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# Time effect on coenzyme $Q_{10}$ loading and stability of micelles based on glycosylated casein via Maillard reaction

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ABSTRACT

Protein application as nano-structured delivery system of bioactive compounds is limited by their insolubility at around its isoelectric point (pI). The objective of this work was to investigate the possibility of glycosylated casein by Maillard reaction with dextran for delivering coenzyme $Q_{10}$. It was found that glycosylated casein encapsulating coenzyme $Q_{10}$ were able to exhibit a better stability at pH around the pI. The relationship between Maillard reaction time and the properties of glycosylated casein micelles was investigated with fluorescence probe pyrene (FPP), dynamic light scattering (DLS) and transmission electron microscopy (TEM). The hydrophobic microdomain formed in the glycosylated casein micelles was time dependent and showed the largest microenvironment polarity by reacting for 20 h. The z-average diameter of glycosylated casein micelles prepared by reacting for 4–24 h was in the range of 190–210 nm. The glycosylated casein micelles size were dependent on the grafting degree. During storage at 40 °C, the micelles formed by glycosylated casein with low graft degree tended to aggregate followed by the leaking of the encapsulated coenzyme $Q_{10}$. The optimum Maillard reaction time of glycosylated casein with preferable coenzyme $Q_{10}$ delivery ability and micelle stability were 20–24 h. Findings from this work might be used to develop nano-structured delivery system with appropriate stability in acidic food and beverages.



## 1. Introduction

In recent years, growing concern has been focusing attention on the promotion of health and prevention of chronic disease through the dietary intake of bioactive compounds. This interest has led to numerous attempts to fabricate food-grade nano-structured delivery system with specific properties, e.g., enhanced solubility, stability and bioavailability (Babazadeh, Ghanbarzadeh, & Hamishehkar, 2016; Cho et al., 2014; Xia et al., 2015). Milk proteins hold a great promise due to their excellent biocompatibility and functional properties consequently they are ideal materials for loading and delivering bioactive compounds (Li, Ma, & Cui, 2014; Livney, 2010; Matalanis, Jones, & McClements, 2011). Casein is a milk protein which self-assembly into very stable micelles and this could be exploited to deliver unstable and hydrophobic nutrients, such as vitamin $D_2$ (Menéndez-Aguirre et al., 2014) and β-carotene (Jarunglumlert, Nakagawa, & Adachi, 2015; Nakagawa, Jarunglumlert, & Adachi, 2016; Saiz-Abajo, Gonzalez-Ferrero, Moreno-Ruiz, Romo-Hualde, & Gonzalez-Navarro, 2013). However, the acidic isoelectric point (pI) of casein limits its solubility and functional properties in low pH range (Kyuya Nakagawa, 2013), serving as a challenge in the utilization of the material in food industry.

Protein glycosylation by Maillard reaction (MR) is frequently used to improve protein functionality with the aim of obtaining new functional food ingredients with improved solubility, emulsification and loading capacity (Chen et al., 2013; Spotti et al., 2014; Zhang, Guo, Zhu, Peng, & Zhou, 2015). According to the literature, the Maillard reaction routes and products are dependent on the reaction conditions, such as pH, temperature, time, and water activity (Liu, Ru, & Ding, 2012). Nanoparticle stabilized with casein

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and dextran conjugate prepared by maillard reaction have been developed to inhibit protein precipitation, protect nutraceuticals from degradation and increase their bioaccessibility (Penalva et al., 2015; Xue, Tan, Zhang, Feng, & Xia, 2014). Lutein was successfully encapsulated in the casein-dextran conjugate nanoparticles by oil in water nanoemulsion method (Gumus, Davidov-Pardo, & McClements, 2016).

Coenzyme $Q_{10}$ ($CoQ_{10}$, ubiquinone-10) is an endogenous synthesized antioxidant existing in all cell membranes of the human body and plays a crucial role in the production of adenosine triphosphate (ATP) (Acosta et al., 2016). Studies revealed that its deficiency is often associated with several disorders such as diabetes, cardiovascular and neurodegenerative disease (Choi et al., 2012; Fakin, Kerin, & Neubauer, 2011; Gao et al., 2012; Lee, Huang, Chen, & Lin, 2012). The application of $CoQ_{10}$ is limited by its extremely poor solubility in water. In order to improve the solubility, *in vitro* dissolution and bioavailability, $CoQ_{10}$ have been encapsulated in numerous delivery systems, such as nanoliposomes (Xia, Xu, & Zhang, 2006; Xia, Xu, Zhang, & Zhong, 2007; Xia, Xu, Zhang, Zhong, & Wang, 2009), simple emulsion using Calcium caseinate, lecithin and oil (Stratulat et al., 2013), nanoemulsion prepared with OSA − starch (Cheuk et al., 2015), spray drying microencapsulation using soyprotein isolate, pea protein isolate, skimmed milk powder and OSA-starch (Zhao & Tang, 2016).

However, the phospholipids for fabricating liposomes delivery system are prone to acid hydrolysis, and application of high shear and heat during processing can destroy the liposomal membrane causing losses of loaded bioactive compounds (Gibis, Ruedt, & Weiss, 2016; Tan, Feng, Zhang, Xia, & Xia, 2016). OSA-starch stabilizes emulsion had a limited protection of $\beta$-carotene to oxidation and it should be used at low concentration to avoid high viscosity difficulty during spray drying (Sweedman, Tizzotti, Schafer, & Gilbert, 2013). Protein stabilized emulsions have a thin interfacial membrane and are susceptible to high temperature, salt content and flocculate at pH near isoelectric point (Liang, Wong, Pham, & Tan, 2016). Covalent bonding of dextran and casein through maillard reaction could protect and increase the bioaccessibility of $CoQ_{10}$.

The purpose of the present study was to investigate the effect of Maillard reaction time on microstructure evolution and loading behavior of glycosylated protein. In order to achieve the objective of the study, we produced glycosylated casein with different Maillard reaction time by dry heating and $CoQ_{10}$ was loaded as the model nutrient. The dependence of the micelle size, microstructure and loading ability on grafting degree was characterized with dynamic light scattering and transmission electron microscopy. The hydrophobicity of micelles was explored with pyrene fluorescence.

## 2. Materials and methods

### 2.1. Materials

Casein (molecular mass 25–30 kDa, 99% purity) (technical grade), standard $CoQ_{10}$ (98% purity), pyrene (fluorescence grade) and Trinitrobenzene sulfonic acid (TNBS) were from Sigma Chemical Co. (St. Louis MO, USA). $CoQ_{10}$ material (98.0%–101.0% purity) was purchased from Nisshin Pharma Inc. (Tokyo, Japan). Dextran (molecular mass 20 kDa, 97% purity) and the other solvents/chemicals used were of analytical grade and were obtained from China Medicine (Group) Shanghai Chemical Reagent Co. (Shanghai, China).

### 2.2. Preparation of glycosylated casein

A model reaction system was modified from the procedure by (Clark and Tannenbaum, 1970). Appropriate amounts of casein (5.0 g) and dextran (20 kDa, 35 g) were dissolved in 0.667 M phosphate buffer (PBS), the final concentration of casein was 8 mg/mL. The solution was lyophilized with stoppering tray dryer (LabConoco, USA). Then the frozen-dried powder was passed through 120 mesh sieve and placed in petri dishes sealed with puncture foil in the dark. In order to obtain the Maillard reaction (MR) samples, the powder was reacted at 60 °C for a certain time at a relative humidity of 78.9% in a desiccator containing saturated KBr solution. The samples were achieved at every 4 h intervals, placed in an ice-bath to cool before storage at 4 °C. The reacted mixtures were then dispersed in distilled water to a concentration of 0.25% (w/v) and prefiltered through a 0.45 μm pore membrane filter (Millipore Corp., Bedford, MA, USA). The solid phase was then conducted with a 20 mL Microsep Advance Centrifugal Device (molecular weight-cut off of 100,000; Pall Corporation, MI, USA) at 1000 rpm for 30 min until 1/8 of the initial volume remained, followed by the addition of deionized water to the original volume. This procedure was repeated three times, and then the retentates were freeze-dried and then stored at 4 °C.

### 2.3. Preparation of $CoQ_{10}$-loaded micelles

The $CoQ_{10}$-loaded micelles were prepared by ethanol injection and ultra-high pressure homogenization method. Glycosylated casein was dissolved in deionized water (55 °C) to prepare solutions with casein concentration 1 mg/mL. Then $CoQ_{10}$ ethanol solution (2 mg/mL) was rapidly injected using a syringe as a pump into 20 mL of protein solution at 55 °C with magnetic stirring. After agitation for 30 min, the ethanol was removed by rotary evaporation (55 °C, 0.1 MPa) to form an aqueous dispersion of micelles. The micelles were placed in an ice bath to cool down and adjusted to pH 4.6 with 1.0 mol/L NaOH and 1.0 mol/L HCl. The samples proceeded ultra-high pressure homogenization with a pressure of 1050 bar (cycle once) and then for the 1400 bar (cycle twice) by using NS1001-L2K mechanical homogenizer (Niro-Soavi, S.p.A., Parma, Italy) and then stored at 4 °C.

### 2.4. Size distribution analysis of micelles

Dynamic laser light scattering (DLS) was used to determine the particle size distribution, including z-average diameter (Dz) and polydispersity index (PDI) using Zetasizer Nano ZS 90 (Malvern Instruments, Worcestershire, UK) equipped with a He/Ne laser ($\lambda = 633$ nm) and scattering angle 90°. Aliquots of 1 mL micelles were diluted to 10 mL with the same buffer solution to avoid multiple scattering phenomena due to interparticle interaction. Immediately, the diluted sample was transferred into the polystyrene cuvette for size determination at 25 ± 0.1 °C, and then the z-average diameter (Dz) and particle size distribution (polydispersity index, PDI) were recorded. The measurements were performed on three individual samples, and the results given were average.

### 2.5. Measurement of free amino group content

The quantity of available amino groups was determined by the modified trinitrobenzenesulfonic acid (TNBS) method (Adler-Nissen, 1979). The glycosylated casein was solubilized in 3% sodium dodecyl sulfate. Sample (0.25 mL) was mixed in a test tube with 2 mL of phosphate buffer at pH 8.2. One milliliter of 0.1% TNBS solution was added and the test tube was shaken and placed in a water bath at 50 °C for 60 min covered with aluminum foil. After incubation, 4.0 mL of HCl (0.1 M) was added to terminate the reaction, and the test tube was allowed to stand at room temperature