Colloids and Surfaces B: Biointerfaces 173 (2019) 43–51

Contents lists available at ScienceDirect

Colloids and Surfaces B: Biointerfaces

journal homepage: www.elsevier.com/locate/colsurfb

# Formation and characterization of self-assembled bovine serum albumin nanoparticles as chrysin delivery systems

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ARTICLE INFO

*Keywords:*
Bovine serum albumin
Chrysin
Nanoparticle
Self-assembly
Heat treatment
pH
Protein concentration

ABSTRACT

Chrysin (5,7-dihydroxyflavone) (Chrys) is a natural flavone extracted from many plants, and it has been proposed as a bioactive agent for cancer therapy. Nevertheless, its use is limited mainly due to its poor water solubility. Bovine serum albumin (BSA) is a water soluble, biocompatible and non-toxic protein with a promising application in lipophilic bioactive compound delivery. Moreover, BSA is heat sensitive, feature that could be used for producing self-assembled nanoparticle with tailor-made properties. In this contribution, we studied the formation of BSA nanoparticles (BSAnp) by thermal treatment at different conditions of temperature (70 °C/5 min and 85 °C/5 min), protein concentration (1.0-4.0%wt.) and aqueous medium pH values (9.0 and 11.0) in which it is known that BSA is found in different unfolded conformations. Binding of Chrys dissolved in dimethyl sulfoxide (DMSO) was studied by fluorescence titration experiments. Characterization of Chrys-loaded and unloaded BSAnp was performed in phosphate buffered saline (PBS) pH 7.4 by applying a set of complementary techniques: dynamic light scattering (DLS), size exclusion fast protein liquid chromatography (SEC-FPLC) and transmission electron microscopy (TEM). Different populations of BSAnp were obtained, which showed different diameters in the range of 1328 nm, ζ potentials around −10.0 mV, molecular weight in the range of 400–1000 kDa and spherical shape. Chrys encapsulation efficiency (EE. %) was also determined, and values between 44–84% were obtained, which mainly depended on the mode of Chrys binding and physicochemical BSAnp properties. Results highlight the ability of self-assembled BSAnp for Chrys vehiculization in an aqueous medium which could found potential application in antitumor therapies.

## 1. Introduction

In the past decades, there has been a growing interest in the development of nanoparticles as promising carriers for therapeutic agents [1–3] with notable prominence in cancer therapy. Albumin-based nanoparticles have gained much interest in this field due to the wide availability of serum albumin and its inherent properties of biodegradability, biocompatibility, low toxicity and antigenicity, and its natural function as plasma transport protein [4–8]. Among serum albumins, bovine serum albumin (BSA) is a water-soluble protein with a promising application in biochemical, pharmaceutical and food industries [9–11].

BSA is a globular protein of 65.5 kDa, composed by 585 amino acids, with a pI of 4.7 in water at 25 °C. Its secondary structure is comprised of at least 67% α-helix, 10% turns and 23% extended chain without β-sheet elements in physiological conditions. BSA contains three main domains (I, II and III); each one is composed of two subdomains (A and B). The protein has 17 intramolecular disulfide bonds with a free sulfhydryl at Cys34, which confers it a relative stability. Moreover, it is known that BSA has different pH-dependent conformations: at pH values below 3.5, it presents as an extended conformation (E-form), with a transition to a called fast form (F-form) when pH abruptly decreases below 4.0. At pH values between 4.5 and 7.0 is found in a normal form (N-form), and when pH increases BSA unfolds adopting a basic form (B-form) at 8.0–9.0 and finally an aged form (A-form) at pH values above 9.0 [12–14]. The transition involves a

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https://doi.org/10.1016/j.colsurfb.2018.09.046
Received 6 June 2018; Received in revised form 24 August 2018; Accepted 20 September 2018
Available online 21 September 2018
0927-7765/ 

decrease in the content of ordered (secondary) structure. The albumin macromolecule in the N-form is globular, whereas it becomes partly opened in the F-form. The N−F transition involves a decrease in the content of ordered (secondary) structure. Upon the basic transition (or N−B transition) BSA apparently loses some of its rigidity, and the macromolecule has a small increase in its radius [14]. These conformational changes could influence on its aggregation process and on ligands binding properties. Besides, previous studies about structural changes upon heat treatment have been reported that above 65 °C BSA unfolds with a loss of α-helix conformation and an increase of β-sheets formation [15,16]. Hence, as it was mentioned these conformational features could be rationally used in the development of tailor-made nanoparticles.

In this context, numerous studies have been published about BSA-based nanoparticles for drug controlled release [8–11,17–19]. This extensive research has been promoted by the US Food and Drug Administration (FDA) approval of the first protein nanotechnology-based chemotherapeutic, nab-paclitaxel, which is a cremophor-free albumin-bound nanoparticle formulation of paclitaxel with a mean particle size of approximately 130 nm [20]. In general, the best-known methods of albumin nanoparticle preparation are based on emulsion formation, desolvation or coacervation [4,9–11,21]. These methods have limitations such as the use of organic solvents and a final stabilizing step consisting on the addition of glutaraldehyde as a cross-linking agent, which must be then eliminated due to its toxicity. Regarding this, the study of new preparation methods, and the evaluation of the effect of physicochemical parameters on nanoparticle properties are important requirements. In the design of nanodevices as a delivery system is very important to control some parameters such as particle size, surface properties, and release of pharmacologically active agents in order to achieve the drugs site-specific action at a therapeutically optimal rate and dosage regimen.

On the other hand, the cancer chemopreventive and therapeutic properties of natural flavonoids have become an important topic of investigation [22–24]. One of these compounds is chrysin (5,7-dihydroxyflavone), a natural flavone found in many plant extracts, including blue passion flower (*Passiflora caerulea*) and natural products such as honey and propolis. Chrysin (Chrys) chemical structure shares the common flavone structure, which is composed of fused A and C rings, and a phenyl B ring attached to position 2 of the C ring, with hydroxyl groups at position 5 and 7 of ring A (Fig. 1SM). Chrys has been proposed as a natural bioactive agent for cancer therapy since it has shown cytotoxic activity on a wide variety of tumor cell lines [25]: MCF-7 (breast cancer) [26], HeLa (adenocarcinoma), B16-F0 (melanoma), Hep-G2 (human hepatocyte carcinoma) [27], among others, without producing adverse effects on normal cells [28]. Several studies have shown that the increased Chrys cytotoxicity, in comparison with other analogues could be attributable to two hydroxyl groups in AC flavone rings [29]. Although its bioactive effects are diverse, the medical application of Chrys is limited due to its hydrophobic nature, poor bioavailability, poor permeability, poor water solubility, and its metabolism occurs in a short time period without entering into blood circulation. For this reason, extensive research on Chrys vehiculization strategies is currently performed [28,30–33].

In this framework, the aim of this contribution was to study the formation and characterization of self-assembled BSA nanoparticles (BSAnp) able to solubilize Chrys in an aqueous medium. Some considerations about the safety of the used materials were taken to account in the design of BSAnp. So, effect of heating temperature (70 and 85 °C at 5 min), BSA bulk concentration (1.0–4.0% wt.) and an aqueous medium pH value (9.0 and 11.0) were systematically evaluated. Previous knowledge of our team about production of heat-treated protein nanoparticles [34–39] was applied for rational design BSAnp nanoparticles for Chrys vehiculization, which could be relevant for the design of new antitumor therapies.

## 2. Materials and methods

### 2.1. Materials

BSA lyophilized powder was purchased from Sigma Aldrich (USA) and its purity was ≥96% according to agarose gel electrophoresis. Chrys sample was purchased from Sigma Aldrich (USA) and its purity was ≥96.5% according to HPLC. Analytical grade salts for phosphate buffered saline (PBS) preparation ($Na_2HPO_4$, $KH_2PO_4$, NaCl, KCl) were obtained from Cicarelli (Argentina). Phosphate buffered saline (PBS; 10 mM $Na_2HPO_4$, 2 mM $KH_2PO_4$, 137 mM NaCl, 2.7 mM KCl, pH 7.4) was prepared by dissolving the species in Milli-Q water. Buffer pH value was adjusted at 7.4 by using 0.5 M NaOH, and it was filtered through a 0.45 µm filter. Dimethyl sulfoxide (DMSO) was obtained from Biopack (Argentina). Extrinsic fluorescence probe, 1-anilino-8-naphthalene sulfonic acid (ANS), was purchased from Fluka Chemie AG (Switzerland). Solvents used for chromatographic analysis were purchased from Pharmco Aaper and Sintorgan and were of HPLC grade.

### 2.2. Self-assembled BSA nanoparticles preparation

BSA solutions at different concentrations (1.0–4.0%wt.) were prepared by dissolving BSA lyophilized powder in NaCl 50 mM solution. In order to complete the protein hydration, the solution was stirred for 2 h at room temperature (25 °C) and stored at 4 °C overnight. Subsequently, to eliminate any protein aggregates that could be present, BSA solution was filtered using a cellulose ester filter of 0.45 µm pore size (Millipore, USA). BSA concentration was corroborated by measurement of absorbance at 280 nm, using an extinction coefficient of 43,747 $M^{-1}\,cm^{-1}$ which was experimentally determined. The obtained coefficient was in concordance with the value 43,824 $M^{-1}\,cm^{-1}$ reported by Peters et al [40]. Finally, the pH of each solution was adjusted at 9.0 or 11.0 by using 1 M NaOH.

Self-assembled BSA nanoparticles (BSAnp) were prepared by thermal treatment. For this, 2.0 ml of BSA solution (1.0–4.0% wt.) was dispensed into glass tubes (2 cm of diameter and 15 cm of height) and immersed in a water bath (Dalvo instruments, BTMP model) at two different temperatures: 70 °C and 85 °C over 5 min. According to heating temperature and pH conditions, the BSAnp systems were named as follow: BSAnp-70-9, BSAnp-70-11 (temperature: 70 °C, pH 9.0 and 11.0 respectively), BSAnp-85-9 and BSAnp-85-11 (temperature: 85 °C, pH 9.0 and 11.0 respectively). Treatment temperatures were selected according to previous DSC studies in which it is reported that changes in BSA structure begin up to 65 °C [41,42]. After thermal treatment, tubes were immediately cooled using an ice bath. Native BSA (BSAn) solution without thermal treatment was used as control.

### 2.3. Intrinsic and extrinsic fluorescence spectroscopy

BSA conformational changes induced by thermal treatment conditions were explored by fluorescence spectroscopy at room temperature (25 °C) using spectrophotometer (Hitachi F-2000, Japan) and 1 cm quartz cells. The spectral resolution was 10 nm. For intrinsic fluorescence (due to Trp emission) measurements, an excitation wavelength of 295 nm was used. Intrinsic emission spectra were registered between 310 nm and 400 nm. For extrinsic fluorescence measurements, 15 µl ANS stock solution (8 mM in Milli-Q water) was added to 2.0 ml of sample. The saturation concentration of ANS binding sites in BSA was experimentally achieved in the range of 20–80 µM (Fig. 4SM) thus extrinsic fluorescence experiments were carried out within this range. Then, the extrinsic fluorescence spectra were obtained at 390 nm excitation wavelength between 400 and 600 nm [37–39,43]. Both intrinsic and extrinsic fluorescence intensity values at the maximum emission wavelength (FI) were also expressed in terms of relative fluorescence intensity (RFI), being $RFI = FI/FI_0$, where FI is the intrinsic fluorescence intensity of BSAnp and $FI_0$ corresponds to intrinsic