanogels. The scale bar is 200 nm.](image)

The dried nanogels shown in Figure 4 are much smaller than their $D_h$ of 172 nm measured in aqueous solution, indicating the nanogels have a hydrogel structure.

**FA Release from FA/Protein/Polysaccharide Nanogels.** The release of loaded FA in PBS was investigated by dialysis. The control was the free FA which was dissolved in 10 mmol/L of pH 7.4 phosphate buffer, put into the dialysis tube, and dialyzed against PBS. Figure 5 shows the release rate of the FA from the nanogels is similar to the diffusion rate of the free FA before and after the digestion, we can estimate that the polysaccharide layer of the nanogel surface is about 16 nm, which is the same as the polysaccharide layer of the protein/polysaccharide nanogels shown in AFM image (Figure 2D).

![Figure 5. Accumulative release of FA from FA/protein/polysaccharide nanogels in PBS. The free FA dissolved in 10 mmol/L of pH 7.4 phosphate buffer was assayed as a control.](image)

Table 3. DLS Results of FA/Protein/Polysaccharide Nanogels Produced at pH 4.0 and Then Changed to Different pH Values before Storage

<table>
<thead>
<tr>
<th>storage pH</th>
<th>freshly prepared</th>
<th>after 2 months of storage</th>
<th>after 6 months of storage</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$D_h$ (nm)</td>
<td>PDI</td>
<td>$D_h$ (nm)</td>
</tr>
<tr>
<td>2.0</td>
<td>207 ± 3</td>
<td>0.17 ± 0.01</td>
<td>232 ± 4</td>
</tr>
<tr>
<td>3.0</td>
<td>175 ± 2</td>
<td>0.16 ± 0.01</td>
<td>185 ± 4</td>
</tr>
<tr>
<td>3.5</td>
<td>173 ± 1</td>
<td>0.17 ± 0.01</td>
<td>190 ± 1</td>
</tr>
<tr>
<td>4.0</td>
<td>170 ± 4</td>
<td>0.13 ± 0.02</td>
<td>195 ± 5</td>
</tr>
<tr>
<td>5.0</td>
<td>177 ± 5</td>
<td>0.18 ± 0.03</td>
<td>202 ± 6</td>
</tr>
</tbody>
</table>

*a The nanogels were produced with a protein concentration of 5 mg/mL, a polysaccharide concentration of 15 mg/mL, and an FA concentration of 0.25 mmol/L.
FA, suggesting the release of the FA in PBS is very fast. At pH 7.4, FA is soluble in aqueous solution and only about 10% of the FA can bind with the nanogels (Table 2). Therefore, it is reasonable that the nanogels have a rapid FA release behavior at pH 7.4.

Figures 6 and 7 show the fluorescence emission and absorption spectra of free FA at pH 7.4, respectively. For the FA released from the nanogels at pH 7.4, the fluorescence spectrum indicates that only about 17% of the FA molecules remain in their natural structure after the irradiation. We also investigated the nanogel sample fluorescence quantum yield because of intra-molecular fluorescence quenching.37 After the irradiation, FA fluorescence at 450 nm increases (Figure 6) due to the formation of PGA ((p-aminobenzoyl)-l-glutamic acid) and FPT (6-formylpterin) as a result of the cleavage of the C9–N10 bond (Scheme 1), followed by the photoinduced conversion of FPT to PCA (pterin-6-carboxylic acid). The 450 nm intensity ratio of the FA fluorescence after UV radiation to that before UV radiation (I_{UV}/I) is 89 for a 0.25 mmol/L FA solution (Table 4). This ratio is smaller than the ratio of 123 reported in the literature.7 The reason may be that we used a glass bottle instead of a quartz cuvette. The HPLC result reveals that about 98% of the FA has decomposed after the UV irradiation (data not shown). The absorption spectrum shown in Figure 7 also changes after the FA was exposed to UV radiation. The optical density at the wavelength of <270 nm decreases, while the absorption peak at 280 nm and the shoulder at 300 nm decrease, and the peak at 350 nm shifts to shorter wavelengths as reported in the literature.8

Table 4. FA Degradation Degrees in Different Samples after UV Radiation

<table>
<thead>
<tr>
<th>Sample</th>
<th>FA concn (mmol/L) and pH value during UV radiation</th>
<th>I_{UV}/I</th>
<th>Degradation degree (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FA</td>
<td>0.25, pH 7.4</td>
<td>89 ± 3</td>
<td>100</td>
</tr>
<tr>
<td>FA + 15 mg/mL polysaccharide</td>
<td>0.25, pH 7.4</td>
<td>29 ± 4</td>
<td>33 ± 5</td>
</tr>
<tr>
<td>FA + 5 mg/mL protein</td>
<td>0.25, pH 7.4</td>
<td>24 ± 2</td>
<td>27 ± 3</td>
</tr>
<tr>
<td>FA/protein/polysaccharide nanogels</td>
<td>0.25, pH 4.0</td>
<td>15 ± 2</td>
<td>17 ± 2</td>
</tr>
</tbody>
</table>

The degradation degree was obtained by measuring the 450 nm intensity of FA fluorescence before and after UV radiation. The FA was released at pH 7.4 and then isolated via ultrafiltration for fluorescence measurement.6 Intensity ratio of FA fluorescence at 450 nm after UV to that before UV. The nanogels were produced with a protein concentration of 5 mg/mL, a polysaccharide concentration of 15 mg/mL, and an FA concentration of 0.25 mmol/L.

A mixed solution of FA and the protein was irradiated at pH 7.4. After the irradiation, the FA in the solution was isolated by ultrafiltration for fluorescence measurement. Interestingly, although the protein binds only about 10% of the FA at pH 7.4, the FA degradation degree is only 27% compared with the free FA (Table 4). This result is similar to the report of Vorobey et al. that the FA photodegradation decreased drastically in the presence of HSA although only less than 15% of the FA was bound to HSA.11 The soy polysaccharide has only about 6.3% protein; compared with the soy protein, a higher concentration is needed for the soy polysaccharide to achieve a similar effect of protecting FA from photodegradation as shown in Table 4.

The FA/protein/polysaccharide nanogel solution was radiated at pH 4.0. After the irradiation, the nanogel solution was adjusted to pH 7.4 to release and then isolate FA. The nanogels can effectively protect the FA from photodegradation; the fluorescence spectrum indicates that only about 17% of the loaded FA degraded after the UV radiation (Figure 6 and Table 4). The absorption spectrum of the FA after irradiation and then release and isolation shown in Figure 7 does not change much in intensities and peak wavelengths compared with the spectrum of the released FA without irradiation. This result indicates most of the FA molecules remain in their natural structure after the irradiation. We also investigated the nanogel solution after 1 month of storage. The fluorescence intensity of the FA released and then isolated from the stored nanogels is...
the same as the intensity of the FA from freshly prepared nanogels. That is to say, the FA molecules in the nanogels keep their natural structure during storage.

Figure S3 of the Supporting Information shows the FA/protein/polysaccharide nanogel solution is not transparent, which may screen the UV irradiation. Therefore, the nanogel solution was diluted 10 times with the same pH aqueous solution. After the dilution, the nanogel solution is transparent. Table S shows that about 55% of the FA degraded after dilution and then irradiation, higher than the 17% degradation without the dilution (Table 4). However, when the diluted nanogel solution contains 15 mg/mL free polysaccharide, which does not change the transparency significantly, the FA degradation decreases to 29% after the irradiation. Similarly, the FA degradation decreases to 21% when the diluted nanogel solution contains 5 mg/mL free soy protein (Table 5). The data in Table S demonstrate that the increase of soy protein or soy polysaccharide concentration can reduce the FA photodegradation.

Table 5. FA Degradation Degrees in Different Samples after UV Irradiation

<table>
<thead>
<tr>
<th>sample</th>
<th>FA concn (mmol/L)</th>
<th>pH value during UV radiation</th>
<th>I_UV/I_0</th>
<th>degradation degree (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FA</td>
<td>0.025, pH 7.4</td>
<td>87 ± 2</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>diluted nanogel solution ± 5 mg/mL soy protein</td>
<td>0.025, pH 4.0</td>
<td>48 ± 2</td>
<td>55 ± 2</td>
<td></td>
</tr>
<tr>
<td>diluted nanogel solution + 15 mg/mL soy polysaccharide</td>
<td>0.025, pH 4.0</td>
<td>25 ± 3</td>
<td>29 ± 4</td>
<td></td>
</tr>
<tr>
<td>diluted nanogel solution + 5 mg/mL soy protein</td>
<td>0.025, pH 7.4</td>
<td>22 ± 4</td>
<td>25 ± 5</td>
<td></td>
</tr>
<tr>
<td>FA (without oxygen)</td>
<td>0.25, pH 7.4</td>
<td>18 ± 2</td>
<td>21 ± 3</td>
<td></td>
</tr>
</tbody>
</table>

Stable FA-loaded protein/polysaccharide nanogels were fabricated by breaking down the original aggregates of soy protein, binding soy protein with soy polysaccharide and FA at pH 4.0, and gelation of soy protein. The nanogels have a polysaccharide surface that makes the nanogels dispersible in acidic conditions where FA is insoluble and soy protein forms precipitates after heating. The nanogels protect the loaded FA from decomposition in the presence of heat, light, and oxygen at acidic conditions and release naturally structured FA at neutral pH.

■ ASSOCIATED CONTENT

 Supporting Information

 SDS–polyacrylamide gel electrophoresis analysis of the soy protein, influence of the preparation conditions on the stability of soy protein/polysaccharide complex nanogels, pyrene fluorescence measurements, and digital photographs of various samples. This material is available free of charge via the Internet at http://pubs.acs.org/.

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 Notes

 The authors declare no competing financial interest.

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■ REFERENCES


