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Effects of Mass Ratio, pH, Temperature, and Reaction Time on Fabrication of Partially Purified Pomegranate Ellagitannin−Gelatin Nanoparticles

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ABSTRACT: Nanoparticles were fabricated using self-assembly between partially purified ellagitannins (PPE) and gelatin. The factors affecting fabrication of nanoparticles, including PPE-to-gelatin mass ratio, pH, temperature, and reaction time, were investigated and the characteristics of formed nanoparticles, including sizes, zeta-potentials, and loading efficiency, were assessed. Nanoparticles that were fabricated using PPE-to-gelatin mass ratio from 1:5 to 6:5 had particle sizes from 121.5 to 129.0 nm. Increasing the ratio to 9:5 caused a drastic increase of particle size (620.7 nm) and was accompanied by formation of precipitation in the colloidal system. Nanoparticles fabricated in the pH range 4.0 to 5.3 (gelatin solution) had particle sizes ranging from 20.6 to 193.9 nm and zeta-potential between +14.7 and +23.8 mV, respectively. Loading efficiency of punicalagin A and B in the nanoparticles under these pH values ranged from 29.5% to 84.3% and from 10.6% to 73.9%, respectively. Extreme pH of gelatin solutions (pH 1.0, 2.0, 3.0, or 11.0) hindered the formation of nanoparticles due to possible hydrolyzation of ellagitannins and weakened affinity between ellagitannins and gelatin. Although gelatin at isoelectric point (pH 6−7) provided more hydrophobic sites to interact with ellagitannins, weakened zeta-potentials resulted in poor stability of nanoparticle suspension. Nanoparticles formed between 25 to 50 °C had particle size below 500 nm, whereas lower temperatures increased the size of nanoparticles. Nanoparticles were formed after 12 h of reaction time, and the nanoparticle colloidal suspension remained stable for 4 days. PPE−gelatin nanoparticles fabricated using PPE-to-gelatin mass ratio below 7:5, pH 4.0, temperature 25−40 °C, and reaction time 1.5 days had smaller particle sizes, higher zeta-potentials, and good loading efficiency.

KEYWORDS: pomegranate ellagitannins, punicalagin, gelatin, nanoparticles

INTRODUCTION

Nanoparticle fabrication is of particular interest for nutritional sciences due to the unique benefits of nanosized carriers to increase oral bioavailability of loaded bioactive ingredients.[1−3] In the gastrointestinal tract, enterocytes and microfold cells in epithelial wall absorb nanoparticles by endocytosis.[2,4] Nanoparticle endocytosis was affected by particle size, zeta-potential, and morphology. It was reported that particle size below 500 nm significantly improved the oral bioavailability of ingredients loaded on nanoparticles.[2,5,6] Zeta-potentials, or surface charge, of nanoparticles affects the stability of nanoparticle suspension system and their absorption in the gastrointestinal tract. It was known that zeta-potential of more than ±30 mV enhanced the stability of nanoparticles in suspension.[7−9] Positive zeta-potential stimulated the entrapment of nanoparticles in epithelial walls due to ionic interactions between nanoparticles and mucin layer on the surface of enterocytes and microfold cells, which prolongs retention time of nanoparticles on epithelial walls.[4] Spherical nanoparticles can be recognized and captured by receptors on the surface of epithelial cells, causing increased absorption rate compared to nanoparticles of different morphologies.[2,10]

Nanoparticles are fabricated using two basic approaches: "top-down" and "bottom-up".[2,11] The "top-down" approach adopts shear or particle collisions as the energy sources to cut the larger entities into nanoscale aggregates, whereas the self-assembly of smaller molecules is the primary mechanism for the "bottom-up" method. Self-assembly between molecules takes advantage of intermolecular affinity.[12,13] Particle size, zeta-potential, and loading efficiency of self-assembled particles are controlled by reaction conditions. In this study, partially purified pomegranate ellagitannin (PPE)−gelatin nanoparticles were fabricated under different PPE-to-gelatin mass ratios, pH conditions, temperatures, and reaction times to study their effects on particle characteristics, including particle size, zeta-potential, and loading efficiency of ellagitannins. The objective of this study was to identify reaction conditions that can result in smaller particle sizes, positive zeta-potentials, and stable nanoparticle colloidal suspension.

MATERIALS AND METHODS

Chemicals. Pomegranates were purchased from a local grocery store. Gelatin type A, formic acid, ethanol, and methanol were products of Fisher Scientific (Pittsburgh, PA). Amberlite XAD-16N resin was a product of Rohm and Haas (Midland, Michigan). Punicalagin and ellagic acid were purchased from Quality Phytochemicals LLC (Edison, NJ) and Sigma-Aldrich (St. Louis, MO), respectively. Partially purified pomegranate ellagitannins (PPE) were prepared according to a published method with minor modification.[14,15] It contained 16.6% of punicalagin A, 32.6%

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of punicalagin B, 0.3% of ellagic acid−hexoside, and 0.8% of ellagic acid. Other compounds in PPE were not characterized.

Fabrication of PPE−Gelatin Nanoparticles. Both gelatin type A (0.5 g) and PPE powder (0.5 g) were dissolved in deionized water (1000 mL) to a concentration of 0.5 mg/mL. The pH value of deionized water was 5.5, and it dropped to 5.3 and 4.2, respectively, after gelatin and PPE were dissolved. The PPE solution was mixed with gelatin solution at different reaction conditions for the self-assembly to occur. The resultant nanoparticle suspensions were centrifuged at 12,000 rpm for 5 min to separate suspension into supernatant and nanoparticles in sediment. Concentrations of punicalagin A and B in the supernatant were quantified using HPLC for the calculation of their loading efficiency.

To study the effects of PPE-to-gelation mass ratios on nanoparticle fabrication, 1, 2, 3, 4, 5, 6, 7, 8, or 9 mL of PPE solution was mixed with 5 mL of gelatin solution. This gave a PPE-to-gelatin mass ratio 1:5 to 9:5. Nanoparticles were fabricated at 25 °C for 2 days. To study the effects of pH on nanoparticle fabrication, HCl (3 mol/L) and NaOH (6 mol/L) solutions were added drop by drop to adjust the pH value of the gelatin solution to 1.0, 2.0, 3.0, 4.0, 5.3, 6.0, 7.0, or 11.0. A PPE solution of 1, 5, or 7 mL was added into 5 mL of gelatin solutions. Size, zeta-potentials, and loading efficiencies of resulting nanoparticles were measured after 2 days of reaction time. For effects of temperature on nanoparticle fabrication, PPE solutions of 1, 5, or 7 mL was mixed separately with 5 mL of gelatin solution. The suspensions were incubated at different temperatures from 5 to 50 °C for 2 days. Then, characteristics of these nanoparticles were measured. For effects of reaction time on nanoparticle fabrication, PPE solutions of 1, 5, or 7 mL were added into 5 mL of gelatin solution at 25 °C. The characteristics of the nanoparticles were measured at 0.5, 1, 1.5, 2, 3, and 4 days, respectively. Except for gelatin solutions which were used to study pH effects, the pH of gelatin and PPE solutions was not adjusted (5.3 and 4.2, respectively).

Transmission Electron Microscopy. PPE−gelatin nanoparticles were fabricated using a PPE-to-gelatin mass ratio 5:5 at 25 °C for 48 h. The pH of gelatin and PPE solutions was 5.3 and 4.2, respectively. The morphology was measured on a JEOL 200CX TEM (JEOL Inc., Tokyo, Japan).

Particle Size Analysis. Mean particle size and size distribution of fresh suspensions was measured by dynamic light scattering (DLS) using Nanotrac ULTRA with an external probe (Microtrac Inc., Largo, FL). Samples were measured in triplicate, and six readings were obtained for each replicate to calculate the average particle size.

Zeta-Potential Measurement. Zeta-potentials were determined using Brookhaven ZetaPlus (Brookhaven Instrument Corp., Holtsville, NY). The following parameters were used for zeta-potential measurement: zeta-potential model, Smoluchoswki; medium, aqueous; temperature, 22.0 °C; viscosity, 0.955 cP; refractive index, 1.334; dielectric constant, 79.63. Triplicate assay was conducted and for each sample ten readings were obtained for each replicate to calculate the average zeta-potential.

Loading Efficiency Assessment by HPLC-ESI-MS^n. An Agilent 1200 HPLC system consisting of an autosampler, a binary pump, a column compartment, and a diode array detector (Agilent Technologies, Palo Alto, CA) was interfaced to a HCT ion trap mass spectrometer (Bruker Daltonics, Billerica, MA). PPE solution and supernatant from PPE−gelatin nanoparticle suspension were filtered through 0.45 μm filter units, and 30 μL was injected without further purification, respectively. An Agilent Zorbax ODS column (4.6 mm × 25 cm) was used for separation of ellagitannins. The binary mobile phase consisted of (A) formic acid:water (2:98 v/v) and (B) formic acid: methanol (2:98 v/v). A 65 min gradient was as follows: 0−30 min, 1−20% B linear; 30−45 min, 20- 40% B; 45−60 min, 40−95% B; 60−65 min, 95−1% B; followed by 5 min of re-equilibration of the column before the next run. The detection wavelength on the diode array detector was 378 nm. Electrospray ionization in negative mode was performed using nebulizer 65 psi, dry gas 11 L/min, drying temperature 350 °C, and capillary 4000 V. The full scan mass spectra was measured from m/z 100 to 2000. The retention time for punicalagin A, punicalagin B, ellagic acid−hexoside, and ellagic acid were 14.48, 19.93, 42.50, and 49.83 min. Punicalagin A and B were quantified using external standard. Data were collected and calculated using Chemstation software (Version B. 01.03, Agilent Technologies, Palo Alto, CA). The loading efficiency was calculated as (amount of ellagitannin embedded into PPE−gelatin nanoparticles)/(amount of ellagitannin used for nanoparticle fabrication).

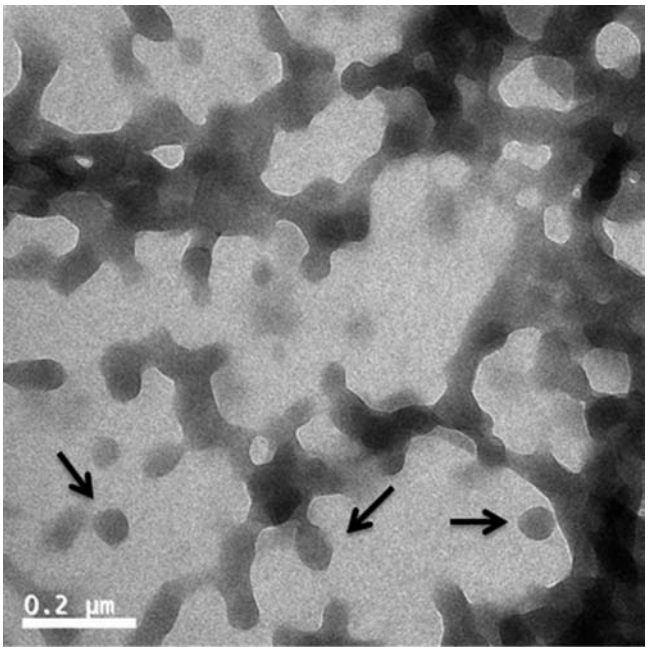

Figure 1. Morphology of PPE−gelatin nanoparticle, measured by transmission electron microscopy, fabricated using PPE-to-gelatin mass ratio of 5:5 at 25 °C for 48 h. The pH of gelatin and PPE water solutions was 5.3 and 4.2, respectively. Nanoparticles in colloidal suspension are denoted by arrows.

Data Grafting. Samples were analyzed in triplicate, and the average values were used. Data were expressed as mean ± standard deviation. Figures were drafted using Sigma-Plot (Version 10.0, Systat Software, Chicago, IL).

RESULTS

Transmission Electron Microscopy. Self-assembly between PPE and gelatin in aqueous medium resulted in the formation of nanoparticles. Nanoparticles fabricated using PPE-to-gelatin mass ratio 5:5 had spherical morphology (Figure 1).

Effects of PPE-to-Gelatin Mass Ratios. The average particle sizes of the nanoparticles from the PPE-to-gelatin mass ratio from 1:5 to 6:5 were in a range of 121.5 to 129.0 nm. A noticeable increase of nanoparticle size was observed as PPE-to-gelatin mass ratio increased to 7:5 and 8:5, but the particle sizes were still below 272.5 nm. The average particle size was dramatically elevated to 620.7 nm at a mass ratio of 9:5. Turbidity in the suspension started to appear at this mass ratio. This observation suggested that there was a critical point when using PPE and gelatin to prepare nanoparticles. At such a point, binding sites on gelatin were saturated by ellagitannins. Adding

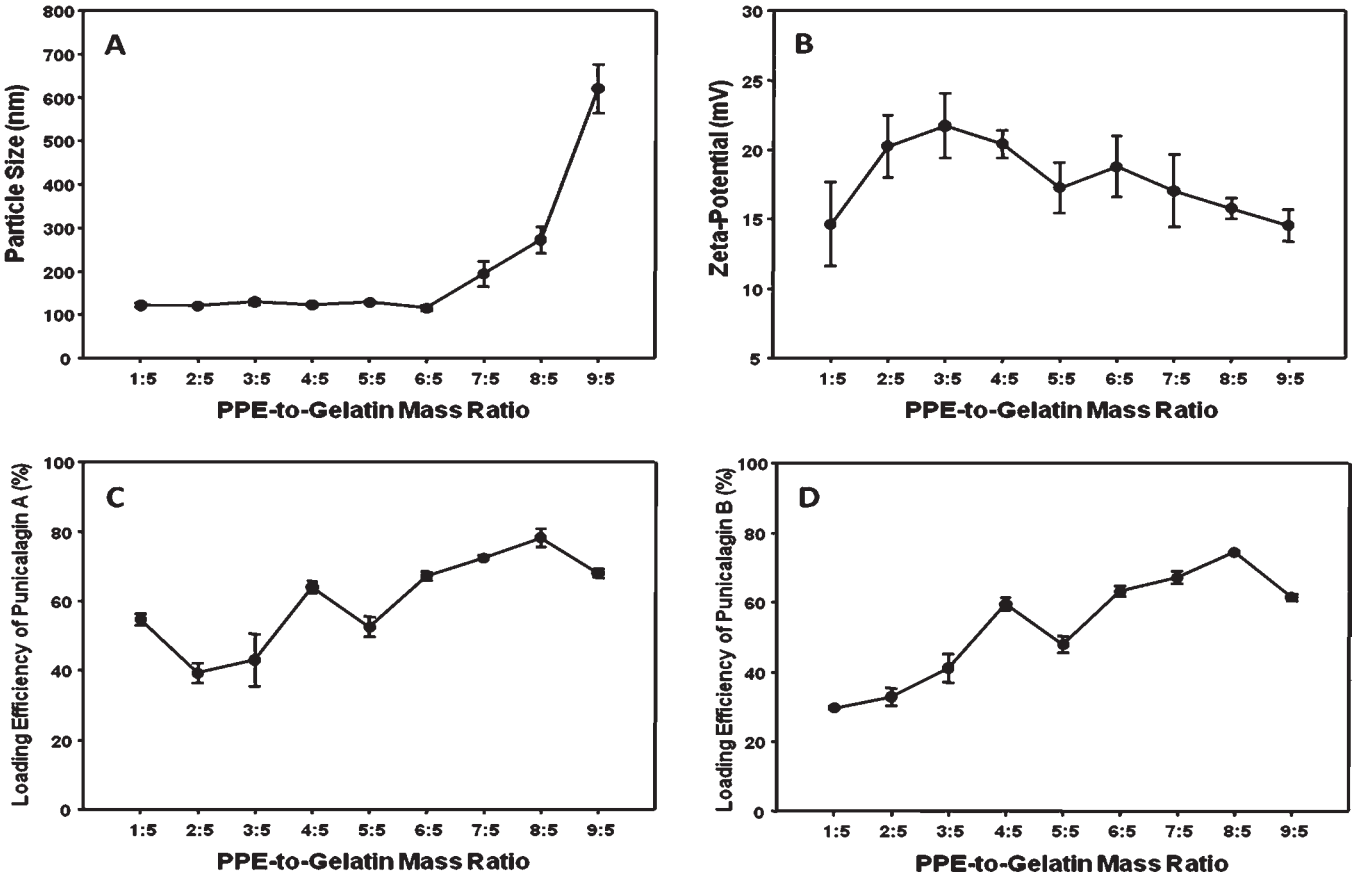

Figure 2. PPE−gelatin nanoparticle characteristics on (A) particle size, (B) zeta-potentials, and loading efficiency of (C) A and (D) B under the conditions of PPE-to-gelatin mass ratio from 1:5 to 9:5 at 25 °C for 2 days. The pH of gelatin and PPE water solutions was 5.3 and 4.2, respectively.

more tannin molecules caused precipitation due to particle aggregation. As a result, the colloidal system lost stability and precipitation started to occur. The critical point for PPE and gelatin appeared to be between mass ratio 8:5 and 9:5.

Zeta-potentials of the nanoparticles increased and reached a peak of +21.7 mV at a mass ratio of 3:5. It dropped to +15.8 mV and +14.5 mV when the ratios were increased to 8:5 and 9:5, respectively (Figure 2 B).

Loading efficiency of punicalagin A was 39.3% at a mass ratio of 2:5 and increased to reach a peak of 78.2% at a mass ratio of 8:5 (Figure 2C). A similar trend was observed for punicalagin B. Its loading efficiency was elevated from 29.6% to 74.5% when the ratio increased from 1:5 to 8:5 (Figure 2D). The characteristics of nanoparticles that were fabricated in deionized water were slightly different from the earlier study, where the pH of DI water was slightly different.[15]

Effects of pH. When gelatin solutions with pH 1.0, 2.0, 3.0, or 11.0 were used for fabrication, particle sizes around 0 nm were observed at three mass ratios. This indicated that no nanoparticles were formed in these pH conditions and the PPE and gelatin existed in a true solution instead of a colloidal suspension (Figure 3 A). At pH 4.0, the size of nanoparticles at mass ratio 1:5, 5:5, and 7:5 was 20.6, 43.9, and 67.0 nm, respectively. At pH 5.3, the size of nanoparticles was 121.5, 129.0, and 193.9 nm, respectively. However, the average sizes of nanoparticles at mass ratio 5:5 and 7:5, at pH above 6.0, were dramatically elevated to 752.0 and 1307.3 nm, respectively. The pH range of 4.0 to 5.3 gave rise to nanoparticles with smaller sizes.

The zeta-potential of PPE−gelatin nanoparticles was significantly affected by pH (Figure 3 B). The zeta-potential of nanoparticles using gelatin solution with pH 4.0 and 5.3 showed +26.1 and +14.7 mV at a mass ratio of 1:5, +23.8 and +17.3 mV at a mass ratio of 5:5, and +15.0 and +17.0 mV at a mass ratio of 7:5, respectively. Nanoparticles fabricated at pH 6.0 and 7.0 had zeta-potential −1.5 and +1.1 mV from mass ratio 1:5, −1.3 and +1.0 mV from of mass ratio 5:5, and +0.9 and −0.7 mV from mass ratio 7:5, respectively. Such low zeta-potentials suggested that surface charges of nanoparticles were close to neutral. Low zeta-potential decreases the stability of nanoparticles in colloidal systems. Nanoparticles that were fabricated using gelatin solution with pH from 4.0 to 5.3 showed higher surface charges and better stability.

In the pH range from 4.0 to 7.0, increases were observed in loading efficiency of punicalagin A and B from nanoparticles fabricated under these mass ratios (Figure 3 C and D). For punicalagin A, its loading efficiency in nanoparticles was increased, from 48.8% to 54.0% at a mass ratio of 1:5, from 29.5% to 84.3% at a mass ratio of 5:5, and from 44.1% to 70.7% at a mass ratio of 7:5, respectively. Punicalagin B was embedded into nanoparticles, from 10.6% to 21.1%, from 23.1% to 73.9%, and from 33.9% to 63.5%, respectively.

Effects of Temperatures. At a mass ratio of 1:5, nanoparticles fabricated at different temperatures (25−50 °C) had similar particle sizes from 114.2 to 121.5 nm (Figure 4A). Particle sizes with mass ratios of 5:5 and 7:5 were highest at 5 °C. The sizes decreased to below 316.4 nm when the temperature was 25 °C or higher.

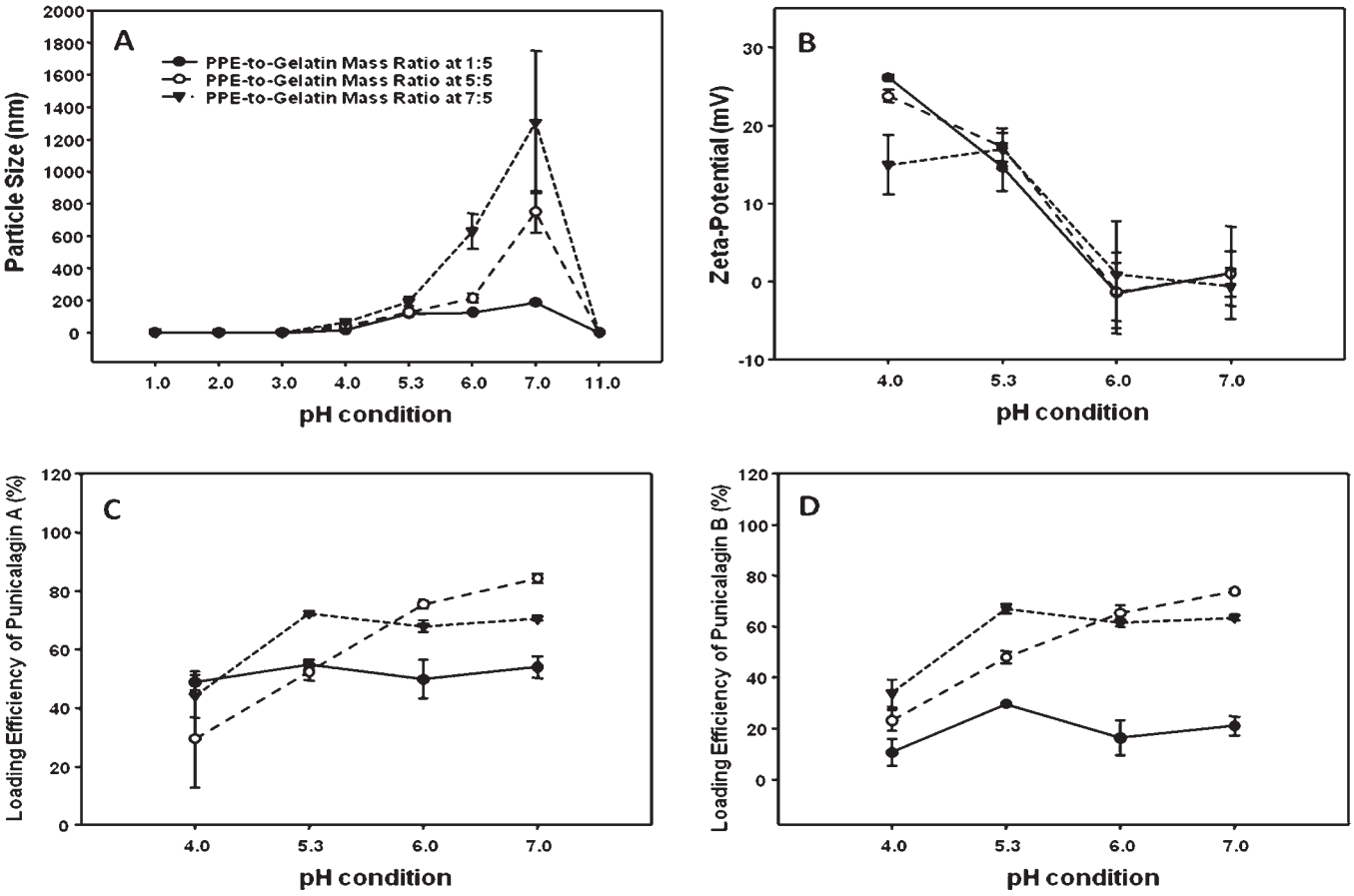

Figure 3. PPE−gelatin nanoparticles characteristics on (A) particle size, (B) zeta-potentials, and loading efficiency of (C) A and of (D) B under the conditions of PPE-to-gelatin mass ratio at 1:5, 5:5, and 7:5, the pH of gelatin solutions was 1.0, 2.0, 3.0, 4.0, 5.3, 6.0, 7.0, and 11.0, temperature at 25 °C, and reaction time of 2 days. The pH of gelatin water solution was 4.2.

Nanoparticles fabricated below 25 °C under these mass ratios showed stable positive-charge properties (Figure 4 B). Nanoparticles formed at 5, 10, 15, and 25 °C, respectively, had zeta-potentials from +14.7 to +17.2 mV at a mass ratio of 1:5, from +17.3 to +19.3 mV at a mass ratio of 5:5, and from +14.2 to +17.0 mV at a mass ratio of 7:5. However, fluctuations in the zeta-potentials were observed above 25 °C. The zeta-potential of nanoparticles fabricated under 7:5 dropped dramatically from +17.0 to −0.9 mV.

Elevation of temperature ranging from 5 to 50 °C slightly increased loading efficiency of punicalagin A and B (Figure 4 C and D). The increase was more pronounced for punicalagin A at a mass ratio of 1:5.

Effects of Reaction Time. Nanoparticles were fabricated under the mass ratio of 1:5 after 12 h, and particle size remained similar to 4 days (137.9 nm) (Figure 5 A). Under a mass ratio of 5:5, nanoparticles with particle size of 75.0 nm were fabricated after 12 h of interaction, and size of 96.3 nm was observed by 4 days. Nanoparticles at mass ratio of 7:5 had particle size of 124.6 nm after 12 h. However, it dramatically increased to 193.9 nm after 2 days and 923.0 nm after 4 days, respectively.

Particle surface-charge showed little change at different reaction time (Figure 5 B). Zeta-potentials of the nanoparticles, in reaction time from 0.5 to 4 days, fluctuated between +14.7 and +18.4 mV under a mass ratio of 1:5, between +17.3 and +19.7 mV under a mass ratio of 5:5, and between +15.5 and +17.6 mV under a mass ratio of 7:5, respectively.

In nanoparticles made at a mass ratio of 1:5, loading efficiency of punicalagin A and B remained 68.6% and 38.0% by 4 days, respectively (Figure 5 C and D). Nanoparticle suspensions at a mass ratio of 5:5 had a loading efficiency of 67.4% for punicalagin A and 57.2% for punicalagin B at 12 h of interaction, and loading efficiency of 88.3% and 78.9% was obtained after 4 days, respectively. From 78.3% to 85.8% of punicalagin A and from 70.3% to 73.1% of punicalagin B was observed in loading efficiency from 0.5 to 4 days, respectively.

DISCUSSION

Fabrication of tannin−protein nanoparticles depends on affinity between tannins and proteins. The structure of the protein molecule determines the degree of affinity.[13,16,17] It was known that the protein with compact tertiary structure provides less hydrophobic sites, constraining interaction with tannin molecules and thus resulting in poor affinity for tannin molecules.[13] However, gelatin is a proline-rich protein with extended random coil conformation. Hence, gelatin provides more interaction sites for tannin molecules, eventually promoting higher affinity for tannin molecules.[16,17] Assembly of tannin−protein particles is also directly determined by the structures of tannin molecules. Tannin molecular weight is a critical factor. Tannins with a higher molecular weight possess larger structure and more hydrophobic sites, which results in

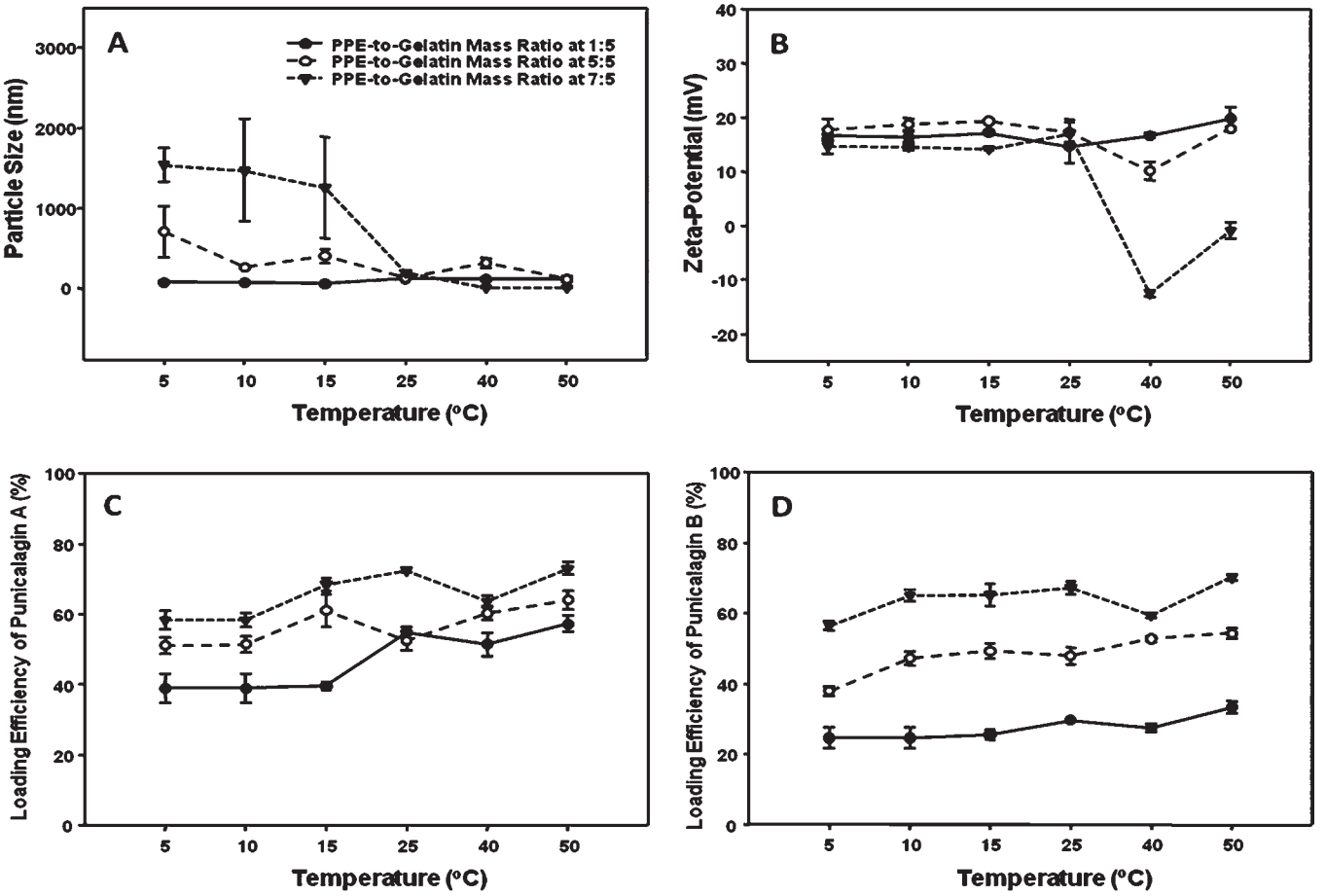

Figure 4. PPE−gelatin nanoparticle characteristics on (A) particle size, (B) zeta-potentials, and loading efficiency of (C) A and of (D) B under PPE-to-gelatin mass ratio at 1:5, 5:5, and 7:5 at 5, 10, 15, 25, 40, or 50 °C for 2 days. The pH of gelatin and PPE water solutions was 5.3 and 4.2, respectively.

increased affinity toward protein.[18] Among four ellagitannins that were identified in PPE, punicalagin A and B were predominant in amount and also had much higher molecular weight (1084) than ellagic acid−hexoside (464) and ellagic acid (302).[15] Punicalagins were able to bind with gelatin to form nanoparticles, whereas ellagic acid−hexoside or ellagic acid could not. Moreover, tannin with higher molecular weight differs in conformation. Change of conformations with temperature and pH caused variable alteration of hydrophobic sites and thus increased or decreased affinity toward gelatin.[13]

Self-assembly of tannin and protein is based on interaction among them via hydrogen bonding and hydrophobic interaction as driving forces.[19,20] The same amount of gelatin was used in the reaction system to provide the same number of hydrophobic sites. Different amounts of PPE were added into the system to test effects of mass ratio. When a small amount of tannins was added in the system, limited numbers of phenolic hydroxyl groups from tannin molecules were present to interact with excessive amount of protein molecules. In this case, one tannin molecule can bind with two or more gelatin molecules. After more PPE was added, more hydroxylic groups from the tannins were brought into the system, increasing interaction between tannin and gelatin molecules. As a result, a single protein molecule could carry more tannin molecules and higher loading efficiency was observed (Figure 1 C and D). However, when the amount of PPE reached the critical point, interaction sites on gelatin molecules became saturated by tannin molecules. PPE that exceeded this stoichiometric value formed an extended network of hydrogen bonds among nanoparticles, which resulted in their aggregation in the suspension.[12,21] Therefore, fabrication of nanoparticles should be conducted below the critical point. Nanoparticles at mass ratios below 6:5 had similar particle sizes. However, lower loading efficiencies were observed in lower mass ratios, such as 2:5, 3:5, which indicated that one tannin molecule might assemble with two or three gelatin molecules during the fabrication process. Larger particle sizes were observed from the particles under mass ratio above 8:5, which suggests that the aggregation occurred when ellagitannin molecules exceeded the saturated value of the network of hydrogen bonds among the nanoparticles. It was proposed that nanoparticles with zeta-potential above ±30 mV provided the best stability. Lower zeta-potentials means weak repulsion between particles and can promote aggregation of nanoparticle and precipitation.[9] Particles fabricated using mass ratio above 8:5 suggested nanoparticles had very low zeta-potential. This may promote aggregation of particles and increased particle sizes.

The pH is a critical factor that affects the structures, the conformations, and charge properties of gelatins and tannins, respectively. Ellagitannins can be hydrolyzed into ellagic acid in extreme pH conditions.[22,23] Hydrolyzed ellagitannins will

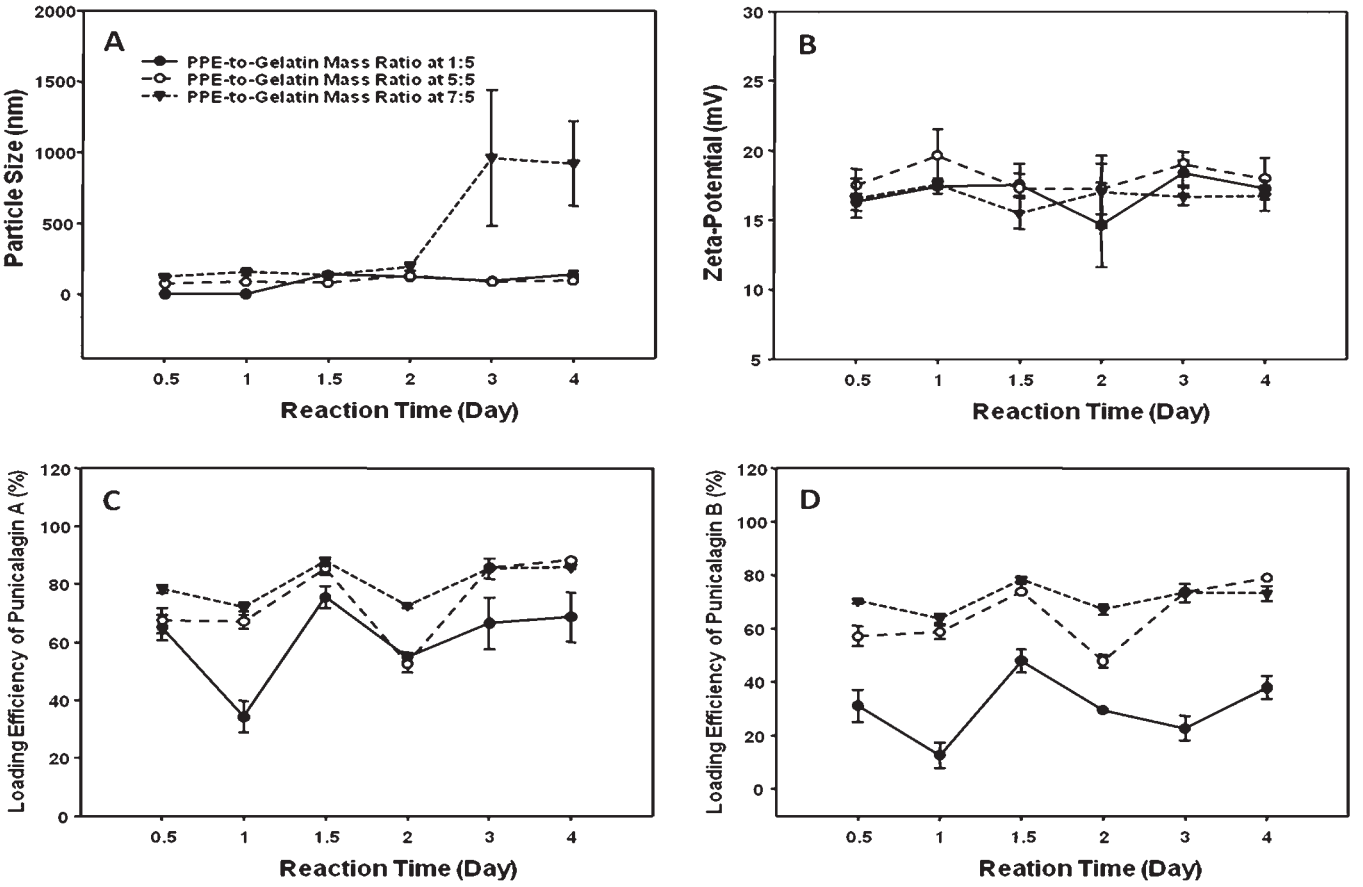

Figure 5. PPE−gelatin nanoparticle characteristics on (A) particle size, (B) zeta-potentials, and loading efficiency of (C) A and of (D) B under PPE-to-gelatin mass ratio at 1:5, 5:5, and 7:5, at 25 °C for 0.5, 1, 1.5, 2, 3, or 4 days. The pH of gelatin and PPE water solutions was 5.3 and 4.2, respectively.

lose the capacity to bind with gelatin. This may explain the absence of nanoparticles when using gelatin solution of these pH conditions (pH 1.0, 2.0, 3.0, and 11.0). Gelatins at different pH conditions possess different numbers of ionizable sites.[24] Gelatin at isoelectric point provides the most binding sites, which will promote self-assembly between gelatin and tannin.[12] However, stability of nanoparticle suspension relies critically on charge repulsions among nanoparticles. Zero repulsions among particles are anticipated when zeta-potentials of nanoparticles are neutral, promoting the aggregation of particles and thus leading to precipitation.[7−9] The isoelectric point of gelatin is around pH 6.0 to 7.0. Our study showed that particles fabricated using gelatin solution with pH from 4.0 to 5.3 had small particle sizes and positive zeta-potential. However, dramatic increases of particle size were observed in pH 6.0 and 7.0. This was consistent with weak zeta-potentials and declined repulsion among particles.

Excessively high temperature alters the structural conformations of ellagitannins and gelatins.[24,25] In our study, PPE−gelatin nanoparticles were able to be fabricated at 50 °C, suggesting that this temperature has little negative influence on molecular binding. It was known that the coil conformation of gelatin became looser and more extended with the increase of temperature.[12] Higher temperature exposes more hydrophobic groups from gelatins to bind with more ellagitannin molecules. As a result, loading efficiency of tannin molecules was increased.[12,24] Particles fabricated under mass ratio of 1:5, 5:5, and 7:5, within the temperature range from 25 to 50 °C, had small particle size and higher loading efficiency, respectively. When interaction happens at lower temperatures, compact coil conformation of gelatin provides less hydrophobic sites for interaction with tannin molecules. As a result, larger particles were formed. Particle sizes prepared from the mass ratio of 5:5 and 7:5 were above 500 nm when the temperature was below 25 °C.

When PPE and gelatin are mixed, interaction between them occurs rapidly until their hydrophobic sites are saturated.[13,17] The suspension will be stable if no more tannin molecules participate in it to induce particle aggregation. In our study, nanoparticle suspensions at mass ratios of 1:5 and 5:5 had favorable nanoparticle characteristics after 0.5 day reaction and remained stable until 4 days. Nanoparticle suspension from the mass ratio of 7:5 had constant particle characteristics by 2 day reaction, but increased particle size with large variation. It indicated that assembly of particles was promoted and eventually led to precipitation.

In conclusion, PPE-to-gelatin mass ratio determined characteristics and stability of nanoparticle suspension. The critical point for PPE−gelatin nanoparticles was at a mass ratio of 8:5. Nanoparticles prepared in pH range from 4 to 5 had particle sizes and zeta-potentials that favor higher bioavailability of loaded ellagitannins. Reaction temperature from 25 to 50 °C had a positive influence on nanoparticle characteristics. Self-assembly occurred rapidly between gelatin and ellagitannin, and fabrication was done after 12 h and remained stable after 4 days.

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