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4 **Development of Tyrosinase-aided Crosslinking Procedure for Stabilizing Protein**
5 **Nanoparticles**

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20 **ABSTRACT**

21 Crosslinking is essential for maintaining the integrity of protein-based nanoparticles against
22 environmental fluctuations. However, conventional crosslinkers (e.g. glutaraldehyde) are
23 usually toxic and environmental unfriendly. In this study, a tyrosinase-aided crosslinking
24 procedure was developed and compared to the conventional glutaraldehyde-based process.
25 Sodium caseinate (SC) nanoparticles were synthesized by ionic gelation or desolvation under
26 aqueous or alcoholic condition, respectively. The nanoparticles were stabilized by different
27 crosslinkers and challenged under simulated environmental stress, including acidification and
28 antisolvent removal. A simple based on dynamic light scattering (DLS) was developed to
29 evaluate the particle integrity, using the retention of particle size and count rate as indicators.
30 Satisfying crosslinking was observed for the ionic gelation procedure in the presence of
31 tyrosinase (10 U/mol SC) combined with two phenols (catechol or chlorogenic acid, 2.5
32 mol/mol SC). In comparison, a higher dose of glutaraldehyde (7.5 mol/mol SC) was required
33 for achieving a comparable crosslinking effect. For the desolvation procedure, the efficacies
34 for both glutaraldehyde and tyrosinase-phenol blends decreased, although they were still
35 significant, and glutaraldehyde exhibited better crosslinking effect. The results from the DLS
36 study were in consistence with those obtained from scanning electron microscopy. Overall,
37 tyrosinase-aided oxidation is a competitive, low-toxicity approach for crosslinking
38 protein-based nanostructures.

39 **Keywords:** Crosslinkers, Protein nanoparticles, Tyrosinase, Phenolic compounds,

40 Glutaraldehyde, Sodium caseinate

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1. Introduction

Biopolymer-based nanoparticles have been reported extensively as delivery systems for enhancing the bioavailability and efficacy of nutraceuticals or drugs (Elzoghby, Samy, & Elgindy, 2012; Ron, Zimet, Bargarum, & Livney, 2010; Teng, Luo, & Wang, 2013; Zhong, Tian, & Zivanovic, 2009). These nano-sized vehicles offer a number of advantages, such as higher stability, increased solubility, prolonged gastric retention time, enhanced tissue permeability, and elevated cellular uptake. Compared with the systems formed by synthetic polymers, biopolymer-based systems exhibit high biocompatibility and thus low toxicity (Ting, Jiang, Ho, & Huang, 2014). Proteins are amphiphilic biopolymers which show adequate interaction with both the solvent and entrapped compounds (Marty, Oppenheim, & Speiser, 1978). Moreover, proteins are capable of forming nanoparticles under aqueous, alcoholic, and apolar procedures (Jahanshahi & Babaei, 2008), and they can be chemically modified through simple procedures to gain additional benefits such as targeted delivery. These characters make proteins versatile candidates for the protection and delivery of various nutraceuticals.

Despite the abovementioned merits, proteins possess flexible conformation which is subject to change under environmental fluctuation. For instance, nanoparticles synthesized via ionic gelation may be dissociated as the solvent composition (pH, ionic strength, etc.) varies (Shi, Zhou, & Sun, 2005; Surh, Decker, & McClements, 2006; Teng, Luo, & Wang, 2012), and those prepared by organic solvent desolvation are prone to disintegration as the antisolvent is removed (Teng, et al., 2012). Chemical crosslinkers are capable of stabilizing nanoparticles

via covalent bonds, but their application is impeded by several drawbacks. For instance, glutaraldehyde as a dialdehyde polymerizes under certain conditions and crosslinks proteins by reacting with multiple lysine residues (Migneault, Dartiguenave, Bertrand, & Waldron, 2004). It is by now the most widely employed crosslinker for protein-based nanoparticles (Dreis, et al., 2007; Leo, Vandelli, Cameroni, & Forni, 1997) and hydrogels (Hwang & Damodaran, 1996; Rathna & Damodaran, 2001) due to its low cost, simple reaction procedure, and desirable crosslinking efficacy (Migneault, et al., 2004). However, glutaraldehyde is potentially harmful to human because of its considerable toxicity, including acute toxicity, respiratory irritation, and mutagenic and carcinogenic effects (Takigawa & Endo, 2006). On the other hand, genipin as a naturally occurring crosslinking agent has been studied extensively for crosslinking nanoparticles (Elzoghby, Samy, & Elgindy, 2013; Won & Kim, 2008), hydrogels (Silva, Saint-Jalmes, de Carvalho, & Gaucheron, 2014; Song, Zhang, Yang, & Yan, 2009), and films (Bigi, Cojazzi, Panzavolta, Roveri, & Rubini, 2002; González, Tártara, Palma, & Igarzabal, 2015). However, the optimal dose of genipin for stabilizing protein-based systems is as high as 22 mg/ml (Liang, Chang, Lin, & Sung, 2003), which adds to the cost of nanoparticle preparation. Moreover, the toxicological profile of genipin at such a high level has not been established (Wang, Lau, Loh, Su, & Wang, 2011). As a consequence, the development of environmentally friendly and low-toxicity crosslinkers is imperative for the application of protein nanoparticles in the food and pharmaceutical industry.

In the past few decades, protein crosslinking by enzymes (e.g., transglutaminase, laccases,

84 peroxidases, sulfhydryl oxidases, etc.) has attracted much attention in food industry (Lantto,
85 2007). Tyrosinases are dicopper enzymes belonging to the polyphenol oxidase (PPO) group,
86 and they exist widely in fungi, plants, bacteria and mammals (Halaouli, Asther, Sigoillot,
87 Hamdi, & Lomascolo, 2006). Tyrosinases play an important role in several biological
88 processes, such as the enzymatic browning in fruit and vegetables, as well as the
89 sclerotization in insects (Loizzo, Tundis, & Menichini, 2012; Sugumaran, Hennigan, &
90 O'Brien, 1987). In the presence of oxygen and certain phenols (e.g., catechol), tyrosinases are
91 capable of catalyzing two sequential reactions, i.e., the formation of o-diphenols by the
92 hydroxylation of monophenols (monophenolase activity), and the conversion of o-diphenols
93 into corresponding electrophilic o-quinones (diphenolase activity) (Kubo, et al., 2003). The
94 formed o-quinones are unstable intermediates that participate in several non-enzymatic
95 reactions with the nucleophilic functional (sulfhydryl, amine, amide, hydroxyl, carboxyl,
96 indole, and imidazole) groups in amino acids and proteins, resulting in covalent crosslinking
97 of protein molecules (Bittner, 2006; Hopkins & Kramer, 1992). In addition, the native
98 tyrosinal residues on certain protein molecules can also be oxidized in the presence of
99 tyrosinase, forming effective crosslinkers without added phenols (Thalmann & Lötzbeyer,
100 2002). So far, tyrosinase has been reported to crosslink several proteins including lysozyme
101 (Leatham, King, & Stahmann, 1980), casein (Hurrell, Finot, & Cuq, 1982), and whey
102 proteins (Thalmann, et al., 2002). The structures generated via tyrosinase-aided crosslinking
103 include protein oligomers (Thalmann, et al., 2002) and hydrogels (Kim, et al., 2014).
104 However, no systematic investigation has been reported on the successful crosslinking of

protein-based nanoparticles using tyrosinase.

In this study, sodium caseinate (SC) was chosen as a model protein based on two considerations: the abundance in reactive residues (tyrosine, lysine, cysteine, etc.) (Jollès & Alais, 1962) and the simple procedures for particle preparation. Two strategies including ionic gelation and ethanol desolvation were applied to produce primary protein nanoparticles, which were then crosslinked by tyrosinase (alone or with added natural phenols) or glutaraldehyde. The particle size, morphology, and surface charge were characterized by dynamic light scattering (DLS), scanning electron microscopy (SEM), and zeta potential measurement, respectively. Thereafter, the crosslinked particles were subjected to an environmental stress, either acidification for the aqueous procedure or solvent evaporation for the alcoholic process, in order to dissociate the non-covalently crosslinked nanoparticles. The efficacies of different crosslinkers were then evaluated by comparing the particle size and count rate before and after the application of environmental stress. The effects of reagent type and concentration on the crosslinking efficacy were compared in details, and possible mechanisms underlying the observed data were discussed.

2. Materials and methods

2.1 Materials

Sodium caseinate (SC) and tyrosinase from mushrooms (TYR, lyophilized powder, ≥ 1000 unit/mg solid) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Catechol (CAT), chlorogenic acid hemihydrate (CA, $\geq 98.0\%$ purity), methyl gallate (MG, $\geq 98.0\%$ purity) and

gallic acid (GAc, 98% purity) were obtained from VWR-International (Radnor, PA, USA).

Calcium chloride, glutaraldehyde, and all other reagents were of analytical grade.

2.2 Determination of crosslinking effect on SC nanoparticles

The SC nanoparticles were prepared through two different procedures, as described below.

Aqueous procedure (ionic gelation, **Fig 1a**). SC was dispersed in water (deionized water,

same hereinafter) at 2 mg/mL, stirred at 400 rpm overnight at room temperature, and adjusted

to pH 6.4 with 1 mol/L HCl. Calcium chloride (CaCl_2) was then added to resultant dispersion

to achieve 0.4 mg/mL. The mixture was diluted by two-fold with water and readjusted to pH

6.4 with 1 mol/L NaOH. These procedures yielded an opaque dispersion containing ionically

crosslinked SC nanoparticles, which was defined as the primary dispersion. Chemical

crosslinking solutions were prepared by dissolving glutaraldehyde (GA, 0.5 mg/mL),

tyrosinase (TYR, 5 kU/mL, aqueous solution, same hereinafter), or phenols (CAT, CA, MG,

and GAc, 0.5 mg/mL) in water. All dispersions were pre-warmed at 37 °C in a water bath for

30 min. Chemical crosslinking was initiated by introducing different crosslinking solutions to

the primary dispersion at predetermined mixing ratios (0.5 to 10 mol phenols or

glutaraldehyde per mol SC, or 10 U TYR per mol SC). The vials containing the resultant

mixtures were incubated at 37 °C in a reciprocal water shaking bath (120 rpm) without

covering to achieve sufficient phenol oxidation. After 4 h of crosslinking, the mixtures (pH

~6.0 after reaction) were adjusted to pH 6.4 using 1 mol/L NaOH. The particle size and count

rate (total amount of photons scattered by the particles) of the mixtures were analyzed by

dynamic light scattering (DLS). Detailed procedure for DLS will be introduced in Section 2.3.

Afterwards, the samples were acidified to pH 3.0 using 1 mol/L HCl to weaken the electrostatic attraction between SC and Ca^{2+} , and the resulting dispersions were subjected to the same DLS measurement. A primary dispersion containing no chemical crosslinker (control) was also prepared, acidified, and subjected to the same DLS analysis.

Alcoholic procedure (ethanol desolvation, **Fig 1b**). SC was dissolved in water at an initial concentration of 40 mg/mL, and the dispersion was equilibrated at 1000 rpm for 2 h. Ethanol as an antisolvent was added dropwise into the dispersion to achieve an SC concentration of 2 mg/mL and ethanol content of 95% (volumetric ratio). The mixture was equilibrated for 30 min under constant stirring at 950 rpm, after which an opaque dispersion containing self-assembled SC nanoparticles (defined as the primary dispersion) was formed. Chemical crosslinkers were dissolved in water and incubated as described for the aqueous procedure, after which they were mixed as follows. For tyrosinase-aided crosslinking, the dispersion of tyrosinase (10 U per mol SC) was mixed with different levels of phenols (0.5 to 10 mol per mol SC). The resulting mixtures were incubated in a water shaking bath (37°C, 120 rpm), without capping on the containing vials. After 1 h of oxidation, the mixtures were combined with the primary dispersion to initiate the crosslinking. For GA-aided crosslinking, different levels (0.5 to 10 moles per mole SC) of GA were mixed with the primary dispersion directly without adding tyrosinase. The ethanol content in the resulting mixtures ranged from 84% to 87%, and they were adjusted uniformly to 80% by adding appropriate amount of water to each mixture. A control sample containing no GA, tyrosinase, or phenols was also prepared. All mixtures were further incubated in the water shaking bath (37°C, 120 rpm) for 12 h,

cooled down to room temperature, and analyzed by DLS. Subsequently, the samples were evaporated at 50 °C under a constant air flow using an N-EVAP111 Nitrogen Evaporator equipped with OA-SYS Heating System (Organomation ASSOC. INC., Berlin, MA, USA). Such a process led to the removal of added ethanol, which was then replaced by the same volume of deionized water. Finally, the same DLS analysis was performed on the alcohol-free dispersions.

2.3 Determination of particle sizes and count rates

The particle size and count rate were measured by DLS using a BI-200 SM Goniometer Version 2 (Brookhaven Instrument Corp., Holtsville, NY, USA) equipped with a 35mW He-Ne laser beam at a wavelength of 637 nm and a scattering angle of 90°. The particle dispersions in both water and aqueous ethanol were analyzed without dilution. To obtain accurate results, the refractive index of protein (1.490) and the viscosity of water (0.8904 cp) or aqueous ethanol (1.598 cp for 80% ethanol, v/v) were employed. A laser power of 10 mW and an aperture pinhole size of 400 µm were adopted consistently. All analyses were conducted at 25 °C for 1 min. The particle sizes were calculated by the BIC Dynamic Scattering Software (Brookhaven Instrument Corp., Holtsville, NY, USA) using the cumulant algorithm. The quadratic mean diameter was reported as the particle size.

2.4 Determination of zeta potential

The nanoparticle dispersions in deionized water were analyzed for their electrophoretic mobility by laser Doppler velocimetry using a Nano ZS90 Zetasizer (Malvern Inc., Malvern, UK) with compatible fold capillary cuvettes offered by Malvern Inc. (Malvern, UK). For SC

nanoparticles formed through ionic gelation, each sample was divided into two aliquots. One aliquot was measured directly at pH 6.4. The other one was adjusted to pH 3.0 using 1 M HCl and subjected to dialysis centrifugation (5, 000 g, 30 min) using a Pall centrifugal tube (10 K MWCO, Pall Corp., Port Washington, NY, USA) to remove dissociated Ca^{2+} . Then, the retentate containing SC nanoparticles was restored to its original volume by adding water and re-adjusted to pH 6.4 before measurement. For particles prepared by desolvation, only the samples obtained after ethanol removal were analyzed. Each sample was measured for three times, and at least 12 runs were performed for each measurement. The data were then converted to zeta potentials using the Smoluchowski model.

2.5 Scanning electronic microscopy (SEM)

The morphology of SC nanoparticles was examined by scanning electron microscopy (SEM). For the nanoparticles prepared by ionic gelation, each sample was adjusted to pH 3.0 using 1 M HCl and subjected to dialysis centrifugation (5, 000 g, 30 min) using a Pall centrifugal tube (10 K MWCO, Pall Corp., Port Washington, NY, USA) to remove dissociated Ca^{2+} . For those prepared via ethanol desolvation, the samples were evaporated under a constant air flow to remove the added ethanol and supplemented with water to restore their original volumes. Afterwards, thirty microliters of the nanoparticle dispersions was drawn and cast-dried on an aluminum pan. The dried samples were then cut into appropriate sizes and adhered to a 1 inch specimen stub with conducted carbon tapes (Electron Microscopy Sciences, Ft. Washington, PA, USA). Thereafter, the samples were coated with a thin layer (<20 nm) of gold and

platinum using a sputter coater (Hummer XP, Anatech, CA, USA) and observed under the SEM (Hitachi SU-70 Pleasanton, CA, USA). Representative images were presented.

2.6 Statistics

All measurements were conducted in triplicate. The experimental results were presented as means $\pm$ standard error. To identify significant differences, the data were subjected to the analysis of variance ($p < 0.05$) using the Origin 7.5 software (OriginLab Corp., MA, USA).

3. Results and discussion

3.1 Chemical crosslinking effect on SC nanoparticles in aqueous solution

A schematic illustration on the nanoparticle crosslinking mechanism is shown in **Fig. 2A**. Native SC (pI=4.6) carries weak negative charge at mildly acidic pH (e.g., pH 6), and it is prone to self-assembly due to the associative driving forces, including intermolecular hydrogen bonds, electrostatic attraction, and hydrophobic interaction (Arvanitoyannis & Biliaderis, 1998; Dickinson & Golding, 1997). Such a process is enhanced by Ca^{2+} , which bridges adjacent SC molecules through electrostatic attraction. (Dickinson & Golding, 1998). However, when the pH of the nanoparticle system is decreased from 6.4 to 3.0, SC becomes positively charged, and its electrostatic interaction with Ca^{2+} is therefore significantly weakened (Surh, et al., 2006). As a result, a considerable portion of the formed nanoparticles disintegrates into individual molecules or smaller aggregates. Chemical crosslinkers, on the other hand, form covalent bonds that are stable against acidic pH. Therefore, the nanoparticles are anticipated to maintain their structure and integrity.

228 The effects of different crosslinking agents (5 mol/mol SC) on the stability of SC
229 nanoparticles formed via ionic gelation are presented in **Fig. 3**. Here we employed two
230 parameters, i.e., the percentages of particle size (defined as PS %) and count rate (defined as
231 CR%) at pH 3.0 compared to those at pH 6.4, as indicators for the particle integrity. The
232 rationale is that if the dispersion containing the primary nanoparticles kept stable after
233 acidification, then the average size and count rate should remain unchanged, making the PS%
234 and CR% close to 100%. For the samples without chemical crosslinkers, the average particle
235 size increased to at most 339% of the original values after acidification, whereas the count
236 rate decreased to as low as 7%. According to our previous studies, these changes suggested
237 the significant dissociation of nanoparticles, especially the ones with smaller sizes (Teng, et
238 al., 2012). Moreover, in the absence of chemical crosslinkers, a similar change was observed
239 for the samples both with and without CaCl_2 . This phenomenon suggested Ca^{2+} is not an
240 effective crosslinker to stabilize SC nanoparticles at acidic pH.

241 On the contrary, when glutaraldehyde (5 mol/mol SC) was added, a PS% and CR% of 110%
242 and 96% respectively were achieved, which suggested a strong structure-maintaining ability
243 of GA. However, GA is known for its potential toxicity (Takigawa, et al., 2006), and its
244 application in food industry should be avoided when possible. Therefore, in the following
245 paragraphs, tyrosinase-aided phenol oxidation was evaluated as another potential approach to
246 GA-aided crosslinking.

247 We firstly assessed whether phenols or tyrosinase alone could act as an effective crosslinker
248 for SC nanoparticles. In the presence of tyrosinase (10 U/mol SC), a PS% of 290% and a CR%

of merely 7% were observed upon acidification (**Fig. 3**). These results suggested that, similar to calcium, tyrosinase could not prevent the dissociation of the primary nanoparticles when added alone. In our preliminary study, a considerable amount of precipitate was observed as the dose of tyrosinase increased to 40 U/mol SC (data not shown), whereas the crosslinking efficacy remained unsatisfactory. These phenomena implied that tyrosinase alone tended to promote the crosslinking on the surface of SC nanoparticles, bridging multiple adjacent particles to form precipitates. On the other hand, the substrates buried inside the nanoparticles might not have reacted effectively with tyrosinase, as indicated by the extensive particle disintegration. A possible explanation for such a selective reactivity lay in the compact conformation of the nanoparticles, which prevented the bulky tyrosinase sterically from accessing the substrate in the hydrophobic cores. This may explain why tyrosinase works satisfactorily on less compact structures, such as protein monomers (Thalmann, et al., 2002) and hydrogels (Kim, et al., 2014), but was unable to crosslink protein nanoparticles effectively.

Likewise, the addition of phenols (5 mol per mol SC) without tyrosinase resulted in a PS% and CR% of 240% and 10%, respectively, which suggested the unsatisfactory particle stabilizing effect of phenols (**Fig. 3**). The affinity between proteins and phenols has been studied extensively and harnessed to prepare various nanocomplexes (Shpigelman, Cohen, & Livney, 2012) and films (Arrieta, Peltzer, del Carmen Garrigós, & Jiménez, 2014; Arrieta, Peltzer, López, et al., 2014). However, these interactions did not exhibit a significant contribution to the stability of SC nanoparticles, which may require more efficient

270 crosslinking to maintain their compact structure. The binding of phenols with protein
271 molecules at acidic and neutral pH originates mainly from non-covalent effects such as
272 hydrogen bonding and hydrophobic interaction. These effects are generally lower in
273 magnitude compared to covalent interactions, and they are more prone to change upon
274 environmental variations such as acidification. Although covalent crosslinks may be formed
275 between proteins and phenols without the presence of tyrosinase, such a process requires
276 relatively high doses (several milligrams per milliliter versus up to 0.25 mg/mL in our study),
277 basic conditions (pH 9 or above), extended time (up to 24 h), and elevated temperature.
278 Therefore, phenolic compounds when employed alone were not ideal crosslinkers for protein
279 nanoparticles, which was confirmed by our study.

280 When tyrosinase and phenols (CAT or CA) were both applied as crosslinkers, the average
281 particle size remained virtually unchanged, and the count rate increased only slightly. In
282 specific, a PS% of 94% and CR% of 125% were observed for tyrosinase (10 U/mol SC, same
283 hereinafter) and CAT (5 mol/mol SC), and a PS% of 98% and CR% of 132% were observed
284 with tyrosinase and CA (5 mol/mol SC). These values were similar with those achieved by
285 GA, indicating the desirable crosslinking efficacy by these tyrosinase-phenol blends. In
286 contrast, MG or GAc (both at 5 mol/mol SC) and tyrosinase nearly failed to maintain the
287 stability of nanoparticles, resulting in a CR% of merely 25% after acidification.

288 Tyrosinase-catalyzed phenolic oxidation is a common phenomenon in many plants. The
289 reactivity of tyrosinase is highly dependent upon the pH and the nature of substituents on the
290 aromatic ring (Yang, Stuart, & Kamperman, 2014). **Fig. 2B** compares the chemical structure

of different phenols utilized in this study. CAT and CA possess an o-benzenediol ring, a well-documented substrate for tyrosinase, which were converted readily into quinones and exhibited satisfactory nanoparticle crosslinking. On the other hand, MG and GAc are conventionally recognized as tyrosinase inhibitors, due to the irreversible association between the three phenolic hydroxyl groups on these compounds and the active site on tyrosinase (Baek, et al., 2008). However, tyrosinase-MG or tyrosinase-GAc blends were reported as the most effective coupling agents for preparing protein-chitosan conjugates (Chen, Vazquez-Duhalt, Wu, Bentley, & Payne, 2001), which was the reason why those formulas were covered in this study. It is worth mentioning that the phenol/tyrosinase ratio applied in Chen's study was one to two orders in magnitude higher than those used in our trials. The extensive hydrogen bonding between MG/GAc and protein, rather than the reactivities of tyrosinase and the formed quinones, might have contributed significantly to the coupling in their study. In our study, no significant crosslinking was achieved using these two phenols with tyrosinase.

3.2 Comparison between glutaraldehyde and tyrosinase-phenol blends (CAT and CA) as crosslinkers in aqueous solution

Based on the results from previous comparison, CAT and CA were selected to determine the optimal working concentration for nanoparticle crosslinking. **Fig. 4A and 4B** presented the PS% and CR% of SC nanoparticles as influenced by the crosslinker concentrations. When GA was used as a crosslinker, the PS% decreased slightly from 146% to 102%, and the CR% increased gradually from 7% to 102% within the concentration range of 0.5 –to 7.5 mol/mol

312 SC. On the other hand, the tyrosinase-phenol blends were capable of achieving 100% PS%
313 and CR% at concentrations above 2.5 mol/mol SC. These results suggested the higher
314 crosslinking efficacies of the tyrosinase-phenol blends. This result may be ascribed to the fact
315 that quinones are able to react with diverse functional groups in proteins as discussed in
316 **Section 1**, whereas GA only forms covalent crosslinks between ϵ -amine groups of lysine or
317 hydroxylysine residues with its aldehyde groups (Damink, et al., 1995). The advantage of
318 tyrosinase-phenol blends is more pronounced when compared with genipin, another
319 nature-occurring crosslinker. The maximum amount of phenol used in our study was
320 calculated as 0.25 mg/mL, which was 100 times lower than the reported value for
321 genipin-aided crosslinking (22 mg/mL) (Liang, et al., 2003). Moreover, only several
322 nanograms of tyrosinase solution was needed for each gram of SC.

323 As the concentration of phenols further increased, the PS% increased to at most 314%
324 (observed at 7.5 mol CA/mol SC), and the CR% increased to as high as 137%. According to
325 these data, both CAT and CA might have induced protein aggregation or precipitation at
326 higher doses (Teng, et al., 2012). Phenolic compounds such as CAT and CA are inclined to
327 self-polymerization at high concentrations in the presence of tyrosinase (Yang, et al., 2014).
328 While monomeric phenols could only react with two adjacent functional groups on the
329 protein, phenolic polymers are capable of forming covalent linkages among multiple reactive
330 groups that are relatively distant from each other. This property, known as multivalency,
331 allows phenolic polymers to bridge multiple protein molecules or nanoparticles and form

large agglomerates.. Further studies are needed to elucidate impact of phenolic polymerization on the stabilization and agglomeration of protein nanoparticles.

3.3 ζ -Potentials of SC nanoparticles in aqueous solution

The zeta potentials of the nanoparticle dispersions were determined as an indicator for their dispersibility. Prior to crosslinking and acidification, the zeta potential of native SC dispersion was approximately -32.4 mV (**Fig. 5**), which was consistent with previously reported results (Ma, et al., 2009). Upon the introduction of CaCl_2 , the zeta potential decreased significantly (in terms of its absolute value, same hereinafter) to -13.0 mV. Divalent cations such as Ca^{2+} are known to adsorb to the surface of negatively charged colloidal particles, lowering their surface charge through salt bridging and ionic screening. When GA or tyrosinase-phenol blends were applied as chemical crosslinking agents, the zeta potential shifted towards more negative values and achieved a maximum (in terms of magnitude) of -16.2 mV. This was possibly attributed to the consumption of the positively charged amine groups by the crosslinkers (Weber, Coester, Kreuter, & Langer, 2000).

As a follow-up study, we attempted to improve the dispersion stability of SC nanoparticles by increasing their zeta potential. The approach we adopted in this study was to weaken the SC- Ca^{2+} ionic association by acidification to pH 3, followed by the removal of the dissociated Ca^{2+} via dialysis centrifugation. After such treatments, the dispersions were re-adjusted to pH 6.4 and measured again for their zeta potentials. As expected, the zeta potentials shifted toward more negative values (**Fig. 5**, gray bars). Nonetheless, the zeta potentials of those samples were still significantly lower than that of the control, which

implied an incomplete removal of the added Ca^{2+} . A possible explanation was that calcium ions were able to associate with the anionic patches (e.g., carboxylic groups) on SC at pH 3, despite the net negative charge of the protein. It should be pointed out that the zeta potential of the SC nanoparticle dispersion is affected significantly by the pH. A more negative zeta potential, indicative for better dispersibility, can be achieved at a higher pH such as pH 7.0.

3.4 Chemical crosslinking effect on SC nanoparticles in aqueous ethanol

Besides ionic gelation, desolvation by organic solvents (e.g., ethanol) is another widely used approach for preparing protein nanoparticles. Thus, the efficacies of different crosslinkers were also evaluated for this procedure. Generally, the addition of an antisolvent (such as ethanol in the case of SC) facilitates protein unfolding and subsequent aggregation via hydrophobic interaction (Lohcharoenkal, Wang, Chen, & Rojanasakul, 2014), while the removal of antisolvent triggers the particle disintegration as a result of weakened hydrophobic interaction and elevated electrostatic repulsion (Teng, et al., 2012), if the particles were not sufficiently stabilized. Similar to the analysis in aqueous solutions, we applied PS% and CR% in this section as indicators for the particle integrity after evaporation. In the absence of chemical crosslinkers, the average particle size of the SC nanoparticles increased to 337% of the original value (**Fig. 6A**), whereas the count rate decreased to 12% concomitantly after ethanol evaporation (**Fig. 6B**). Similar to our previous studies in the aqueous solution, these changes suggested the disintegration of nanoparticles. In a sharp contrast to the case for ionic gelation, neither GA nor the tyrosinase-phenol blends exhibited 100% retention in both particle size and count rate after removal of ethanol. GA (7.5 mol/mol

SC) presented relatively high crosslinking capacity, resulting in a PS% and CR% of 209% and 101%, respectively. When tyrosinase-phenol blends (7.5 mol phenol/mol SC, 10 U tyrosinase/mol SC) were employed as crosslinkers, the PS% of all samples ranged between 243% and 329%, while the highest CR% was merely 35% for CAT, followed by MG (12%), CA (6%), and GAc (4%).

3.5 Comparison of glutaraldehyde and tyrosinase-phenol blends as crosslinkers in aqueous ethanol

The dose dependence of crosslinking efficacy of GA and tyrosinase-phenol blend was investigated and summarized in **Fig.7**. Although better crosslinking was achieved as the crosslinker concentration increased, as indicated by decreasing PS% and increasing CR%, none of the crosslinkers exhibited satisfactory crosslinking effect (100% PS% and CR%). As mentioned in **Section 1**, the protein crosslinking is typically initiated by the electrophilic addition of o-quinones (as well as GA) on the nucleophilic functional groups in protein molecules. Such process produces a negatively charged and highly reactive intermediate (Kutyrev & Moskva, 1991), whose stability is critical for the subsequent crosslinking reaction. Since ionic intermediates are more stable in polar solvents (e.g., water) while destabilized in less polar environment (e.g., aqueous ethanol), the efficacies for all crosslinkers were reduced in the desolvation procedure compared to those in the ionic gelation process.

In addition, unlike the ionic gelation where tyrosinase-phenol blends demonstrated better performance, GA displayed more noticeable crosslinking (at most 100% CR) in the desolvation process than CAT (at most 42% CR) and CA (at most 12% CR). This was

probably because GA remained stable in ethanol, thus being able to crosslink protein nanoparticles over the whole incubation period. On the contrary, tyrosinase as a protein is prone to denaturation in ethanol. Therefore, reactive quinones could only be formed during the prior incubation in water, and no quinone could be replenished under alcoholic condition after being consumed by the protein. Another important factor was the dramatic decrease in solvent polarity during the desolvation process. This change has a negative impact on the association between proteins and phenols (Teng, Li, & Wang, 2014), which could possibly lead to lower crosslinking efficacies.

Lastly, distinct crosslinking behaviors were found between CAT and CA, which was also opposite to our previous observation in aqueous procedure. Under the alcoholic condition, a large amount of aggregates was observed in the presence of CA, as evidenced by high turbidity and count rate (preliminary data not shown). Moreover, the majority of those aggregates disintegrated upon the removal of ethanol, leading to a low percentage of the residual count rate. A possible explanation is the weak associative interactions that maintained the aggregate structure, which needs further validation.

3.6 ζ -Potentials of SC nanoparticles in aqueous ethanol

The zeta potentials of nanoparticle dispersions prepared via ethanol desolvation were evaluated and presented in **Fig.8**. The added ethanol was removed and replaced by DI water prior to the measurements. A control sample was prepared by subjecting the dispersion without added crosslinkers to ethanol desolvation and evaporation. This sample exhibited slightly lower zeta potential compared to native SC dispersion, probably due to the partial

exposure of the hydrophobic peptide chains caused by the added ethanol. Interestingly, the application of chemical crosslinkers did not cause a significant ($P < 0.05$) change in the zeta potential, which was opposite to what was found in the aqueous procedure. Insufficient crosslinking reaction, as was discussed in Section 3.5, might have accounted for such a phenomenon.

3.7 SEM Analysis

The stabilities of SC nanoparticles crosslinked under different conditions were further assessed by SEM. Without chemical crosslinking, only a small number of nanoparticles remained detectable after acidification (for ionic gelation, **Fig. 9A**) or ethanol evaporation (for desolvation, **Fig. 9B**). When different types and amounts of crosslinkers were applied, an increased portion of the nanoparticles remained stable. The results observed under SEM were generally supportive of those obtained by DLS, in that the samples showing a PS% and CR% closer to 100% also exhibited more intact particles in the SEM images.

In conclusion, four natural phenolic compounds combined with tyrosinase were investigated as potential crosslinkers for SC nanoparticles. In the ionic gelation procedure under aqueous condition, SC nanoparticles dissociated upon acidification but remained remarkably stable in the presence of tyrosinase (10 U/mol SC) and CAT or CA (2.5 mol/mol SC). Only a few nanograms of tyrosinase was required for one gram of SC. Additionally, the optimal dose for phenols was three-fold and over 100-fold lower than those required for GA and genipin, respectively. However, the tyrosinase-phenol blends did not perform as well as GA in the desolvation process carried out in aqueous ethanol, possibly due to the destabilization of both

tyrosinase and the reactive intermediates in this solvent. Our study demonstrated for the first time the potential of tyrosinase-aided oxidation as an attractive, low-toxicity strategy for stabilizing protein nanoparticles against environmental stress. The same strategy could be implemented not only on SC nanoparticles but also on a wide variety of biopolymer-based delivery systems. Further studies may be focused on the optimization under alcoholic conditions and the application of as-prepared nanoparticles for the encapsulation and delivery of bioactive compounds. The effects of different crosslinkers and crosslinking procedures on the supramolecular structure and digesting/drug releasing profile may also be studied.

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REFERENCE

- Arrieta, M. P., Peltzer, M. A., del Carmen Garrigós, M., & Jiménez, A. (2014). Structure and mechanical properties of sodium and calcium caseinate edible active films with carvacrol. *Journal of Food Engineering*, 114(4), 486-494.
- Arrieta, M. P., Peltzer, M. A., López, J., Garrigós, M. d. C., Valente, A. J. M., & Jiménez, A. (2014). Functional properties of sodium and calcium caseinate antimicrobial active films containing carvacrol. *Journal of Food Engineering*, 121, 94-101.
- Arvanitoyannis, I., & Biliaderis, C. G. (1998). Physical properties of polyol-plasticized edible films made from sodium caseinate and soluble starch blends. *Food chemistry*, 62(3), 333-342.
- Baek, H. S., Rho, H. S., Yoo, J. W., Ahn, S. M., Lee, J. Y., Jeonga-Lee, J.-L., Kim, M.-K., Kim, D. H., & Chang, I. S. (2008). The inhibitory effect of new hydroxamic acid derivatives on melanogenesis. *Bulletin of the Korean Chemical Society*, 29(1), 43-46.
- Bigi, A., Cojazzi, G., Panzavolta, S., Roveri, N., & Rubini, K. (2002). Stabilization of gelatin films by crosslinking with genipin. *Biomaterials*, 23(24), 4827-4832.

- Bittner, S. (2006). When quinones meet amino acids: chemical, physical and biological consequences. *Amino acids*, 30(3), 205-224.
- Chen, T., Vazquez-Duhalt, R., Wu, C.-F., Bentley, W. E., & Payne, G. F. (2001). Combinatorial screening for enzyme-mediated coupling. Tyrosinase-catalyzed coupling to create protein-chitosan conjugates. *Biomacromolecules*, 2(2), 456-462.
- Damink, L. O., Dijkstra, P., Van Luyn, M., Van Wachem, P., Nieuwenhuis, P., & Feijen, J. (1995). Glutaraldehyde as a crosslinking agent for collagen-based biomaterials. *Journal of Materials Science: Materials in Medicine*, 6(8), 460-472.
- Dickinson, E., & Golding, M. (1997). Rheology of sodium caseinate stabilized oil-in-water emulsions. *Journal of Colloid and Interface Science*, 191(1), 166-176.
- Dickinson, E., & Golding, M. (1998). Influence of calcium ions on creaming and rheology of emulsions containing sodium caseinate. *Colloids and Surfaces A: Physicochemical and Engineering Aspects*, 144(1), 167-177.
- Dreis, S., Rothweiler, F., Michaelis, M., Cinatl, J., Kreuter, J., & Langer, K. (2007). Preparation, characterisation and maintenance of drug efficacy of doxorubicin-loaded human serum albumin (HSA) nanoparticles. *International journal of pharmaceutics*, 341(1), 207-214.
- Elzoghby, A. O., Samy, W. M., & Elgindy, N. A. (2012). Albumin-based nanoparticles as potential controlled release drug delivery systems. *Journal of Controlled Release*, 157(2), 168-182.
- Elzoghby, A. O., Samy, W. M., & Elgindy, N. A. (2013). Novel spray-dried genipin-crosslinked casein nanoparticles for prolonged release of alfuzosin hydrochloride. *Pharmaceutical research*, 30(2), 512-522.
- González, A., Tártara, L. I., Palma, S. D., & Igarzabal, C. I. A. (2015). Crosslinked soy protein films and their application as ophthalmic drug delivery system. *Materials Science and Engineering: C*, 51, 73-79.
- Halaouli, S., Asther, M., Sigoillot, J. C., Hamdi, M., & Lomascolo, A. (2006). Fungal tyrosinases: new prospects in molecular characteristics, bioengineering and biotechnological applications. *Journal of Applied Microbiology*, 100(2), 219-232.
- Hopkins, T. L., & Kramer, K. J. (1992). Insect cuticle sclerotization. *Annual review of entomology*, 37(1), 273-302.
- Hurrell, R. F., Finot, P. A., & Cuq, J. L. (1982). Protein-polyphenol reactions. 1. Nutritional and metabolic consequences of the reaction between oxidized caffeic acid and the lysine residues of casein. *Br J Nutr*, 47(2), 191-211.
- Hwang, D.-C., & Damodaran, S. (1996). Chemical modification strategies for synthesis of protein-based hydrogel. *Journal of agricultural and food chemistry*, 44(3), 751-758.
- Jahanshahi, M., & Babaei, Z. (2008). Protein nanoparticle: A unique system as drug delivery vehicles. *African Journal of Biotechnology*, 7(25), 4926-4934.
- Jollès, P., & Alais, C. (1962). Amino Acid Composition of κ -Casein and Terminal Amino Acids of κ -and para- κ -Casein. *Archives of Biochemistry and Biophysics*, 98(1), 56-57.

- Kim, B. J., Oh, D. X., Kim, S., Seo, J. H., Hwang, D. S., Masic, A., Han, D. K., & Cha, H. J. (2014). Mussel-Mimetic Protein-Based Adhesive Hydrogel. *Biomacromolecules*, 15(5), 1579-1585.
- Kubo, I., Kinst-Hori, I., Nihei, K.-I., Soria, F., Takasaki, M., Calderón, J. S., & Céspedes, C. L. (2003). Tyrosinase inhibitors from galls of *Rhus javanica* leaves and their effects on insects. *Zeitschrift für Naturforschung C*, 58(9-10), 719-725.
- Kutyrev, A. A., & Moskva, V. V. (1991). Nucleophilic reactions of quinones. *Russian Chemical Reviews*, 60(1), 72.
- Lantto, R. (2007). Protein cross-linking with oxidative enzymes and transglutaminase: effects in meat protein systems. *VTT Publications*.
- Leatham, G., King, V., & Stahmann, M. (1980). In vitro protein polymerization by quinones or free radicals generated by plant or fungal oxidative enzymes. *Phytopathology (USA)*.
- Leo, E., Vandelli, M. A., Camerini, R., & Forni, F. (1997). Doxorubicin-loaded gelatin nanoparticles stabilized by glutaraldehyde: involvement of the drug in the cross-linking process. *International journal of pharmaceuticals*, 155(1), 75-82.
- Liang, H. C., Chang, W. H., Lin, K. J., & Sung, H. W. (2003). Genipin-crosslinked gelatin microspheres as a drug carrier for intramuscular administration: In vitro and in vivo studies. *Journal of Biomedical Materials Research Part A*, 65(2), 271-282.
- Lohcharoenkal, W., Wang, L., Chen, Y. C., & Rojanasakul, Y. (2014). Protein nanoparticles as drug delivery carriers for cancer therapy. *BioMed research international*, 2014.
- Loizzo, M., Tundis, R., & Menichini, F. (2012). Natural and synthetic tyrosinase inhibitors as antibrowning agents: an update. *Comprehensive Reviews in Food Science and Food Safety*, 11(4), 378-398.
- Ma, H., Forssell, P., Partanen, R., Seppänen, R., Buchert, J., & Boer, H. (2009). Sodium caseinates with an altered isoelectric point as emulsifiers in oil/water systems. *Journal of agricultural and food chemistry*, 57(9), 3800-3807.
- Marty, J., Oppenheim, R., & Speiser, P. (1978). Nanoparticles--a new colloidal drug delivery system. *Pharmaceutica Acta Helveticae*, 53(1), 17.
- Migneault, I., Dartiguenave, C., Bertrand, M. J., & Waldron, K. C. (2004). Glutaraldehyde: behavior in aqueous solution, reaction with proteins, and application to enzyme crosslinking. *Biotechniques*, 37(5), 790-806.
- Rathna, G., & Damodaran, S. (2001). Swelling behavior of protein - based superabsorbent hydrogels treated with ethanol. *Journal of applied polymer science*, 81(9), 2190-2196.
- Ron, N., Zimet, P., Bargarum, J., & Livney, Y. D. (2010). Beta-lactoglobulin-polysaccharide complexes as nanovehicles for hydrophobic nutraceuticals in non-fat foods and clear beverages. *International Dairy Journal*, 20(10), 686-693.
- Shi, Q., Zhou, Y., & Sun, Y. (2005). Influence of pH and Ionic Strength on the Steric Mass - Action Model Parameters around the Isoelectric Point of Protein. *Biotechnology progress*, 21(2), 516-523.

- Shpigelman, A., Cohen, Y., & Livney, Y. D. (2012). Thermally-induced beta-lactoglobulin-EGCG nanovehicles: Loading, stability, sensory and digestive-release study. *Food Hydrocolloids*, 29(1), 57-67.
- Silva, N. F. N., Saint-Jalmes, A., de Carvalho, A. F., & Gaucheron, F. (2014). Development of casein microgels from cross-linking of casein micelles by genipin. *Langmuir*, 30(34), 10167-10175.
- Song, F., Zhang, L.-M., Yang, C., & Yan, L. (2009). Genipin-crosslinked casein hydrogels for controlled drug delivery. *International journal of pharmaceutics*, 373(1), 41-47.
- Sugumaran, M., Hennigan, B., & O'Brien, J. (1987). Tyrosinase catalyzed protein polymerization as an in vitro model for quinone tanning of insect cuticle. *Archives of insect biochemistry and physiology*, 6(1), 9-25.
- Surh, J., Decker, E. A., & McClements, D. J. (2006). Influence of pH and pectin type on properties and stability of sodium-caseinate stabilized oil-in-water emulsions. *Food Hydrocolloids*, 20(5), 607-618.
- Takigawa, T., & Endo, Y. (2006). Effects of glutaraldehyde exposure on human health. *Journal of occupational health*, 48(2), 75-87.
- Teng, Z., Li, Y., & Wang, Q. (2014). Insight into Curcumin-Loaded β -Lactoglobulin Nanoparticles: Incorporation, Particle Disintegration, and Releasing Profiles. *Journal of Agricultural and Food Chemistry*, 62(35), 8837-8847.
- Teng, Z., Luo, Y., & Wang, Q. (2012). Nanoparticles synthesized from soy protein: preparation, characterization, and application for nutraceutical encapsulation. *Journal of agricultural and food chemistry*, 60(10), 2712-2720.
- Teng, Z., Luo, Y., & Wang, Q. (2013). Carboxymethyl chitosan–soy protein complex nanoparticles for the encapsulation and controlled release of vitamin D3. *Food Chemistry*, 141(1), 524-532.
- Thalmann, C., & Lötzbeyer, T. (2002). Enzymatic cross-linking of proteins with tyrosinase. *European Food Research and Technology*, 214(4), 276-281.
- Ting, Y., Jiang, Y., Ho, C.-T., & Huang, Q. (2014). Common delivery systems for enhancing in vivo bioavailability and biological efficacy of nutraceuticals. *Journal of Functional Foods*, 7, 112-128.
- Wang, C., Lau, T. T., Loh, W. L., Su, K., & Wang, D. A. (2011). Cytocompatibility study of a natural biomaterial crosslinker—Genipin with therapeutic model cells. *Journal of Biomedical Materials Research Part B: Applied Biomaterials*, 97(1), 58-65.
- Weber, C., Coester, C., Kreuter, J., & Langer, K. (2000). Desolvation process and surface characterisation of protein nanoparticles. *International Journal of Pharmaceutics*, 194(1), 91-102.
- Won, Y.-W., & Kim, Y.-H. (2008). Recombinant human gelatin nanoparticles as a protein drug carrier. *Journal of Controlled Release*, 127(2), 154-161.
- Yang, J., Stuart, M. A. C., & Kamperman, M. (2014). Jack of all trades: versatile catechol crosslinking mechanisms. *Chemical Society Reviews*, 43(24), 8271-8298.

584 Zhong, Q., Tian, H., & Zivanovic, S. (2009). Encapsulation of Fish Oil in Solid Zein
585 Nanoparticles by Liquid-liquid Dispersion. *Journal of Food Processing and*
586 *Preservation*, 33(2), 255-270.

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Figure captions

Fig.1. Schematic illustration for the preparation of SC nanoparticles (upper: ionic gelation; lower: ethanol desolvation) and determination of crosslinking efficacies. PS: particle size. CR: count rate.

Fig. 2. Illustration on the nanoparticle crosslinking process. Figure 2A describes the ionic and chemical crosslinking processes. Figure 2B presents the chemical structures of different chemical crosslinkers.

Fig. 3. Effect of crosslinking agents on the stability of SC nanoparticles prepared through ionic gelation. The parameters PS% (A) and CR% (B) denoted respectively the percentages of particle size and count rate at pH 3 versus pH 6.4. The molar ratio between the crosslinkers and SC was fixed at 5:1.

Fig. 4. Effect of crosslinker types concentrations on the stability of SC nanoparticles as indicated by PS% (A) and CR% (B). The nanoparticles were prepared through ionic gelation

Fig. 5. Zeta potential of SC nanoparticles prepared through ionic gelation and crosslinked by different chemical agents. The zeta potentials were measured at pH 6.4, with (gray bars) or without (black bars) prior acidification and dialysis (see Section 2.4 for details).

Fig. 6. Effect of crosslinking agents on the stability of SC nanoparticles prepared by ethanol desolvation. The parameters PS% (A) and CR% (B) denoted respectively the percentages of particle size and count rate after ethanol removal versus those before such a treatment. The molar ratio between the crosslinkers and SC was fixed at 7.5:1.

Fig. 7. Effect of crosslinker types concentrations on the stability of SC nanoparticles as indicated by PS% (A) and CR% (B). The nanoparticles were prepared through ethanol desolvation.

Fig. 8. Zeta potential of SC nanoparticles prepared through ethanol desolvation and crosslinked by different chemical agents. The zeta potentials were measured after ethanol removal and water supplementation (see Section 2.4 for details).

Fig.9. SEM images of SC nanoparticles crosslinked by different chemical reagents. The nanoparticles were prepared through ionic gelation (panel A, after acidification to pH 3) or ethanol desolvation (panel B, after ethanol evaporation).

Figure captions

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Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Crosslinkers

Figure 6

Figure 7

Figure 8

Figure 9

(A)

(B)