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One-step fabrication of phytoferritin-chitosan-epigallocatechin shell-core nanoparticles by thermal treatment

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ABSTRACT

The inner surface and outer surface of ferritin cage provide interfaces for the encapsulation and delivery of food nutrients. Traditional methods to fabricate ferritin-nutrients shell-core nanoparticle usually apply acid/alkaline pH transition, which may cause the activity loss of the food nutrients or the formation of insoluble aggregates. In attempt to tackle these limitations, a simple one-step method was utilized to prepare the red bean seed ferritin (RBF)-epigallocatechin (EGC)-chitosan nanoparticle (REC) by thermal treatment at 55 °C. Results indicated that the apoRBF was partially uncoiled with a decrease of 5.3% of α-helix content induced by 55 °C treatment, and the EGC molecules could spontaneously permeate into the inner cavity of the ferritin with an encapsulation ratio of 11.8% (w/w). Meanwhile, the thermal treatment facilitated the chitosan attaching onto the outer surface of the ferritin by electrostatic interactions with a binding constant of $4.7 \times 10^5\ M^{-1}$. Transmission electron microscope and dynamic light scattering results indicated that the REC was mono-dispersedly distributed, with a diameter of 12 nm and a hydrodynamic radius (R_H) of 7.3 nm. In addition, the chitosan decorating onto the apoRBF improved the EGC stability by weakening the degradation of apoRBF against digestive enzymes in simulated gastrointestinal tract. This work is a novel attempt to fabricate shell-core nanoparticle in the encapsulation and delivery of functional molecules based on the ferritin cage in a benign condition without extreme pH changes.

1. Introduction

Ferritins are a kind of protein that can store thousands of iron in their inner cavity (Yang, Zhou, Sun, Gao, & Xu, 2015). Each ferritin consists of same or different twenty-four subunits which assemble into a shell-like structure with inner size of 8 nm and external diameter of 12 nm, respectively. Thanks to its nano-sized inner cavity and the reversible assembly character of the ferritin, small bioactive molecules have been sparked to be encapsulated into the ferritin cage. For example, β-carotene, EGCG, and rutin have been successfully encapsulated into the phytoferritin or recombinant ferritin (Chen et al., 2014; Yang et al., 2015, 2016). After encapsulation, these food nutrient molecules can be functionalized by the valuable tool of ferritin shell to realize the stabilization, solubilization, and targeted delivery.

Traditional methods to fabricate ferritin-nutrients shell-core nanoparticle usually take advantage of the pH transition (pH 2.0/12.0 to neutral condition) to disassemble and resemble the ferritin cage (Yang et al., 2015), which may cause the loss of a large fraction of the samples to insoluble aggregates. After pH transition, the integrity of the reassembled ferritin remains a question (Tetter & Hilvert, 2017). Additionally, the extreme acid/alkaline conditions may abrogate the sensory properties, stability, and bioactivity of the certain pH-sensitive molecules, such as EGCG and anthocyanin. Thus, how to realize the successful encapsulation of the food nutrients in the ferritin cage and meanwhile to maintain the integrity of ferritin structure are challenges. Previous report has revealed that the whole ferritin protein can be stable against heat treatment (80 °C) for 10 min (Stefanini et al., 1996), but some local structures of ferritin such as the pore/channel subdomains are sensitive to

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temperature changes and can be uncoiled upon thermal treatment (Liu, Jin, & Theil, 2003; Theil, Liu, & Tosha, 2008). Inspired by these findings, the thermally induced pore/channels may provide a sally port for the encapsulation of nutrients in a different manner, and the food nutrient molecules are expected to be permeated into the ferritin cavity through these expanded pore/channel structures.

One the other hand, the ferritin instability in the gastrointestinal tract is also a limitation factor that can influence the encapsulation and delivery of the nutrients. The ferritin can be hydrolyzed by the digestive enzymes such as pepsin and trypsin, and the nutrients are easily released to the gastrointestinal tract (Deng et al., 2011; Yang et al., 2015), which is adverse to the stability of the nutrients. In order to improve the physicochemical and functional properties of ferritin and thus to protect the encapsulated molecules, decorating of the outer-surface of the ferritin with the polysaccharide is a favorable direction. For example, the chitosan molecules are natural cationic polysaccharide which has been applied as a good wall material for food component encapsulation due to its non-toxicity and biocompatibility properties (Lee, Yim, Choi, Van, & Ko, 2012; Pillai, Paul, & Sharma, 2009). In addition, the ferritin cage owns a high negative charge density on its external surface and interior surface. Thus, attaching of ferritin with cationic chitosan may be feasible to be realized, and the resulting ferritin-chitosan composites are potential to protect the protein cage and thus to improve the bioavailability of encapsulated bioactive compounds.

To verify above hypothesis, the apo-red bean seed ferritin (apoRBF) (deprived of irons), epigallocatechin (EGC), and chitosan molecules are selected as the candidates. A novel method to fabricate apoRBF-EGC-chitosan shell-core nanoparticle (REC) induced by thermal treatment is investigated for the first time. The effects of thermal treatment on the structure changes of the ferritin, the encapsulation ratio of the EGC, and the interaction between the ferritin and the chitosan are emphasized. The thinking of this work will be useful for fabrication of ferritin-nutrients shell-core nanoparticle by thermal treatment, which is especially suitable for the encapsulation of pH-sensitive molecules without undergoing pH changes.

2. Materials and methods

2.1. Ferritin preparation and Native-PAGE and SDS-PAGE analysis

Red bean seed ferritin (RBF) and apoRBF (deprived of irons) was prepared as previously described (Li, Yun, Yang, & Zhao, 2013). The molecular weight of apoRBF was determined by Native-PAGE using a 12% polyacrylamide gradient gels running at 5 mA for 8 h at 4 °C. SDS-PAGE was performed under reducing conditions in 15% SDS-polyacrylamide gel. Gels were stained with bromophenol blue. The concentrations of ferritin were determined according to the Lowry method with BSA as a standard sample.

2.2. Fabrication of holo RBF and iron release analysis

Holo RBF (1.0 mL) was prepared by adding 700 irons ($FeSO_4$) to each apoRBF solution by 7 increments with every 20 min intervals in 50 mM Mops buffer (50 mM NaCl, pH 7.0). Iron release from Holo RBF was investigated as follows. Briefly, the reaction system (1 mL) contained 1.0 μM holoRBF, 500 μM ferrozine and 50 mM NaCl in 50 mM Mops buffer (pH 7.0). Reactions were carried out at 20 and 55 °C, respectively, and were initiated by the addition of ascorbic acid (1 mM). The formation of $[Fe(ferrozine)_3]^{2+}$ was measured by recording the increase in absorbance at 562 nm, and iron release was measured using $\varepsilon_{562} = 27.9\ mM^{-1}\ cm^{-1}$ (Hynes & Coinceanainn, 2002). The initial rate of iron release (v_o) was calculated as previously described (Hynes & Coinceanainn, 2002). The influence of different temperature (15, 25, 35, 45, 55, and 65 °C) on the v_o was investigated.

2.3. Fluorescence spectrofluorometry analysis

The fluorescence titration experiments were performed by using the RF-5301PC spectrofluorophotometer (Shimadzu, Japan). Two groups of measurements at 55 and 25 °C were respectively carried out by an adding of 0.0–10.0 μL of chitosan molecules to 500 μL of the protein (0.5 μM, pH 6.75) in 50 mM Mops, to generate chitosan/apoRBF ratios of 0, 2, 4, 6, 8, 10, 12, 14, 16, 18, followed by a reaction time of 5 min. Path lengths for excitation and emission were 1.0 and 0.5 cm at wavelengths of 290 and 330 nm, respectively. A slit width of 10 nm and 5 nm was set for excitation and emission, respectively. To obtain the stoichiometry n and binding constant K, the data were fitted to Equation (1) for the binding of chitosan to n independent binding sites on the ferritin (Bou-Abdallah et al., 2008).

$$I = I_0 - \left(\frac{I_0 - I_\infty}{2n[P]_0}\right) \times \left[1/K + [CS]_0 + n[P]_0 - \sqrt{(1/K + [CS]_0 + n[P]_0)^2 - 4n[P]_0[CS]_0}\right] \quad (1)$$

The $[P]_o$ and $[CS]_o$ are the ferritin and chitosan concentrations, and I_o and I_∞ are the fluorescence intensities in the absence and presence of chitosan when the binding sites are fully saturated, respectively. The purity of chitosan used is about 88.13%, and the obtained binding constant and stoichiometry value has been converted by this purity value.

2.4. Preparation of EGC by thermal treatment

EGC (25.0 mg) was dissolved in deionized water (pH 6.75, adjusted by HCl) to make a 1.0 mM stock solution and stored in the dark in an amber bottle at 4 °C. Briefly, the apoRBF (1.0 μM, pH 6.75, 5.0 mL) was pre-incubated with 55 °C for 40 min; subsequently, EGC stock solution and the different chitosan molecules (with molar weight of 4600) was added to the above solution with apoRBF/EGC ratio of 1:140 and apoRBF/chitosan ratio of 1:18, followed by stirring to produce a homogeneous solution. The incubation was performed for 60 min (55 °C), followed by reducing the temperature to 25 °C to induce the formation of RBF-epigallocatechin (EGC)-chitosan nanoparticle (REC). The resulting solution was dialyzed (MW 10 kDa cutoff) against MOPS buffer (50 mM, pH 6.75) with three buffer changes (every 1.5 h intervals) to remove the unbound EGC and chitosan. Finally, the suspension was further filtered through a 0.45-μm hydrophilic cellulose membrane filter to clarify the nanoparticles.

EGC-loaded RBF nanoparticle as a control sample was also prepared by the traditional method by pH changes (Chen et al., 2014). Firstly, the pH value of the apoRBF solution (1.2 μM, 4.5 mL) was adjusted to pH 2.0 with HCl (1 M) for 40 min to disassemble apoRBF into subunits; subsequently, EGC stock solution was added to above solution with an apoRBF/EGC ratio of 1–140, followed by stirring for 60 min (4 °C) in the dark to produce a homogeneous solution. The pH of the resulting mixture was then adjusted to 6.75 with NaOH (1.0 M), followed by incubation at 4 °C for 60 min to induce reassembly and to encapsulate EGC molecules within the ferritin cage to generate the EGC-loaded RBF nanoparticle. Then, a faction of the EGC-loaded RBF nanoparticle was mixed with chitosan molecules for 60 min. The products were then dialyzed (MW 10 kDa cutoff) against MOPS buffer (20 mM, pH 6.75) with three buffer changes (every 1.5 h intervals). Finally, the