

ABSTRACT

Title of Document: POTENTIAL LOW TOXICITY CROSS-LINKER FOR PROTEIN-BASED NANOPARTICLES

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Crosslinking is an essential procedure for maintaining the integrity of protein-based nanoparticles, but the application of toxic crosslinkers is usually undesirable. In this study, a tyrosinase-aided crosslinking procedure was developed and compared to a conventional crosslinker (i.e. glutaraldehyde). Nanoparticles were firstly synthesized from sodium caseinate (SC) in both aqueous and alcoholic solvent systems. The particles were crosslinked by tyrosinase (alone or with added natural phenols) or glutaraldehyde and then examined for their integrity under simulated environmental stress, including pH variation and solvent evaporation. Under aqueous condition, SC nanoparticles were not crosslinked sufficiently by tyrosinase or phenols alone, despite the abundance of tyrosine residues in SC. Conversely, satisfying crosslinking was achieved by tyrosinase combined with two natural phenols (catechol or chlorogenic acid, both at 2.5 mol/mol protein), as evidenced by stable particle size and count rate

under environmental stress. A higher dose of 7.5 mol/mol protein was required for glutaraldehyde to achieve a comparable efficacy. Upon introduction of alcohol, the efficacies for both glutaraldehyde and tyrosinase-phenol mixtures decreased, but glutaraldehyde required lower dose and exhibited more significant crosslinking for achieving same crosslinking efficiency. However, a considerable number of nanoparticles were detected by scanning electron microscopy with both crosslinkers. Overall, tyrosinase-aided oxidation is a competitive, low-toxicity approach for crosslinking protein nanoparticles.

POTENTIAL LOW TOXICITY CROSS-LINKER FOR PROTEIN-BASED
NANOPARTICLES

By

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Chapter 1: Literature Review

1.1 Encapsulation techniques

The demands of bioactive natural compounds with physiological benefits in health improvement and disease prevention have gained much attention recently from the scientific community and food industry. However, the biological efficacies of nutraceuticals in providing health benefits and decreasing illness risk are commonly compromised by their low oral bioavailability due to various factors such as insufficient gastric residence time, poor permeability and/or solubility within the gut, susceptibility to physical conditions encountered in food processing (heat, oxygen light), and instability to changing physiological environments in the gastrointestinal (GI) tract (pH, enzymes, presence of other nutrients) (L. Chen, Remondetto, & Subirade, 2006). Therefore, to enhance the bioavailability and the efficacy of nutraceuticals, various delivery systems with functional properties have been designed to stabilize and deliver bioactive compounds to the physiological target.

Among the developed delivery systems, encapsulation has been identified to be one of the most promising approaches to improve the oral bioavailability of poorly absorbed nutraceuticals (Ting, Jiang, Ho, & Huang, 2014). This technique involves the incorporation of sensitive solids, liquids, or gaseous materials within other less functionally active matrix to produce small capsules with a size range from a few micrometers to a few millimeters (Munin & Edwards-Lévy, 2011). The encapsulated materials, which are commonly called core material, coated material, active agent, fill, internal or payload phase, generally are bioactive molecules (e.g. antioxidants,

minerals, vitamins, phytosterols, lutein, fatty acids, lycopene) and living cells (e.g. probiotics). On the other hand, the packaging substance may be called the coating, capsule, membrane, carrier, shell, wall, external phase, or matrix, which should be food grade, biodegradable and able to provide a physical barrier protecting the bioactive components against its surroundings (Fang & Bhandari, 2010; Zuidam & Shimoni, 2010). Among various materials that are employed for encapsulation, natural or modified polysaccharides are the most widely used in the food applications; gums, proteins, lipids and synthetic polymers are also suitable for encapsulation (F. Gibbs, 1999).

Various possible benefits are related to the application of encapsulation techniques. The main goal of encapsulation is to (1) protect sensitive or unstable core materials from degradation resulted from surrounding environment such as undesirable effects of oxygen, moisture, and light, and (2) control the release availability of the encapsulated compounds and enable target delivery at a particular place within the body. Encapsulation also provides advantages to isolate incompatible components coexist in the same mixture, covert a liquid into an easy handling powder, mask unpleasant odor or taste of the core material, preserve volatile flavors/aromas, improve stability in final products and during processing, adjust properties of active agents, etc. (Zuidam & Shimoni, 2010). By far, numerous strategies have been developed for encapsulation of polyphenol (Anand, Nair, Sung, Kunnumakkara, Yadav, Tekmal, et al., 2010; Deladino, Anbinder, Navarro, & Martino, 2008), herbal extracts (Chan, Yim, Phan, Mansa, & Ravindra, 2010), food-fortifying compounds (vitamins, micronutrients, fish oils, peptides, etc.) (Q. Chen, Wang, Liu, Wu, Kang,

Moore, et al., 2009; Han, Guenier, Salmieri, & Lacroix, 2008; Nesterenko, Alric, Silvestre, & Durrieu, 2014; Zhong, Tian, & Zivanovic, 2009), and probiotics /microbes (lactobacilli, bifidobacteria) (Sohail, Turner, Coombes, Bostrom, & Bhandari, 2011) in food systems and immobilization of cells and enzymes in fermentation processing (Nedovic, Kalusevic, Manojlovic, Levic, & Bugarski, 2011).

1.2 Sodium caseinate-based encapsulated systems

Compared with other materials that have been studied as encapsulants, proteins possess several advantages. Proteins are amphiphilic biopolymers which show adequate interaction with both the solvent and the entrapped compounds (Marty, Oppenheim, & Speiser, 1978). Besides, as naturally occurring polymers, they exhibit low toxicity compared with the systems formed by synthetic polymers due to their biodegradability (Weber, Coester, Kreuter, & Langer, 2000). The numerous functional properties of proteins, including emulsification and gelation (L. Chen, Remondetto, & Subirade, 2006), together with the ease of modification, make proteins attractive candidates as nutraceutical carriers suitable for various encapsulating systems.

Casein makes up the major protein component in milk. It forms polydisperse spherical complexes consisting of casein molecules and colloidal calcium phosphate (CCP) (HadjSadok, Pitkowski, Nicolai, Benyahia, & Moulai-Mostefa, 2008). SC is a mixture of principal casein proteins (α_1 -, α_2 -, β -, and κ -caseins), produced from pH adjustment of acid-coagulated casein by sodium hydroxide (Dickinson & Golding, 1997b; Sánchez & Patino, 2005). The individual SC molecules present an average molar mass weight (Mw) at around 24 kDa (Kumosinski, Pessen, Farrell, &

Brumberger, 1988; Ribadeau, Brignon, Grosclaude, & Mercier, 1972) with an isoelectric point (pI) at pH 4.6 (Lucey, Srinivasan, Singh, & Munro, 2000; Perrechil & Cunha, 2010). This protein aggregates at pH < 5.5 in the presence of salt or when the temperature increase to 50°C to 70°C (HadjSadok, Pitkowski, Nicolai, Benyahia, & Moulai-Mostefa, 2008).

Sodium caseinate (SC) is widely used as an ingredient in food formulations due to its unique functions. The most distinguished property lies in its foaming and emulsifying properties. The structures and amphiphilic characteristics of the individual casein proteins provide caseinate with low interfacial tension during emulsification. Besides, SC shows satisfactory thermal stability, water-binding, fat-binding, thickening, and gelation properties (Dickinson, 1989; Kinsella & Morr, 1984). Its random coil nature assists the self-assemble of SC protein molecules into biopolymer matrix through intermolecular hydrogen bonds and electrostatic, hydrophobic interactions (Dickinson & Golding, 1997b). These properties, together with the abundance in reactive residues (tyrosine, lysine, cysteine, etc.) (Jollès & Alais, 1962), make SC a suitable delivery vehicle for bioactive compounds or drugs.

The advantages of SC allow the application of this food protein as a versatile template for the protection and delivery of nutraceutical compounds in various biopolymer matrix forms. Four typical systems have been reported extensively by recent publications. One of the most important forms is the SC-stabilized nanoemulsions. Yi et al. proposed a beta-carotene in oil-in- water emulsion systems stabilized by SC (Yi, Li, Zhong, & Yokoyama, 2014). At homogenization pressure of 100MPa, the droplet diameters could be less than 200nm. These emulsions were generally stable to

coalescence during 30 days. On the other hand, the nanoemulsions carrying astaxanthin were developed by Anarjan et al. (Anarjan, Mirhosseini, Baharin, & Tan, 2011). The astaxanthin emulsions with desirable physicochemical properties were achieved by using a high-pressure homogenizer at 30 MPa with three passes and then evaporating solvent at 25 °C.

Besides, SC-based composite films were successfully synthesized by Pereda et al. through casting of aqueous suspensions (Pereda, Amica, Rácz, & Marcovich, 2011). The tensile properties and water vapor permeability of these films were evaluated upon the addition of nanocellulose. Similar investigations were conducted by Fabra et al. on SC-based films containing oleic acid-beeswax mixtures (Fabra, Talens, & Chiralt, 2008). Additionally, several studies have been reported extensively on acid-induced SC gels (Koh, Merino, & Dickinson, 2002; Kontogiorgos, Ritzoulis, Biliaderis, & Kasapis, 2006; Matia-Merino, Lau, & Dickinson, 2004; Myllärinen, Buchert, & Autio, 2007). Their microstructural, rheology, and mechanical behavior were systematic evaluated upon various modifications.

Nanoparticles formed by SC were prepared to encapsulate a variety of hydrophobic bioactive compounds in recent studies. Zimet et al. reported the incorporation of docosahexaenoic acid (DHA), an important Omega-3 polyunsaturated fatty acids, into the SC nanoparticles (Zimet, Rosenberg, & Livney, 2011). At room temperature without the addition of calcium and phosphate, the diameter of the formed DHA-loaded SC nanoparticles was 288.9 ± 9.6 nm. The DHA molecules associated with SC at a binding ratio of 3-4 DHA molecules per protein molecule on average and presented a relatively high affinity with $K_b = 8.38 \pm 3.12 \times 10^6 \text{ M}^{-1}$. Similar studies

have also been described on SC-curcumin (Pan, Zhong, & Baek, 2013) and SC-thymol (Pan, Chen, Davidson, & Zhong, 2014) complex synthesized through spray-drying and high shear homogenization, respectively. These nanoparticle systems showed the significantly improved dispersibility and increased solubility of encapsulated bioactive compounds.

In addition, complex encapsulants consisting of SC and various polymers (e.g., proteins, polysaccharides) have been developed to confer desirable water solubility and delivery efficacy. Recently, Luo et al. reported the fabrication of zein nanoparticles coated with SC (Luo, Teng, Wang, & Wang, 2013). As a second layer, SC provided the formed nanoparticles with a hydrophilic shell, resulting in better redispersibility and enhanced cellular uptake. Similar studies have been conducted on chitosan-SC (Anal, Tobiassen, Flanagan, & Singh, 2008) and thymol-loaded zein-SC (Li, Yin, Yang, Tang, & Wei, 2012) nanoparticles, which showed good stability and improved biological activities.

1.3 Chemical and natural crosslinking

Despite the high nutritional value and excellent functional properties, proteins have flexible conformation which is subject to change under environmental fluctuation. For instance, nanoparticles prepared by organic solvent desolvation are prone to disintegration as the solvent is removed, and those 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). Therefore, chemical crosslinkers are frequently used to retain the particle integrity via

covalent bonds. However, conventional and some novel crosslinkers exhibit some drawbacks that impede the application of protein nanoparticles.

Glutaraldehyde as a dialdehyde polymerizes under certain conditions, and the formed polymer 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, Rothweiler, Michaelis, Cinatl, Kreuter, & Langer, 2007; Leo, Vandelli, Cameroni, & Forni, 1997; Teng, Luo, & Wang, 2012) and hydrogels (Hwang & Damodaran, 1996; Rathna & Damodaran, 2001) due to its low cost, simple reaction procedure, and desirable crosslinking efficacy (Migneault, Dartiguenave, Bertrand, & Waldron, 2004). The major drawback of glutaraldehyde crosslinking lies in its considerable toxicity, including acute toxicity, respiratory irritation, and mutagenic and carcinogenic effects (Takigawa & Endo, 2006).

On the other hand, natural crosslinking agents have been studied extensively as an alternative crosslinker. One of the examples, genipin, has been reported with satisfying crosslinking efficacy for protein-based 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 amount of genipin needed for achieving ideal crosslinking is generally as high as 250 mg/ml (Liang, Chang, Lin, & Sung, 2003). This factor, combined with the cost and incomplete toxicological data (Wang, Lau, Loh, Su, & Wang, 2011), poses considerable challenge on the application of genipin. Therefore, the

development of nature and economic friendly crosslinkers is imperative for the application of protein nanoparticles in the food and pharmaceutical industry.

1.4 Enzymatic-catalyzed crosslinking

In the past few decades, protein crosslinking by enzymes (e.g., laccases, peroxidases, sulfhydryl oxidases, etc.) has attracted much attention in food industry (Lantto, 2007). The most common crosslinking is induced by transglutaminase, which has provided a more biocompatible and nature approach for maintenance the structure of biopolymer matrix systems required for delivery of bioactive compounds. Known as a nature enzyme, transglutaminase forms isopeptide bonds within or between proteins by crosslinking of glutamine and lysine residues (Bönisch, Huss, Weitzl, & Kulozik, 2007). Until now, transglutaminase-induced crosslinking has been applied for the formation of various biopolymer matrixes. Tanimoto and Kinsella found that both casein and beta-lactoglobulin form oligomers and polymers by transglutaminase-assisted crosslinking (Tanimoto & Kinsella, 1988). Bönisch et al. investigated the effect of glutathione (GSH) on the synthesis of casein and SCSC micelle crosslinked by transglutaminase (Bönisch, Lauber, & Kulozik, 2007). Eissa and Khan developed low pH whey proteins polymers and gels with desirable textural and rheological properties through transglutaminase-catalyzed crosslinking (Eissa & Khan, 2003). In spite of its wide application in stabilizing hydrogels or preparing protein oligomers, no study has been reported by far on the successful application of transglutaminase (or other enzymes) on the crosslinking of nanoparticles. One of the possible explanations lies in the bulky molecular structure of the enzymes. In a compact polymeric matrix, such as a nanoparticle, the steric hindrance is so significant that

relatively large enzyme molecules are prevented from entering the internal region. As a consequence, crosslinking among adjacent nanoparticles is more likely to occur than that among different functional groups within the same nanoparticle (Thalmann & Lötzbeyer, 2002). Therefore, alternative enzymes or crosslinking techniques are needed to achieve desirable crosslinking efficiency for protein molecules.

Tyrosinases are dicopper enzymes derived from fungi, plants, bacteria and mammals (Jus, Stachel, Schloegl, Pretzler, Friess, Meyer, et al., 2011). They are actively involved in the enzymatic browning of fruit and vegetables as a result of the biosynthesis of melanin pigments from phenols, and they also contribute to sclerotization in insects (Heck, Faccio, Richter, & Thöny-Meyer, 2013). Among various types of tyrosinases, mushroom tyrosinases have achieved much interest in current biotechnology applications. This form of tyrosinase have been reported to induce crosslinking of several proteins like lysozyme (Leatham, King, & Stahmann, 1980), casein (Hurrell, Finot, & Cuq, 1982), and whey proteins (Thalmann & Lötzbeyer, 2002) in the presence of phenols. Possible mechanisms include the enzymatic oxidation of phenolic compounds (including the tyrosine residual in the proteins) into o-quinones, followed by the serial Michael-like addition of the quinones to the amino-sulphydryl or pyrrolidine side chains of the protein (Buchert, Ercili Cura, Ma, Gasparetti, Monogioudi, Faccio, et al., 2010). By now, various studies have been conducted on tyrosinase-aided crosslinking on proteins. Thalmann & Lötzbeyer demonstrated the formation of protein oligomers of whey proteins (α -lactalbumin and β -lactoglobulin) and lysozyme by tyrosinase-catalyzed crosslinking with the addition of caffeic acid under appropriate conditions (Thalmann &

Lötzbeyer, 2002). They even found this type of enzyme may induce a direct crosslinking of α -lactalbumin molecules in the absence of the caffeic acid. On the other hand, Kim et al. reported protein-based hydrogels achieved by tyrosinase-induced crosslinking with higher cohesive strength and longer gelation time (Kim, Oh, Kim, Seo, Hwang, Masic, et al., 2014). However, to the date, no systematic investigation has been reported on the successful crosslinking of protein-based nanoparticles using tyrosinase.

1.5 The objective of this study

In this study, SC was chosen as a model protein based on two considerations, namely, the abundance in reactive residues (tyrosine, lysine, cysteine, etc.) (Jollès & Alais, 1962) and the ease of particle formation. Two strategies including ionic gelation and organic solvent desolvation were applied to produce primary protein nanoparticles, which were then crosslinked by tyrosinase (alone or with added natural phenols) or glutaraldehyde. The particles were characterized by dynamic light scattering (DLS), scanning electron microscopy (SEM), and zeta potential measurement. Thereafter, these particles were subjected to an environmental stress, either pH variation or solvent evaporation, to dissociate the non-covalently crosslinked nanoparticles. The efficacies of different crosslinkers were then evaluated by comparing the particle size and count rate data before and after the application of environmental stress. The aim of this work was to (1) explore the possibility of using tyrosinase-aided phenols as alternative crosslinkers (2) optimize the procedure based on the observed data (3) compare the effects of chemical type and concentration on the crosslinking efficacy .

Chapter 2: Materials and Methods

2.1 Materials

SC and tyrosinase from mushroom (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 separately in two solvents, i.e. water and ethanol. *Aqueous procedure* (Fig. 2.1a). 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 ($\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$) was then added to resultant dispersion at 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 (Fig. 2.2) 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 at 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. The resultant mixtures were incubated at 37°C in a reciprocal water shaking bath (120 rpm). In order to achieve sufficient phenol oxidation, the vials containing the reactants were not covered. After 4 h of crosslinking, the mixture was withdrawn and adjusted to pH 6.4. The particle size and count rate (amount of scattered photons) 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 further adjusted to pH 3.0 using 1 mol/L HCl to weaken the electrostatic attraction between SC and Ca²⁺, and the resulting dispersions were subjected to the same DLS measurement. A primary dispersion containing no chemical crosslinker (control) was also prepared and analyzed by DLS.

Alcoholic procedure (Fig. 2.1b). 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). An opaque dispersion containing desolvated SC nanoparticles (defined as the primary dispersion) was formed after 30 min of equilibration, under constant stirring at 950 rpm. 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 mole SC) was mixed with different levels of phenols (0.5 to 10 moles per mole SC). The resulting mixtures, without lids covering the vials, were incubated in a 37°C water bath shaking at 120 rpm. After 1 h of enzyme-assisted 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. A control sample containing no GA, tyrosinase, or phenols was also prepared. When necessary, predetermined amount of water was supplemented to ensure a consistent water/ethanol ratio for all samples. The resulting mixtures were further incubated at 37°C in a reciprocal water shaking bath (120 rpm) for 12 h, cool down to room temperature, and analyzed by DLS. Subsequently, the samples were heated at 40-50°C and subjected to a constant air flow, using a N-EVAP111 Nitrogen Evaporator equipped with OA-SYS Heating System (Organomation ASSOC. INC., Berlin, MA, USA). Such process led to the removal of added ethanol, which was 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 35mV He-Ne laser beam at a wavelength of 637 nm and a scattering angle of 90°. Particle dispersion in both water and aqueous ethanol were analyzed without dilution. To obtain accurate results, the refractive index of SC protein nanoparticles (1.490) and the viscosity of water (0.8904 cp) or aqueous ethanol (1.598 cp for 80% ethanol, v/v) were employed. The laser power and aperture pinhole size were adopted consistently at 10 mW and 400 µm, respectively. All sample analyses were conducted at 25°C for 1 min. The data were calculated by the BIC Dynamic Scattering Software (Brookhaven Instrument Corp., Holtsville, NY, USA) using cumulant algorithm, and the quadratic mean particle size was reported.

2.4 Determination of zeta potential

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 cross-linked by chemical agents in aqueous solution, 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), in order to remove dissociated Ca^{2+} . Then, the retentate containing SC nanoparticles was re-adjusted to pH 6.4 and analyzed for its zeta potential. For particles prepared via alcoholic procedure, only the samples obtained after evaporation were subjected to zeta potential analysis. 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.

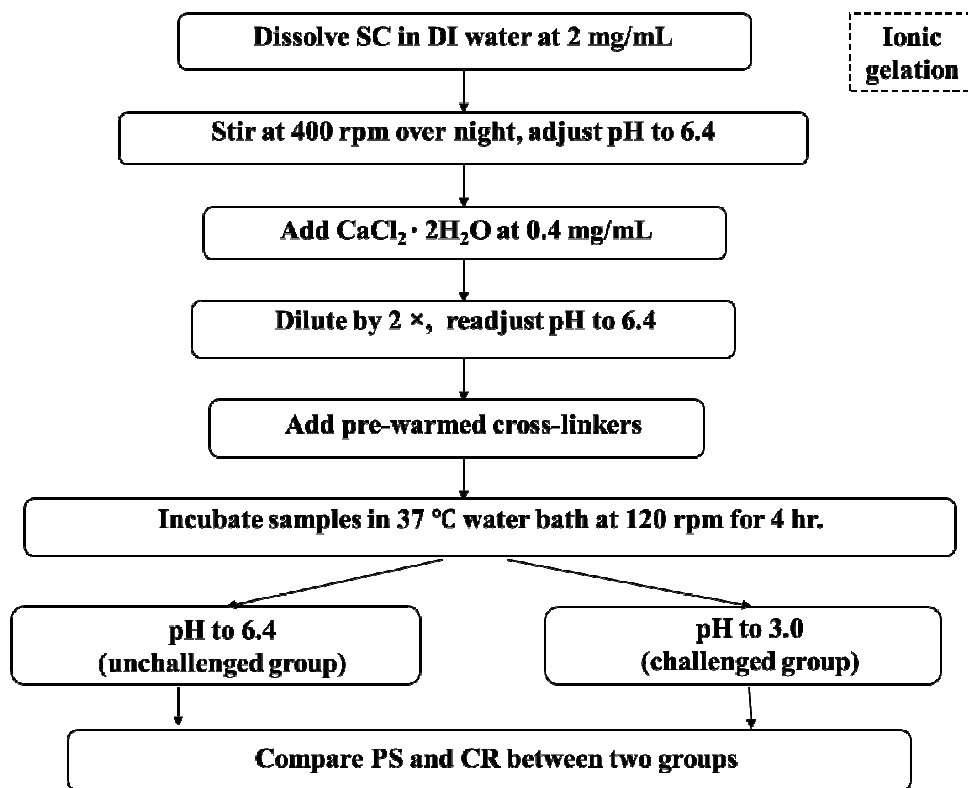
2.5 Scanning electronic microscopy (SEM)

The morphology of nanoparticles was examined by using SEM (Hitachi SU-70 Pleasanton, CA, USA). For SC nanoparticles cross-linked by chemical agents in aqueous solution, 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 particles prepared via alcoholic procedure, samples were evaporated under a constant air flow to remove the added ethanol and replaced with the same volume of deionized

water. After these treatments, thirty microliters of 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 SEM observation. Representative images were reported.

2.6 Statistics

All the measurements were conducted in triplicates. The experimental results were presented as means \pm standard error. For statistical analysis, data were performed by analysis of variance ($p < 0.05$) using the Origin 7.5 software (OriginLab Corp., MA, USA).



a

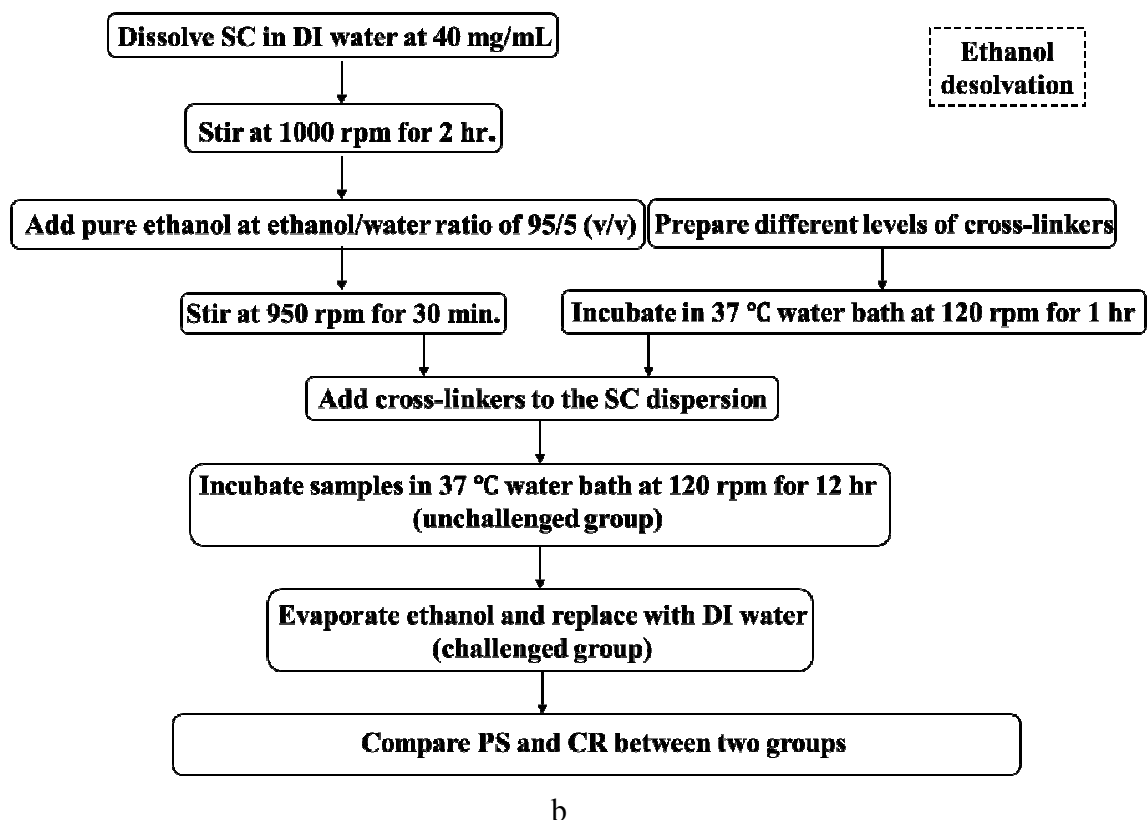


Fig.2.1. Schematic illustration on the aqueous (a) and alcoholic (b) procedures of determination of crosslinking effect on SC nanoparticles. PS: particle size. CR: count rate.

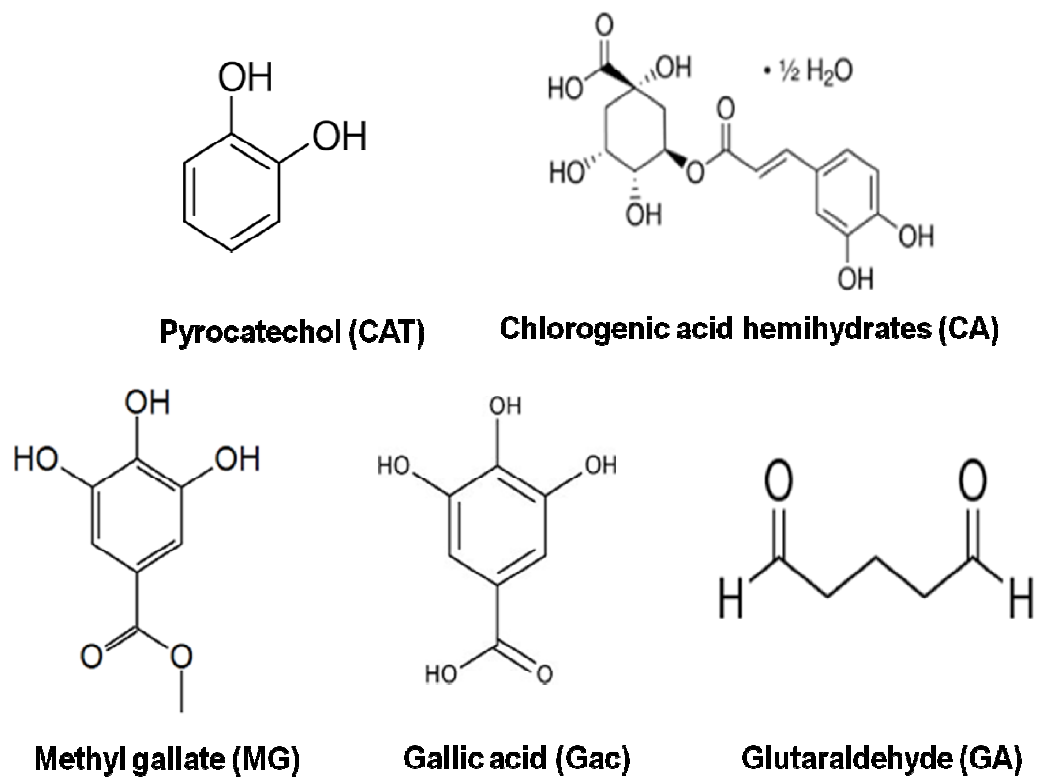


Fig.2.2. Schematic illustration on the chemical structures of phenols (CAT, CA, MG, Gac) and glutaraldehyde (GA).

Chapter 3: Results and Discussions

3.1 Chemical crosslinking effect on SC nanoparticles in aqueous solution

The effect of different crosslinking agents (5 mol/mol protein) on the stability of SC nanoparticles in the aqueous solution is presented in Fig. 3.2. Native SC (pI=4.6) is prone to self-assemble in mildly acidic aqueous solution, forming nanoparticles through intermolecular hydrogen bonds, electrostatic attraction, and hydrophobic interaction (Arvanitoyannis & Biliaderis, 1998; Dickinson & Golding, 1997a). The addition of calcium ion tends to ionically reinforce the self-association of SC particles with the strong crosslinking of calcium ions between the negatively charged carboxyl residues on SC molecules at neutral to basic pH (Dickinson & Golding, 1998). However, when the pH of the nanoparticle system was reduced from 6.4 to 3.0, SC became positively charged, and its electrostatic interaction with Ca^{2+} was therefore weakened significantly (Surh, Decker, & McClements, 2006). As a result, a considerable portion of the formed nanoparticles disintegrated into individual molecules or smaller aggregates. Here we employed two parameters, i.e., the percentages of particle size (defined as PS %) and count rate (defined as CR%, where CR is the total amount of photons scattered by the particles) at pH 3.0 to those at pH 6.4, as indicators for the particle integrity. The rationale is that if the dispersion containing nanoparticles kept stable after acidification, then its average size (Fig.3.1A) and count rate (Fig.3.1B) should remain unchanged. For the samples without chemical crosslinker, the average particle size increased to at most 339% of the original values after acidification, whereas the count rate decreased to as low as 7%. According to previous studies, these changes suggested the significant dissociation of

nanoparticles, especially the ones with smaller sizes (Teng, Luo, & Wang, 2012). In addition, in the absence of chemical crosslinkers, a similar change was observed for the samples both with and without CaCl_2 . This phenomenon suggested Ca^{2+} is not an effective crosslinker to stabilize SC nanoparticles at acidic pH when employed as the only crosslinker.

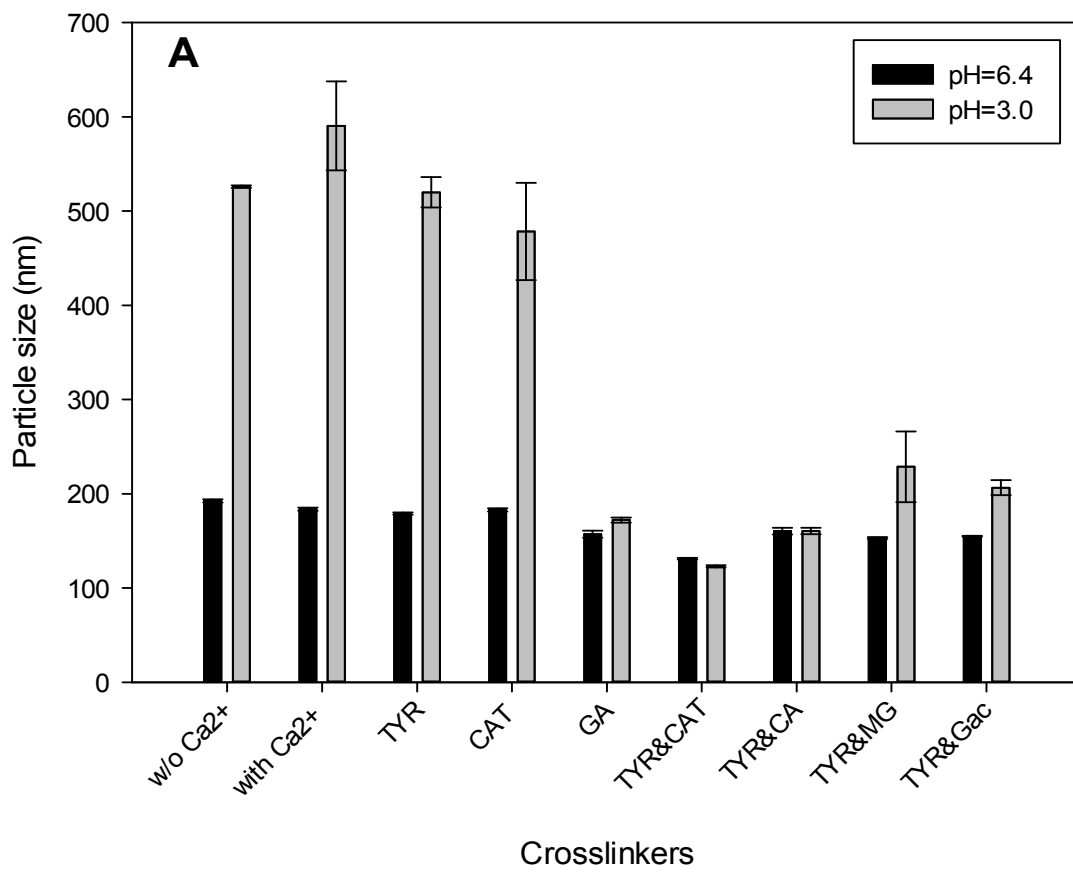
Glutaraldehyde is widely used as a chemical crosslinking agent for protein nanoparticles or hydrogels, as it crosslinks ϵ -amine groups of lysine or hydroxylysine residues covalently with its aldehyde groups (Damink, Dijkstra, Van Luyn, Van Wachem, Nieuwenhuis, & Feijen, 1995). In sharp contrast to the results achieved by using ionic crosslinker only, the PS% and CR% of SC nanoparticles were 110% and 96%, respectively, when glutaraldehyde (5 mol per mole SC) was added. These results suggested a strong structure-maintaining ability of chemical crosslinkers such as glutaraldehyde for SC nanoparticles. Despite of its high crosslinking efficiency, glutaraldehyde is known for its toxicity (Takigawa & Endo, 2006). Therefore, in the following paragraphs, tyrosinase-aided phenol oxidation was evaluated as a potential alternative to glutaraldehyde-aided crosslinking.

We firstly assessed whether phenols or tyrosinase alone could act as an effective crosslinker for SC nanoparticles. In the presence of tyrosinase only, a PS% and CR% of 290% and 7% respectively were observed upon acidification. Similar trend was observed when CAT was added without tyrosinase: the particle size at pH 3.0 was 240% of the original value, and the count rate decreased to 10% concomitantly (Fig. 3.2). These values were similar to the nanoparticles with or without Ca^{2+} crosslinking, indicating that tyrosinase or phenols alone could not prevent the dissociation of

formed nanoparticles. It was noteworthy to mention that a considerable amount of precipitate was observed as the dose of tyrosinase increased to 40 U/mol SC (preliminary data not shown), whereas the crosslinking efficacy remained low. This might be ascribed to the bulky molecule structure of tyrosinase, which might have sterically prevented it from accessing the hydrophobic tyrosine residues buried inside the particles. On the other hand, the tyrosinal side-chains that were exposed at the peripheral area of the particles might be oxidized and thus crosslinked by tyrosinase, forming inter-particle bonds that contributed to the precipitation. Similar result was observed on transglutaminase-induced crosslinking of proteins, particularly for whey proteins, leading to the formation of a large amount of by-products during cheese production (Thalmann & Lötzbeyer, 2002). Therefore, tyrosinase alone could not crosslink protein particles though works on protein oligomers (Thalmann & Lötzbeyer, 2002) or hydrogels (Kim, et al., 2014) as indicated in other studies.

On the other hand, when tyrosinase and phenols (CAT or CA) were applied together as crosslinkers, the average particle size exhibited nearly no change with a slightly increase in count rate. Therefore, PS% of 94% and 98% with CR% of 125% and 132% were observed for tyrosinase-oxidized CAT and tyrosinase-oxidized CA, respectively. These values were similar with nanoparticles cross-linked with glutaraldehyde, which would promote the stabilization of nanoparticles through covalent interactions. In contrast, tyrosinase-oxidized MG and Gac nearly failed to maintain the stability of nanoparticles after the pH adjustment, a PS% around 130% with only about 25% of CR% was presented in the aqueous solution.

CAT and CA are phenolic compounds acting as substrates of polyphenol oxidase (PPO) in many natural systems (Oszmianski & Lee, 1990; Yang, Stuart, & Kamperman, 2014). The tyrosinase-catalyzed oxidation of CAT involves two major steps: (1) the activation of inactive tyrosinase to its react state and (2) the oxidation of catechols by the activated tyrosinase to form electrophilic o-semiquinone and quinone through a two-electron transfer process. The tyrosinase-generated oxidation of catechols is influenced by several factors including pH and the nature of the aromatic ring substituents on catechols (Yang, Stuart, & Kamperman, 2014). On the other hand, chlorogenic acid, known as an ester of caffeic acid and quinic acid, could also be oxidized to quinone by tyrosinase due to its ortho-diphenol structure (Bittner, 2006; Strauss & Gibson, 2004). In contrast, MG and Gac have been found to act as a substrate inhibitor of mushroom tyrosinase (T. Chen, Vazquez-Duhalt, Wu, Bentley, & Payne, 2001), whereas gallic acid was also reported to inhibit the oxidation activity catalyzed by this kind of enzyme and thus reduce the o-quinone produced by the tyrosinase-induced oxidation (Kubo, Chen, & Nihei, 2003). On the basis of the aforementioned reasons, tyrosinase-oxidized both CAT and CA presented a noticeable crosslinking performance compared to that of tyrosinase-oxidized MG and Gac on SC nanoparticles.



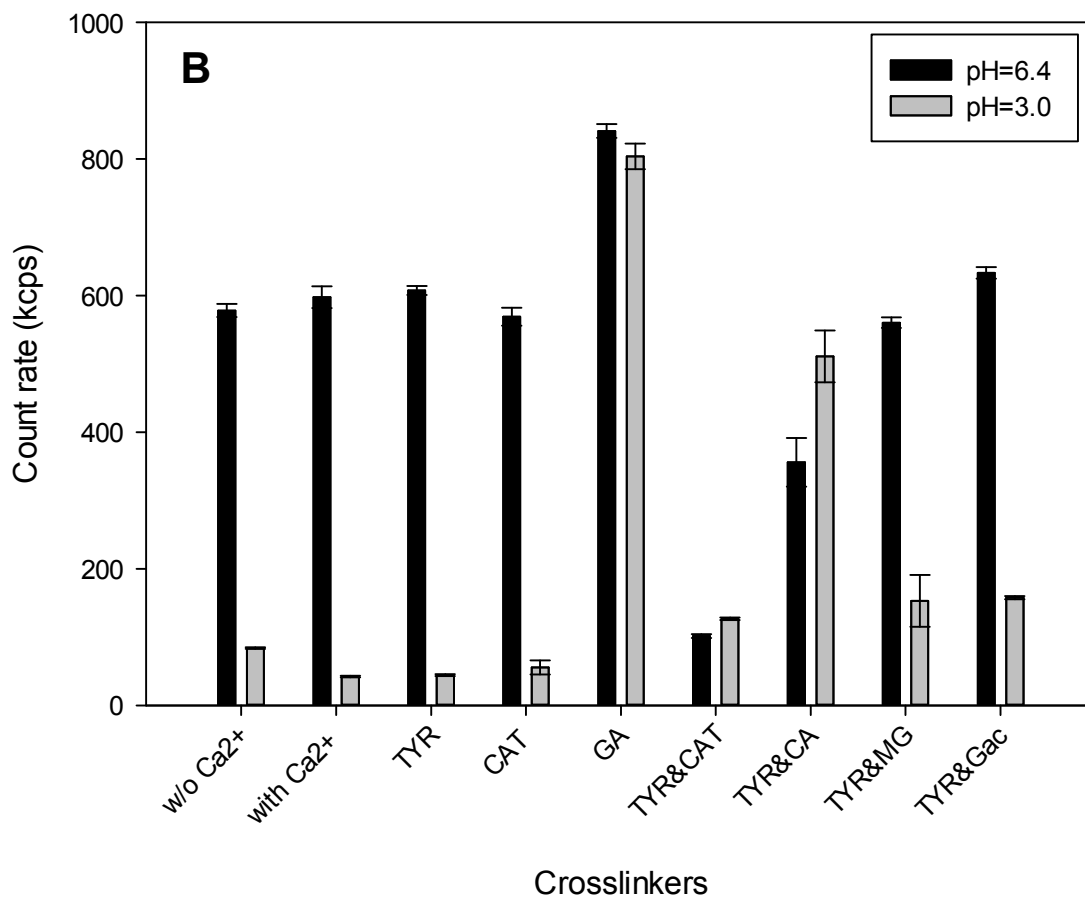
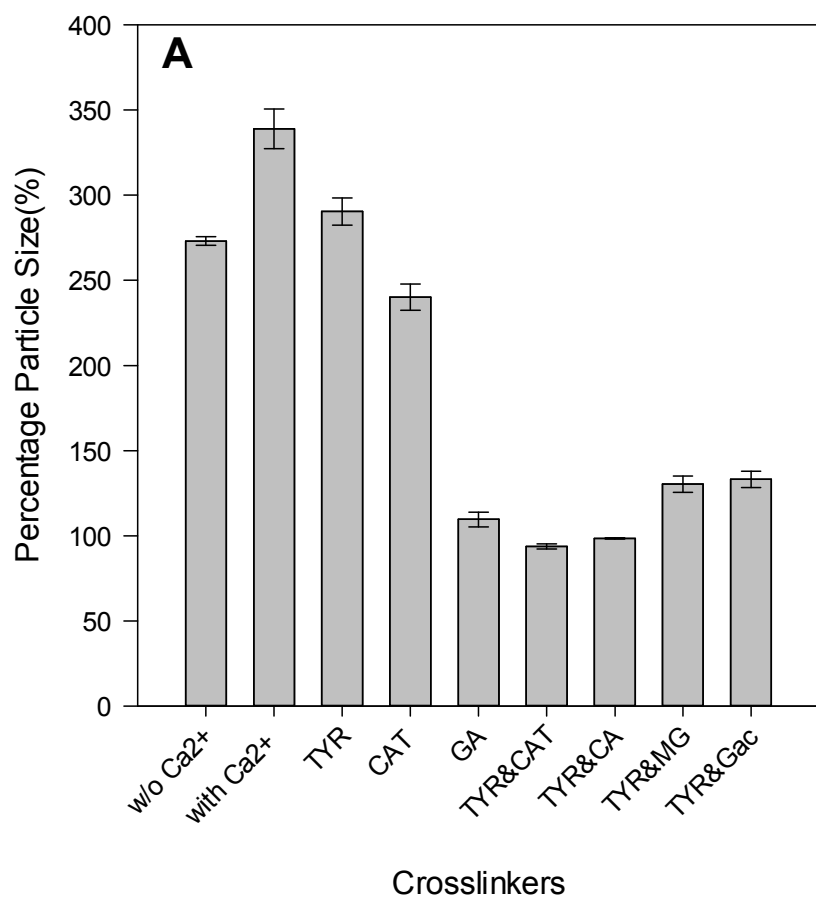


Fig. 3.1. Effect of different crosslinking agents (Ca^{2+} , TYR, CAT, GA, TYR & CAT, TYR & CA, TYR & MG, TYR & Gac) on the particle size (A) and count rate (B) of SC nanoparticles before and after acidification in the aqueous solution (crosslinker concentration = 5 mol/mol protein).



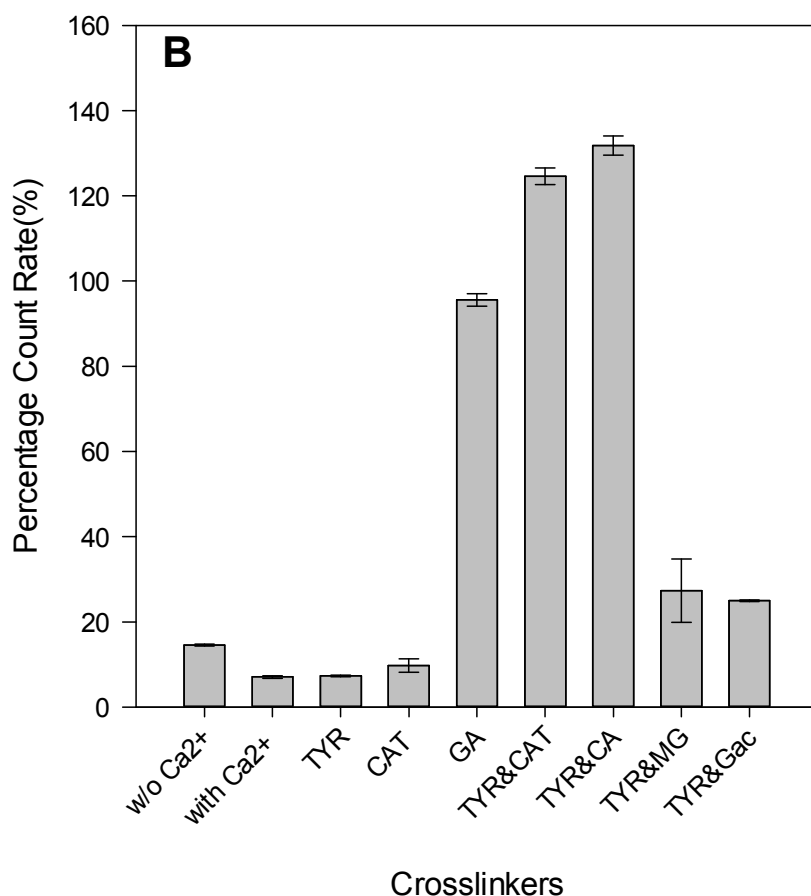


Fig. 3.2. Effect of different crosslinking agents (Ca²⁺, TYR, CAT, GA, TYR & CAT, TYR &CA, TYR & MG, TYR & Gac) on the percentage particle size (A) and percentage count rate (B) of SC nanoparticles in the aqueous solution (crosslinker concentration = 5 mol/mol protein)

3.2 Comparison of glutaraldehyde and tyrosinase-oxidized phenols (CAT and CA) as crosslinkers in aqueous solution

Based on the results from previous comparison, CAT and CA were selected for further investigation at the optimal working concentration. Fig. 3.3A and 3.3B indicated the PS% and CR% of SC nanoparticles as influenced by crosslinker concentrations. As discussed before, the control sample without any chemical

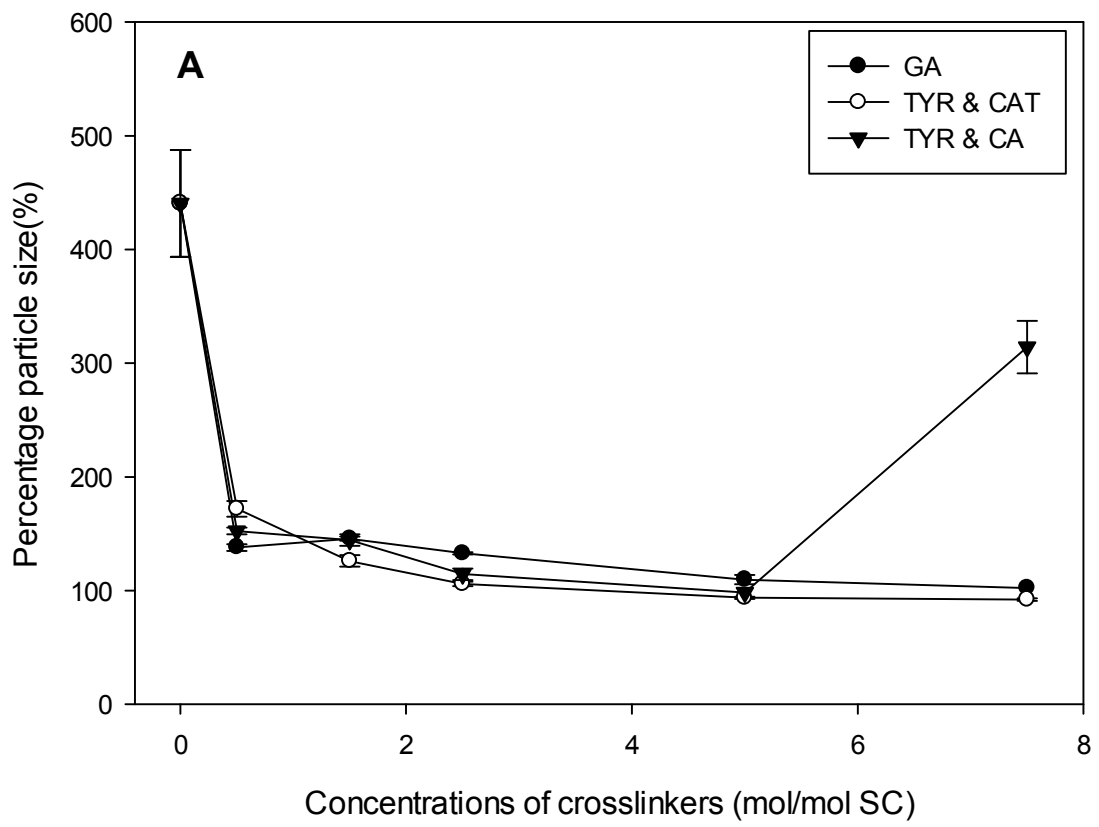
crosslinker showed significant dissociation of nanoparticles, indicated by a high PS% and low CR%. Upon the addition of GA as a crosslinker, the PS% decreased slightly from 146% to 102% and CR% increased gradually from 7% to 102% within the concentration range from 0.5 mol/mol protein to 7.5 mol/mol protein. This result suggested GA was not capable to stabilize the SC nanoparticles until its concentration reached to 7.5 mol/mol protein.

On the other hand, the tyrosinase-oxidized CAT and CA were found incapable of crosslinking SC nanoparticles at concentrations below 2.5 mol/mol SC indicated by relatively high PS% and low CR%. As the concentration of crosslinkers reached to 2.5 mol/mol SC, the average particle size was 106% of the original value with a CR% of 102% when nanoparticles cross-linked by tyrosinase-oxidized CAT. Similar results were observed when tyrosinase-oxidized CA was employed as a crosslinker, showing PS% of 114% and CR% of 116%. These results implied both of the tyrosinase-oxidized CAT and CA would maintain the stability of SC nanoparticles effectively at 2.5 mol/mol SC after acidification. As the concentration of phenols increased further to 5.0 - 7.5 mol/mol SC, the PS% increased to at most 314%, while the CR% increased to as high as 137%. According to these data, both CAT and CA might have induced protein aggregation or precipitation at higher concentrations. However, the significant increase of PS% to 314% corresponding to CA implied that larger aggregates were formed in the presence of CA than with CAT, Further investigation is needed to test such hypothesis.

Tyrosinase is a binuclear copper containing enzyme in the PPO group, widely distributed in nature. In the presence of oxygen, tyrosinase is capable of catalyzing

two distinct reactions, the formation of o-diphenols by the hydroxylation of monophenols(monophenolase activity) and the conversion of these o-diphenols to corresponding electrophilic o-quinone (diphenolase activity) (Kubo, Kinst-Hori, Nihei, Soria, Takasaki, Calderón, et al., 2003). O-quinones are unstable intermediates and reactive agents that can undergo several non-enzymatic reactions with nucleophilic functional groups such as sulfhydryl, amine, amide, hydroxyl, carboxyl, indole and imidazole in amino acids and proteins, resulting covalently crosslinking of protein molecules (Bittner, 2006; Hopkins & Kramer, 1992). Compared to tyrosinase-catalyzed phenolic crosslinkers, GA only forms covalent crosslinks between ϵ -amine groups of lysine or hydroxylysine residues with its aldehyde groups (Damink, Dijkstra, Van Luyn, Van Wachem, Nieuwenhuis, & Feijen, 1995). As a result of that, a higher amount of GA (7.5 mol/mol protein) is required to maintain the stability of SC nanoparticles. Moreover, it is reported that tyrosinase could also catalyzes the oxidation of tyrosine residues on the protein molecules, leading to the formation of direct crosslinking between the proteins (Thalmann & Lötzbeyer, 2002). Therefore, the tyrosinase-oxidized CAT and CA are capable of crosslinking SC nanoparticles effectively at a lower level of 2.5 mol/mol proteins. In a study conducted by Liang, et al. involving development of genipin-crosslinked gelatin microspheres for intramuscular administration, the concentration of genipin applied for crosslinking gelatin microspheres is 250 mg/mL, which is much higher than the amount of phenols used in our study for SC nanoparticle crosslinking (Liang, Chang, Lin, & Sung, 2003).

At higher phenol levels (5.0 - 7.5 mol/mol protein), a tyrosinase-catalysed oxidative self-polymerization might take place. As a result, the protein aggregation or precipitation observed under increased phenol concentration was observed. It might attribute to these phenol-based polymers of different sizes crosslinking with the protein molecules and ultimately form polymeric aggregates (Yang, Stuart, & Kamperman, 2014). Additionally, in comparison with CAT, the relatively large bulky molecular structure of CA makes it more capable of associating the functional groups on the surface of protein nanoparticles and cause crosslinking among adjacent nanoparticles than those among corresponding binding-sites inside the particles. This reason might explain the larger aggregates formed in the presence of CA rather than CAT. Further study needs to be conducted to test this hypothesis.



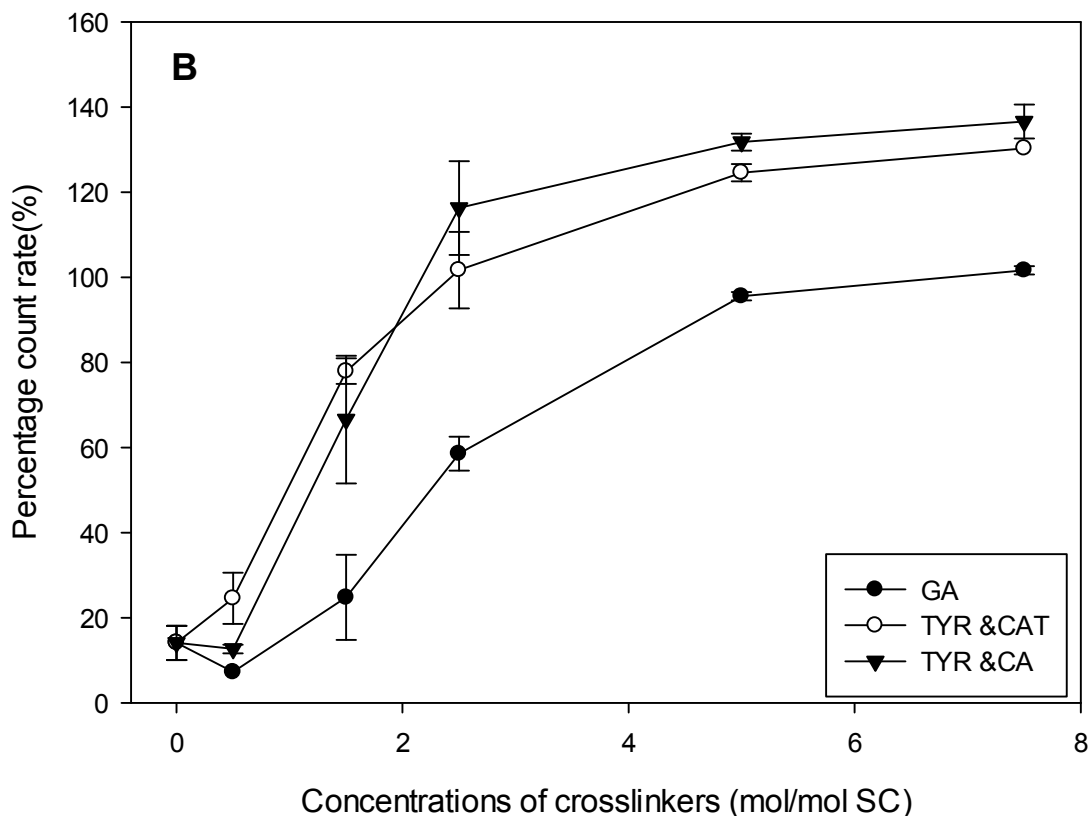


Fig. 3.3. Effect of crosslinker concentrations on the percentage particle size (A) and percentage count rate (B) of SC nanoparticles crosslinked by glutaraldehyde (GA) and tyrosinase-oxidized phenols (TYR&CAT and TYR&CA) in the aqueous solution.

3.3. ζ -Potentials of SC nanoparticles in aqueous solution

With the aim to investigate the impact of acidification on the dispersibility of SC nanoparticles crosslinked by different chemical agents, we measured the zeta potential of the prepared dispersions. Prior to crosslinking and acidification, the zeta potential of native SC dispersion was about -32.4 mV (Fig. 3.4), which was consistent with previously reported results (Ma, Forssell, Partanen, Seppänen, Buchert, & Boer,

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 for their ability to adsorb to the surface of negatively charged colloidal particles, lowering their surface charge through ionic bridging and screening. When GA, tyrosinase-oxidized CAT and CA were applied as chemical crosslinking agents, the zeta potential tended to become more negative and achieved a minimum of -16.2 mV. This was possibly attributed to the reaction between the crosslinkers and the positively charged primary amino groups.

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 lower the pH to 3.0, thus weakening the SC- Ca^{2+} ionic association. The dissociated Ca^{2+} can then be removed via dialysis centrifugation, after which the dispersion was re-adjusted to pH 6.4 for comparison. As expected, the zeta potential shifted toward to more negative values (Fig. 3.4, red bars) after such treatments. It should be mentioned that the zeta potential of the treated samples were still significantly lower than that of the control, probably because of incomplete removal of the added Ca^{2+} . Further improvement might be achieved by adjusting the pH to lower values (such as 2.0). Since the electrostatic interaction between Ca^{2+} and SC is further weakened at this pH, more Ca^{2+} associated with SC is anticipated to be removed by dialysis centrifugation. It is also noteworthy that the zeta potential of the SC nanoparticles depends highly on the pH. A more negative zeta potential, which is indicative for better dispersibility, can be observed at higher pH such as 7.0.

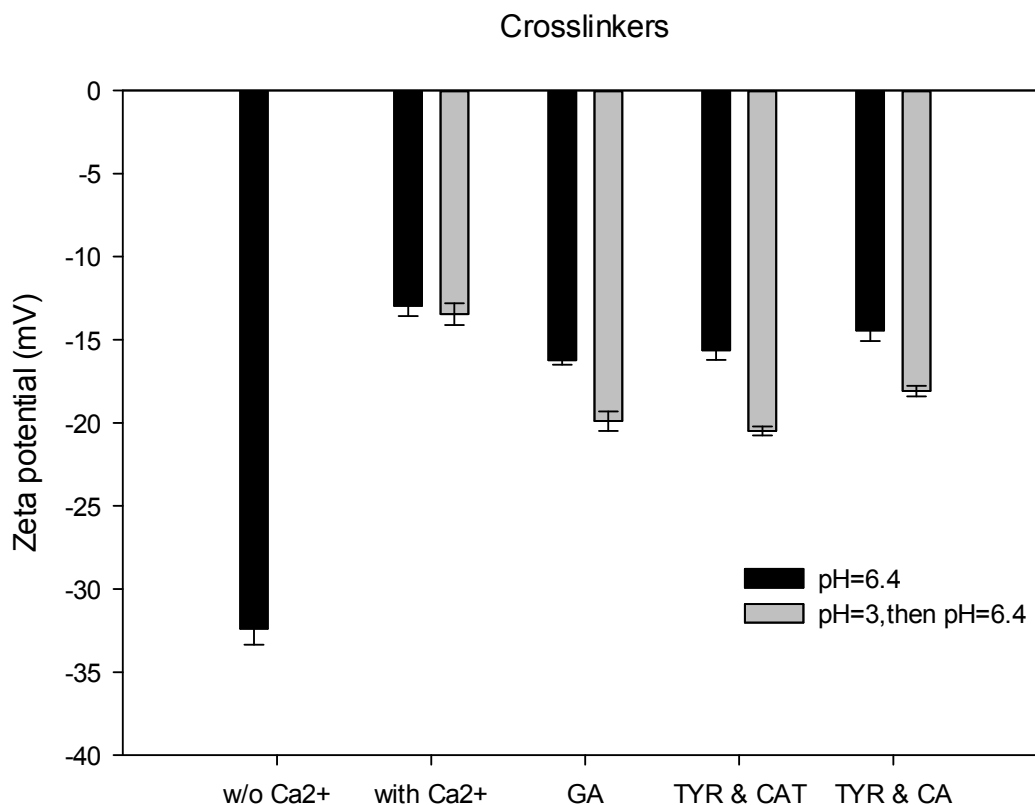


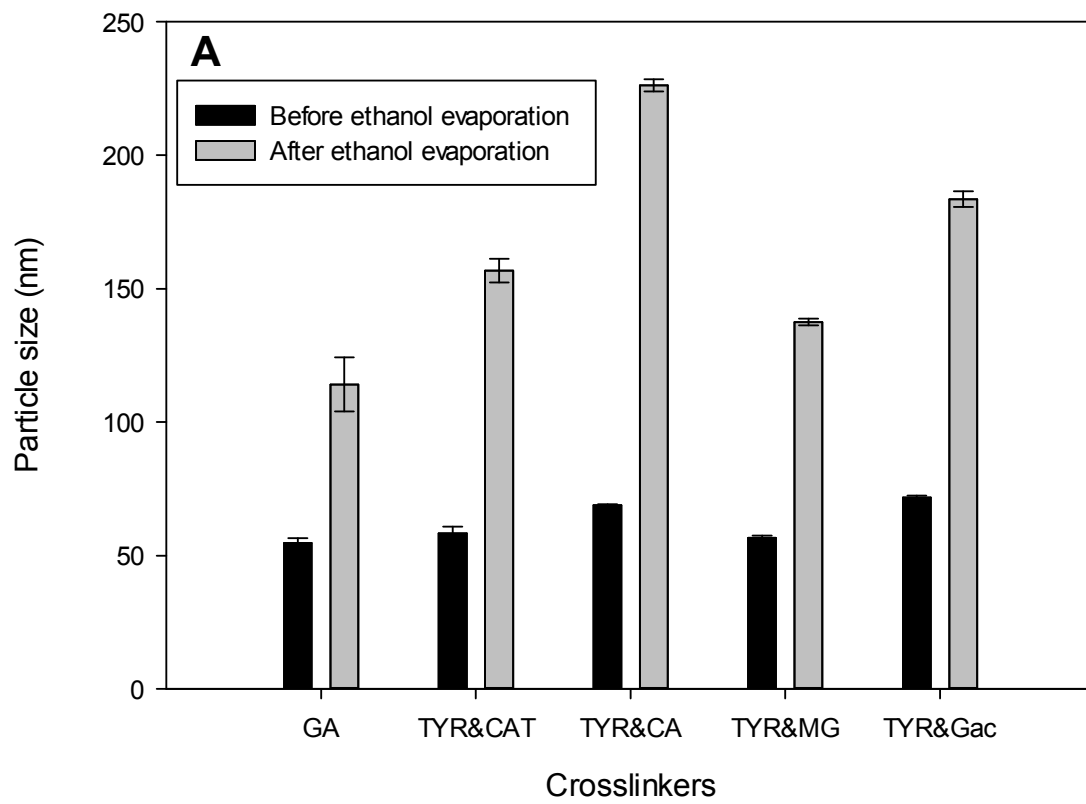
Fig. 3.4. Effect of acidification on the zeta potential of SC nanoparticles crosslinked by different crosslinking agents (Ca^{2+} , GA, TYR & CAT, TYR & CA).

3.4 Chemical crosslinking effect on SC nanoparticles in aqueous ethanol

Besides ionic gelation, organic solvent desolvation is another widely used approach for preparing protein nanoparticles. Therefore, the efficacy of different crosslinkers was also evaluated in an ethanol desolvation procedure. Generally, the formation of SC nanoparticles would be assisted by the ethanol, which results in the exposure of hydrophobic chains in SC molecules and facilitate the hydrophobic interaction between them (Lohcharoenkal, Wang, Chen, & Rojanasakul, 2014). On the other hand, the evaporation of ethanol followed by replacement with water may trigger

particle disintegration. Possible mechanisms include the burial of apolar chains that leads to weakened hydrophobic interaction, as well as increased surface charge resulting in elevated electrostatic repulsion (Teng, Luo, & Wang, 2012). Similar to the analysis in aqueous solutions, we applied two parameters, i.e., the percentages of particle size and count rate of nanoparticle dispersions after evaporation and water supplementation to those observed before such treatments, as indicators for the particle integrity. Following the similar rationale, we considered that if the particles in dispersion kept stable after ethanol evaporation, then their average size (Fig.3.5A) and count rate (Fig.3.5B) should remain unchanged.

In the absence of chemical crosslinkers, the average particle size of the SC nanoparticles increased to 337% of the original value (Fig. 3.6A), whereas the count rate decreased to 12% concomitantly after ethanol evaporation (Fig. 3.6B). Similar to our previous studies in the aqueous solution, these changes suggested the disintegration of nanoparticles. Conversely, GA (7.5 mol/mol protein) exhibited a significant crosslinking effect, resulting in a PS% and CR% of 209% and 101%, respectively. When tyrosinase-oxidized phenols (7.5 mol/mol protein) were employed as crosslinkers, the average particle size of all samples increased to 243%-329%. Meanwhile, the highest CR% achieved by all tyrosinase-oxidized phenols was merely 35% for CAT, followed by MG (12%), CA (6%), and Gac (4%). According to these results, none of the phenols was an effective crosslinker as GA for SC nanoparticles, which was in a sharp contrast to the observation under aqueous condition.



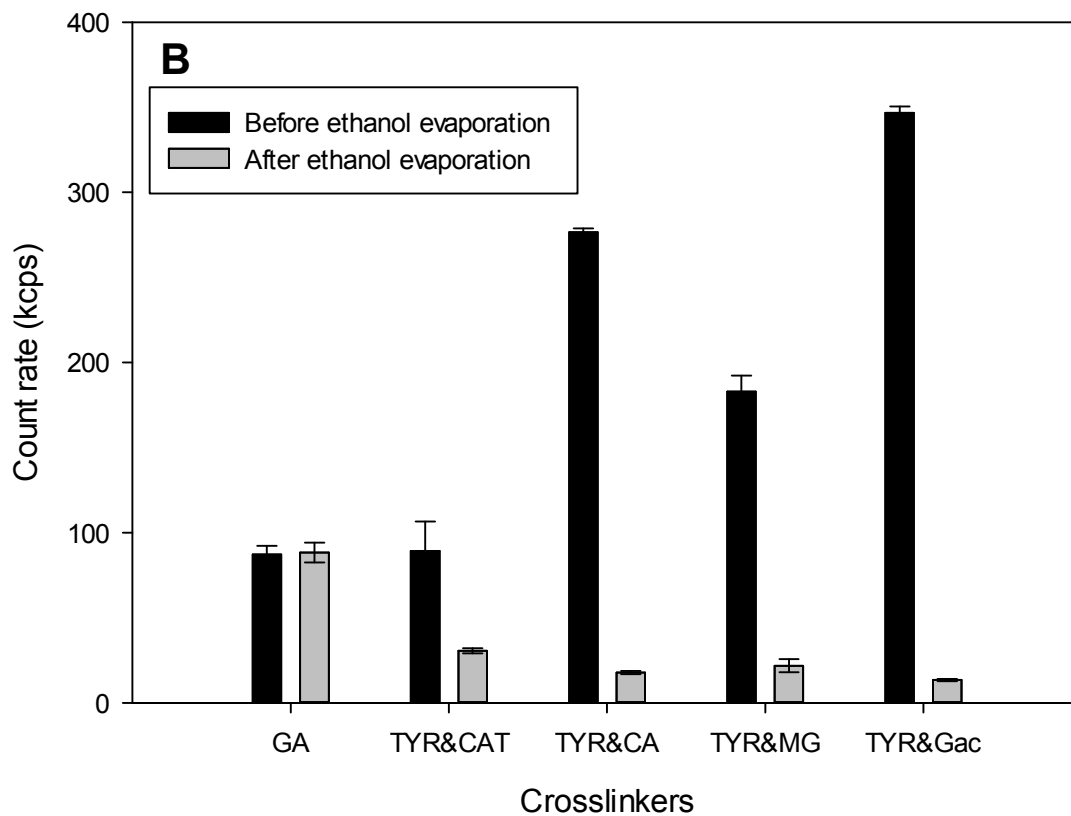
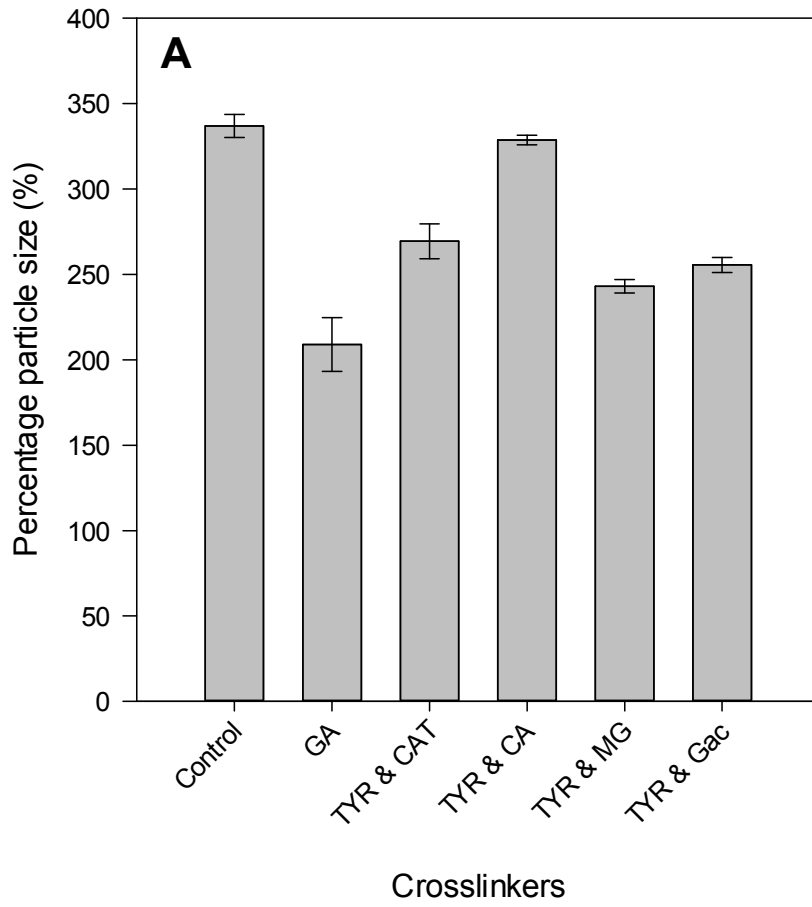


Fig. 3.5. Effect of crosslinking agents (GA, TYR & CAT, TYR & CA, TYR & MG, TYR & Gac) on the particle size (A) and count rate (B) of SC nanoparticles before and after ethanol evaporation in the aqueous ethanol (crosslinker concentration = 7.5 mol/mol protein).



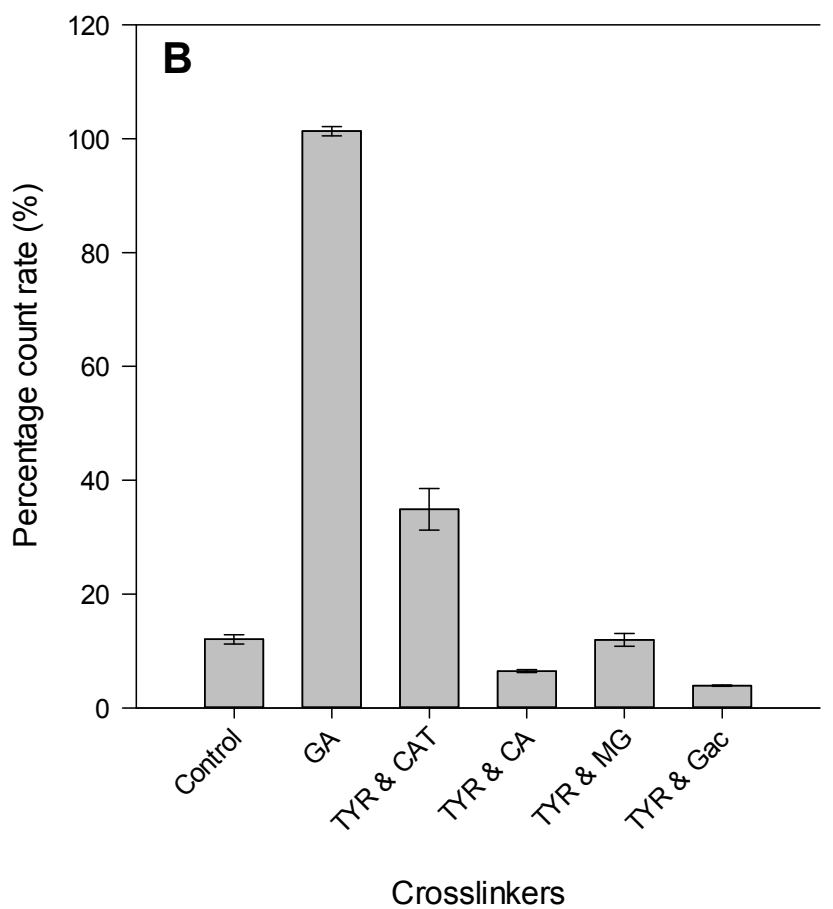
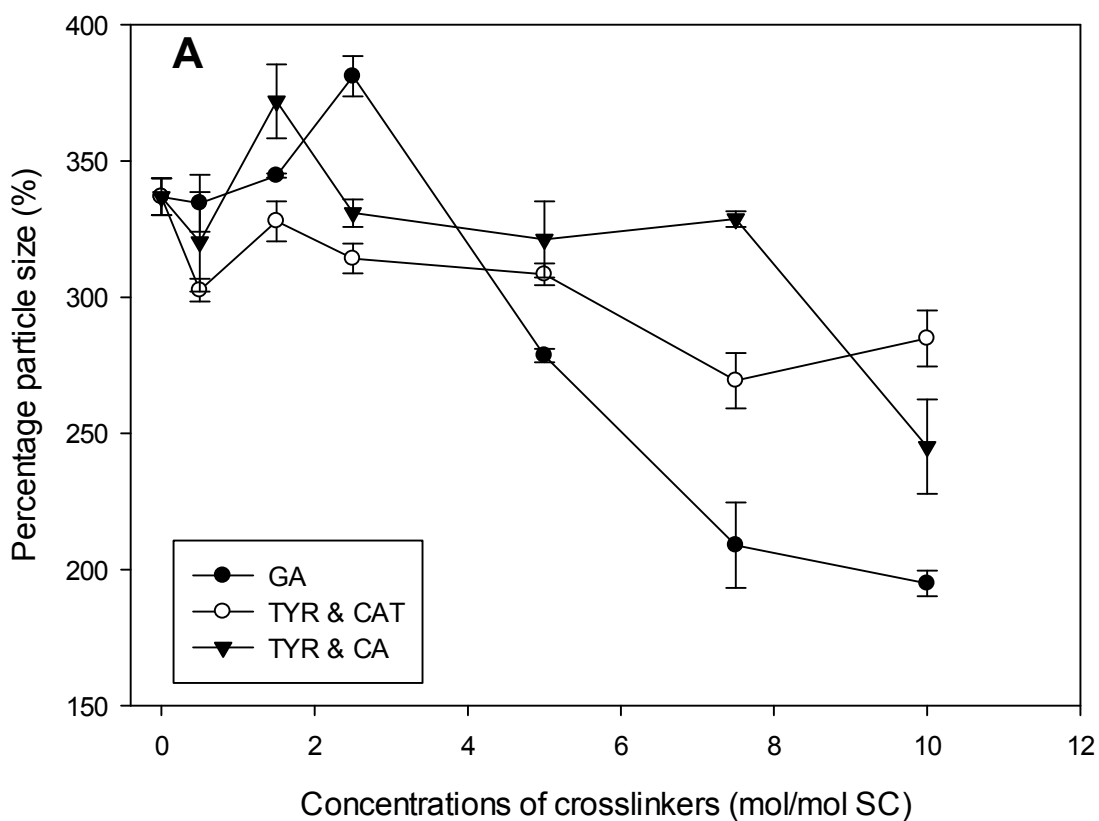


Fig. 3.6. Effect of crosslinking agents (GA, TYR & CAT, TYR & CA, TYR & MG, TYR & Gac) on the percentage particle size (A) and percentage count rate (B) of SC nanoparticles in the aqueous ethanol (crosslinker concentration = 7.5 mol/mol protein).

3.5 Comparison of glutaraldehyde and tyrosinase-aided oxidation of phenols (CAT and CA) as crosslinkers in aqueous ethanol

Since tyrosinase-oxidized phenols (CAT and CA) were not as effective as GA for crosslinking SC nanoparticles in aqueous ethanol, further investigation was conducted to find their optimal working concentrations. The effect of crosslinker type and concentration was summarized in Fig. 3.7, where three noteworthy phenomena were observed. First, although better crosslinking was observed as the crosslinker concentration increased, as indicated by decreasing PS% and increasing CR%, none of the crosslinker exhibited satisfactory crosslinking efficiency (100% PS and CR). In the process of protein nanoparticles crosslinking, the nucleophilic function groups in protein molecules bind to electrophilic o-quinone formed from tyrosinase-oxidized phenols by nucleophilic addition with formation of intermediates. These intermediates are highly unstable in aqueous ethanol and consequently affect the further addition of another protein molecule (Kutyrev & Moskva, 1991). Second, unlike the aqueous procedure where two phenols demonstrated better performance, GA displayed more noticeable crosslinking (at most 100% CR) than CAT (at most 42% CR) and CA (at most 12% CR). This result was probably ascribed to the fact that although tyrosinase-oxidized phenol crosslinking might be interrupted by the formation of unstable intermediates in the aqueous ethanol, GA could continue crosslinking protein molecules under this condition, and as crosslinking process goes on, the consumption of GA shifts the reaction equilibrium towards the side that could promote the protein molecule crosslinking. Therefore, GA presents a higher crosslinking ability compared with tyrosinase-oxidized phenol compound.

Third, distinct crosslinking behaviors were found between CAT and CA, which was also opposite to our previous observation in aqueous procedures. Under 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). However, a large proportion of those aggregates disintegrated upon the removal of ethanol, leading to a low percentage of residual count rate. A possible explanation is the weak associative interactions between SC nanoparticles that maintained the aggregate structure, which needs further validation.



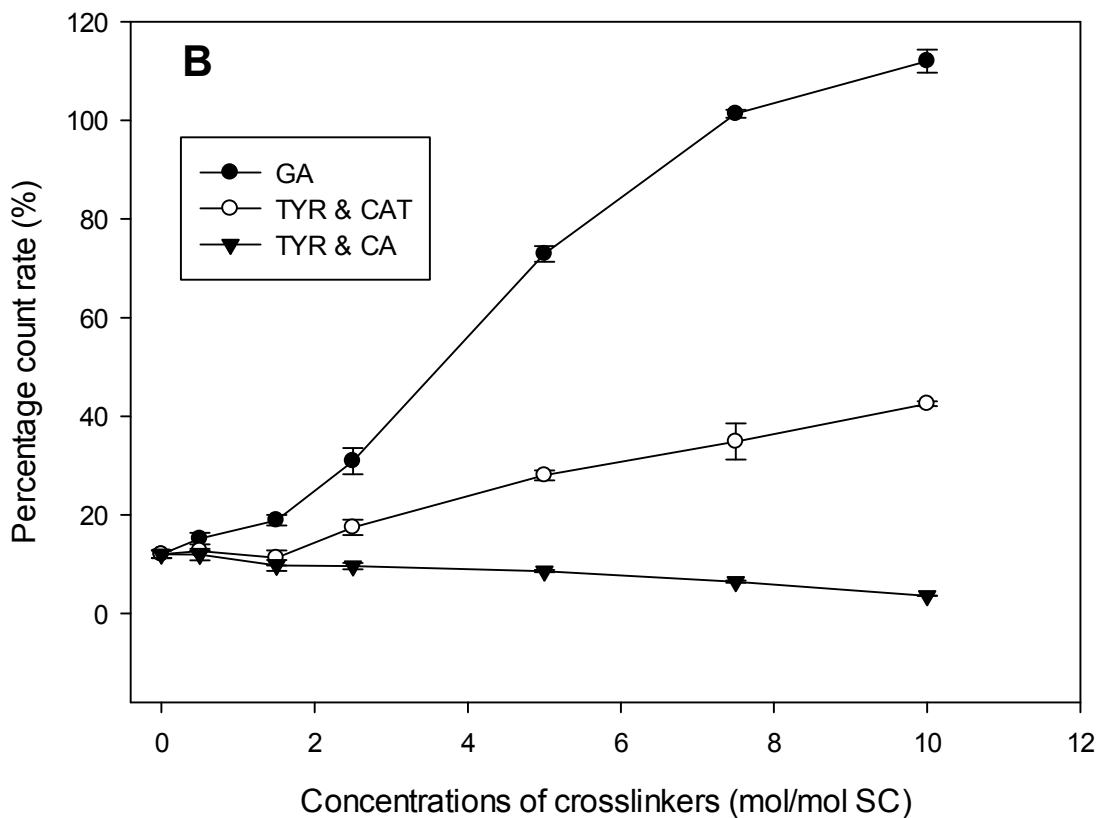


Fig. 3.7. Effect of crosslinker concentrations on the percentage particle size (A) and percentage count rate (B) of SC nanoparticles crosslinked by glutaraldehyde (GA) and tyrosinase-oxidized phenols (TYR&CAT and TYR&CA) in the aqueous ethanol.

3.6 ζ -Potentials of SC nanoparticles in aqueous ethanol

To illustrate the effect of different crosslinkers on the surface charge of SC nanoparticles formed in the aqueous ethanol, the zeta potentials of these nanoparticle dispersions were evaluated and presented in Fig. 3.8. The ethanol was removed and replaced by DI water prior to the measurements. The dispersion of native SC exhibited a zeta potential of -35.1 mV, which was in accordance with previous results obtained from nanoparticles in aqueous solution. The addition of ethanol could impair the electrolysis of the charged groups in SC protein molecules and thus lead to a decreased in zeta potentials of SC nanoparticle dispersions. 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 provide a reasonable explanation to such a phenomenon.

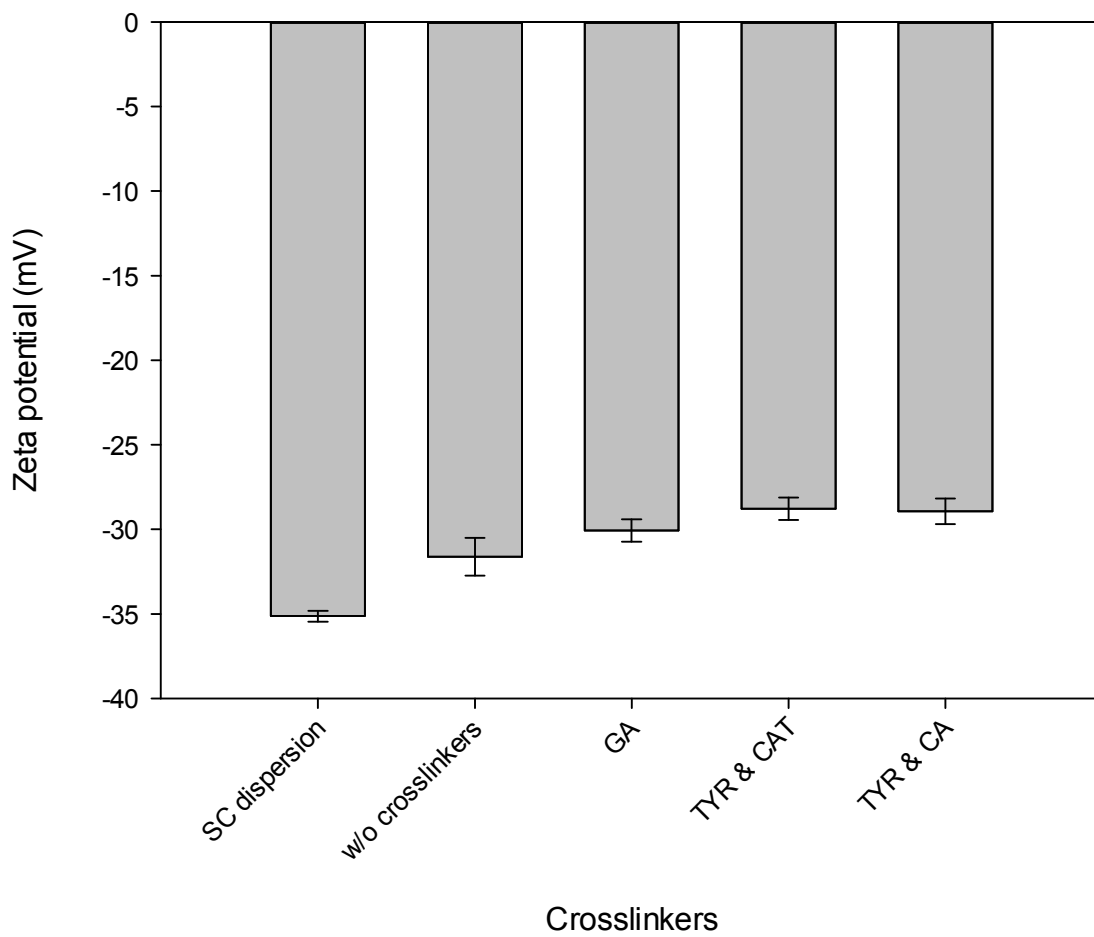


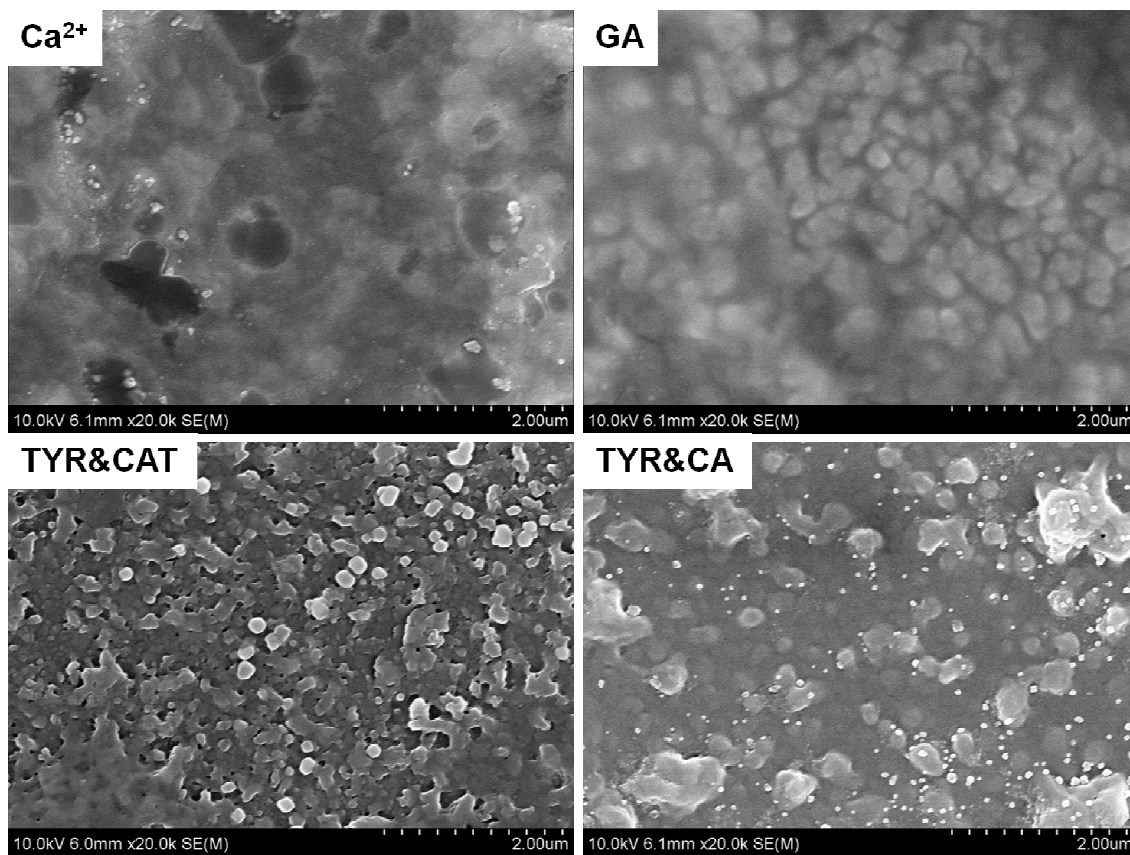
Fig. 3.8. Effect of different crosslinkers (GA, TYR & CAT, TYR & CA) on the zeta potential of SC nanoparticles dispersions in the aqueous ethanol.

3.7 Morphology Analysis

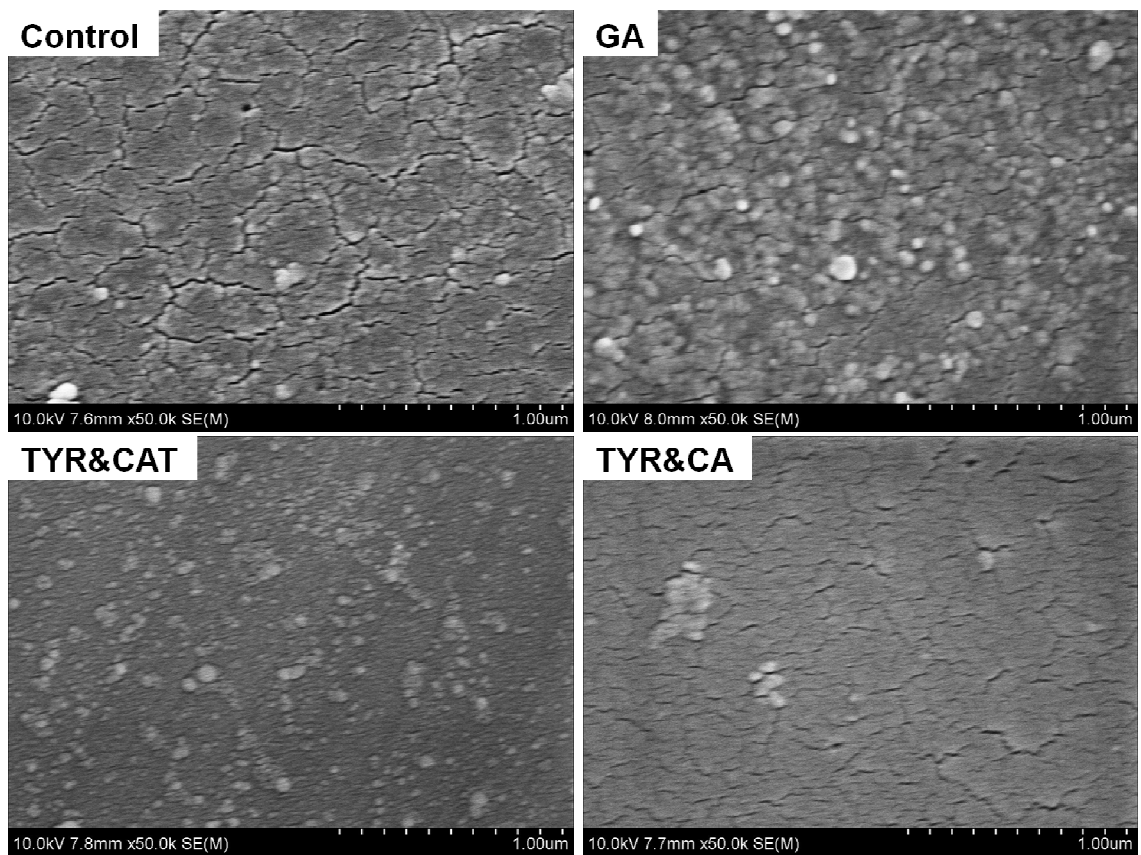
The morphology of SC nanoparticles prepared in aqueous solution and aqueous ethanol solution with different crosslinkers was examined by SEM. When SC nanoparticles were synthesized by aqueous procedure (Fig. 3.9A), in the absence of chemical crosslinkers, only a small number of nanoparticles were observed after acidification. This result supported the assumption that a considerable portion of nanoparticles dissociated into individual molecules, Ca^{2+} alone was not capable to stabilize SC nanoparticles at acidic pH. As crosslinking was performed with 5mol/mol SC glutaraldehyde, a larger portion of nanoparticles was observed after acidification. Similar images were obtained when SC nanoparticles were crosslinked with tyrosinase-oxidized CAT or CA at the same concentration. These results were in accordance with that presented in previously section 3.1, suggesting a strong structure-maintaining ability offered by chemical crosslinking agents.

As SC nanoparticles were prepared by alcoholic procedure (Fig. 3.9B), without chemical crosslinkers, the portion of nanoparticles appeared very small after ethanol evaporation. This result illustrated the previous inference that the dissociation of nanoparticles might be induced by the evaporation of ethanol followed by replacement with water. After 7.5 mol/mol protein GA was applied as a crosslinker, a considerable number of nanoparticles were observed under SEM, showing a significant crosslinking ability of GA. In contrast, the proportion of SC nanoparticles decreased significantly for tyrosinase-oxidized CAT crosslinking at the same concentration, followed by nanoparticles crosslinked with 7.5 mol/mol protein tyrosinase-oxidized CA. These images of SC nanoparticles obtained after ethanol

evaporation was in agreement with previous studies in section 3.4, which explained none of the phenols could contribute satisfactory crosslinking effects to SC nanoparticles.



(A)



(B)

Fig. 3.9. SEM images of SC nanoparticles prepared through aqueous (panel A, after acidification) or alcoholic (panel B, after ethanol evaporation) procedure.

Chapter 4: Conclusions and Recommendations

Conclusions

In the present study, the application of natural phenol compounds as alternative crosslinkers for protein nanoparticles was investigated. Two strategies including ionic gelation and organic solvent desolvation were employed to synthesis SC nanoparticles, which were further crosslinked by tyrosinase (alone or with added natural phenols) or glutaraldehyde and subjected to an environmental stress (pH variation or solvent evaporation) to disintegrate the non-covalently crosslinked nanoparticles. From the aqueous procedure, the observations achieved after acidification suggested that addition of tyrosinase alone could not lead to satisfactory crosslinking for SC nanoparticles. The most effective crosslinking capacity was obtained when 2.5 mol/mol SC protein tyrosinase-oxidized catechol (CAT) or chlorogenicacid (CA) was applied for crosslinking, and then followed by tyrosinase-oxidized gallic acid and methyl gallate. This result was better than that observed with glutaraldehyde, which achieved the same crosslinking capacity at a higher concentration of 7.5 mol/mol protein. In a sharp contrast to aqueous procedure, for the alcoholic procedure, none of the tyrosinase-oxidized phenols could maintain the stability of SC nanoparticles after ethanol evaporation, even with a concentration as high as 10 mol/mol protein, the most crosslinking capacity achieved by tyrosinase-oxidized CAT still exhibited unsatisfactory crosslinking effect as indicated by a high PS% (285%) and low CR%(42%). This results suggested none of the phenols was as effective as GA for crosslinking SC nanoparticles under alcoholic condition, which presented a more significant crosslinking ability at concentration of 7.5 mol/mol

protein. The results from this study for the first time demonstrated the crosslinking capacity of tyrosinase and polyphenols on the development of protein nanoparticles with reduced toxicity.

Recommendations for future work

This study for the first time demonstrated the crosslinking capacity of tyrosinase and polyphenols in the preparation of protein nanoparticles, and it shed some light on the development of biopolymer-based encapsulants with reduced toxicity. The future study will focus on the encapsulation of nutraceuticals in SC nanoparticles using tyrosinase-generated phenolic compounds as crosslinkers. The encapsulation efficiency, in vitro releasing profile, and potential toxicity of these nanoparticles will be evaluated and reported.

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