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Stimuli-responsive nanoparticles by thermal treatment of bovine serum albumin inside its complexes with chondroitin sulfate

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A B S T R A C T

We combine electrostatic polysaccharide/protein complexation with subsequent thermal denaturation of proteins to produce chondroitin sulfate/bovine serum albumin nanoparticles that do not lose their integrity at neutral and basic pH. Light scattering at a wide angular range shows that nanoparticle size (radius of gyration and hydrodynamic radius) and molar mass may be tuned by the CS/BSA mass ratio while the irreversible protein-protein contacts upon temperature treatment endow the complexes with properties of nanogels. These nanoparticles respond to changes of pH by reversing their surface charge at pH 5.3 while their size responds to the solution ionic strength and pH. The conformational changes of bovine serum albumin upon thermal treatment are confirmed by spectroscopy methods. The ability of the nanoparticles to encapsulate bioactive substances is demonstrated by loading the nutraceutical β-carotene. The methodology of this work may be applied to other polysaccharide/protein systems to produce multi-functional stimuli-responsive nanoparticles for nanodelivery in food science and biomedical applications.

1. Introduction

The development of polymeric nanoparticles (NPs) for food science, medicine and biotechnology is continuously advancing in terms of possibilities for encapsulation and interactions with bioactive substances (Koseva, Rydz, Stoyanova, & Mitova, 2015; Zhao et al., 2016; Raveendran, Rochani, Maekawa, & Kumar, 2017). Polymeric NPs have stimuli responsive properties and can interact with charged and hydrophobic components. Self-assembly can be used for association of amphiphilic block polyelectrolytes (Elezaby, Gad, Metwally, Geneidi, & Awad, 2017) into micellar nanostructures (Papagiannopoulos, Meristoudi, Pispas, & Radulescu, 2016; Yang, Zheng, Guo, Qian, & Zhang, 2011) and oppositely charged polyelectrolytes into complexes and coacervates (Blocher & Perry, 2017; Gucht, Spruijt, Lemmers, & Cohen Stuart, 2011). Examples from complexation include chitosan/carboxylated ι-carragenan complexes that have been used for insulin delivery (Sahoo et al., 2017) and poly (ethylene glycol)-chitosan-graft-spermine complexes with DNA that were demonstrated to inhibit tumor growth (Kim et al., 2012). Polyelelectrolyte/protein (Cooper, Dubin, Kayitmazer, & Turksen, 2005) and especially polysaccharide/protein (Comert, Malanowski, Azarikia, & Dubin, 2016) complexation has been extensively studied and the effects of protein charge distribution, pH, and ionic strength have been explored in detail. Proteins are themselves complex macromolecules that are characterized by a charge patch and hydrophobic domains and can themeselves act as versatile nanocarriers. The great possibilities that open by using polysaccharide/protein complexes (Devi, Sarmah, Khatun, & Maji, 2017) are supported by the fact that polysaccharides are stable, safe, biodegradable and non-toxic (Liu, Jiao, Wang, Zhou, & Zhang, 2008).

Another important aspect is the response of globular protein conformation on external triggers and especially temperature. It finds important applications in protein drugs and blood clotting and therefore has been studied for many decades (Privalov, 1979; Schön, Clarkson, Jaime, & Freire, 2017). Unfolding of the cysteine-containing pocket enables disulfide bridges and subsequent protein-protein aggregation (Wetzel et al., 1980). Temperature-induced structural changes in bovine serum albumin (at 65 °C) have been reported partially reversible (Takeda, Wada, Yamamoto, Moriyama, & Aoki, 1989). In a detailed ATR-FTIR study (Lu et al., 2015) it was proposed that irreversible intermolecular β-sheets are formed at 50–52 °C and are enhanced up to 80–85 °C with a second distinct transition at ~80 °C. Most of the helical structure is disrupted in the region between 50 and 100 °C. The recovered helical content (at 25 °C, after the thermal treatment) becomes lower as the thermal denaturation temperature increases (Moriyama et al., 2008).

Thermal treatment of polysaccharide/protein complexes has been demonstrated to produce NPs with tunable size and physicochemical properties (Owen Griffith Jones & McClements, 2010). In particular the thermo-irreversibility of the protein transition has been proposed for fabrication of biopolymer nanopaticles (Owen G. Jones, Decker, & McClements, 2010). In this framework, proteins not only introduce multifunctional properties to the complexes but also act as versatile structural components. Indeed, loading of hydrophobic nutraceuticals to β-lactoglobulin/polysaccharide complexes has been optimized for

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clear acid beverages (Ron, Zimet, Bargarum, & Livney, 2010) and encapsulation of β-carotene in thermally treated β-lactoglobulin/pectin complexes has been proposed (Gutiérrez et al., 2013).

In this work we use the complexation of chondroitin sulfate (CS) with BSA in acidic conditions, by citric acid which is a non-toxic chemical, in order to form well-defined biocompatible NPs. We study the size, mass and surface charge of the complexes by light scattering techniques and show that electrostatic complexation leads to NPs with controllable size at low CS content. The integrity of untreated NPs is strongly pH dependent as at neutral pH both the protein and the polysccharide are negatively charged. When the NPs are thermally treated the denaturation of BSA causes irreversible changes that render the NPs resistant to pH-induced disintegration. The thermally treated NPs are responsive to salt content and pH. The ability of the thermally treated NPs to act as nanocarriers of nutracuricals is proved by the effective encapsulation of β-carotene. This study demonstrates the use of proteins as both building blocks and nanocarriers inside multifunctional nanoparticles and can be used as a guide for other systems of polysaccharide/protein pairs and bioactive substances.

2. Materials and methods

2.1. Materials and samples preparation

Porcine chondroitin sulfate in the sodium salt form (Na-CS) with $M_w \sim 23{,}000$ gmol^{-1} (PDI ~ 2) was provided from Bioiberica. Bovine serum albumin (BSA) was purchased from Sigma-Aldrich and was used without further treatment. β-Carotene and citric acid (CA) were purchased from Sigma Aldrich. Stock aqueous solutions from the separate components i.e. CA (1.0 mgml^{-1}), CS (1.0 mgml^{-1}) and BSA (10 mgml^{-1}) were prepared in distilled water and kept overnight to dissolve and equilibrate at 4 °C. Salt concentration was set by adding the desired volume of NaCl (1M). The final volume ratios of the mixtures to obtain CS/BSA complexes were obtained by mixing proper volumes of stock solutions and distilled water under gentle stirring. Water was first mixed with the Na-CS solution and CA solution was subsequently added to final pH 4.2. Desired volumes of BSA stock solutions were added in the end. The temperature treatment protocol consisted of placing sealed vials of solutions of CS/BSA complexes in an oven at 90 °C for 2 h and then allowing to cool at room temperature in air. NaOH was used to set pH. All measurements were performed at 25 °C unless mentioned otherwise.

For loading the bioactive compound, β-carotene was dispersed in acetone at 5 mgml^{-1} and 20 μl from this dispersion were added to 1 ml of the temperature-treated NPs solution at neutral pH conditions. The mixtures were left in a fume cupboard for the acetone to evaporate and subsequently gently shaken overnight at room temperature. Free β-carotene was extracted from the NPs solution by adding 200 μl n-hexane and vortexing for a couple of minutes. Twenty μl of the supernatant n-hexane phase were subsequently diluted with n-hexane and assayed by UV analysis in order to obtain the amount of non-encapsulated bioactive compound.

2.2. Light scattering

Light scattering (LS) was performed on an ALV system (ALV-CG-3 goniometer/ALV-5000/EPP multi tau digital correlator) with a He—Ne laser (λ = 632.8 nm). The time-averaged scattered intensity is collected and reduced to the Rayleigh ratio $R(q)$ for SLS (Chu, 1991) in static light scattering (SLS). The weight average molecular weight M in combination with the form factor $P(q)$ of the scattering particles is extracted from equation (1).

$$\frac{Kc}{R(q)} = \frac{1}{MP(q)} \tag{1}$$

Where c is the mass solution concentration, q is scattering wave vector ($q = \frac{4\pi n_0}{\lambda} sin\frac{\theta}{2}$) and K is the LS contrast factor $K = \frac{4\pi^2 n_0^2}{N_A \lambda^4}(\partial n/\partial c)^2$. In K the parameter n_0 is the solvent's refractive index and $\partial n/\partial c$ the refractive index increment of the scattering molecules in the specific solvent. We used $\frac{\partial n}{\partial c} = 0.18\ ml g^{-1}$ which is near the values of both CS and BSA.

$$\frac{R(q)}{Kc} = M \cdot e^{-\frac{1}{3}q^2 R_g^2 + B\cdot(q^2)^2} \tag{2}$$

In the standard Guinier approximation (Borsali & Pecora, 2008) for the form factor $P(q) = e^{-\frac{1}{3}q^2 R_g^2}$ the radius of gyration R_g of the scattering particles is obtained. We used a quadratic approximation (in q^2) for the form factor (equation (2)) so that the whole series of Guinier plots could be fitted (see Results and Discussion).

In dynamic light scattering (DLS) (Berne & Pecora, 2000) the scattered light intensity autocorrelation functions $g_2(\tau)$ are transformed into the field autocorrelation functions $g_1(\tau)$ by the Sieget relation $g_2(\tau) - 1 = \beta |g_1(\tau)|^2$ where β is a normalization constant and τ the lag-time. The characteristic relaxation time τ_c(q) of $g_1(\tau)$ was extracted by cumulant analysis. The characteristic relaxation rate $\Gamma(q) = 1/\tau_c(q)$ was fitted by equation (3) (see Results and Discussion about the use of a quadratic formula) to obtain the diffusion coefficient D.

$$\Gamma(q) = D\cdot q^2 + C\cdot(q^2)^2 \tag{3}$$

Stokes-Einstein relation (equation (4)) leads to the hydrodynamic radius R_h (η is the solvent viscosity).

$$R_h = \frac{k_B T}{6\pi\eta D} \tag{4}$$

The shape factor defined as $\rho = R_g/R_h$ is a quantity that characterizes the shape of the scattering particles. Field autocorrelation functions were analyzed by the CONTIN algorithm to extract the distribution of hydrodynamic radii. Static (SLS) and dynamic (DLS) light scattering data were collected over a wide angular range from θ = 30° to θ = 130°.

2.3. Electrophoretic light scattering

A Zetasizer Nano-ZS by Malvern Instruments Ltd. was used for electrophoretic light scattering (ELS). Zeta potential calculation was made by the Henry equation under the Smoluchowski approximation. Reported ζ values are averages of 10 measurements taken at scattering angle θ = 173° and measurements were performed at 25 °C.

2.4. Circular dichroism

Circular dichroism (CD) measurements were performed with a Jasco J-815 CD spectrophotometer with a peltier model PTC-423S/15 thermo stabilizing system. The solutions were loaded on 1-mm quartz Suprasil cells. Aqueous solutions were diluted five times so that BSA concentration was at 0.2 mgml which was the optimum value for adequate CD signal and relevance with the conditions of LS experiments. The contents of α-helix, β-sheet and random coil conformations were estimated by K2D3 software (Louis-Jeune, Andrade-Navarro, & Perez-Iratxeta, 2012). The spectra were accumulated by averaging 4 successive runs.

2.5. Fourier transform infrared spectroscopy

Infrared spectra (FTIR) were recorded on a Bruker Equinox 55 Fourier Transform Instrument, equipped with an attenuated total reflectance (ATR) diamond accessory, from SENS-IR, and a press. The dried solutions were placed at the center of the sample holding device and 64 scans were performed in the range 500–5000 cm^{-1}, at a resolution of 2 cm^{-1}. Two measurements on different loaded samples were performed to confirm reproducibility.