Colloids and Surfaces B: Biointerfaces 159 (2017) 759–769

Contents lists available at ScienceDirect

Colloids and Surfaces B: Biointerfaces

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

Full Length Article

Nanoparticulation of bovine serum albumin and poly-D-lysine through complex coacervation and encapsulation of curcumin

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ARTICLE INFO

Article history:
Received 25 December 2016
Received in revised form 29 July 2017
Accepted 25 August 2017

Keywords:
Soluble coacervate nanoparticles
Polyelectrolytes
Electrostatic interactions
Coacervation method
DLS
Curcumin encapsulation
SEM
Biopolymers
Colloid nanoparticles

ABSTRACT

Soluble coacervate nanoparticles were fabricated by mixing bovine serum albumin (BSA) and poly-D-lysine with low (LMW-PDL) and high molecular weights (HMW-PDL). The particle size was influenced by molecular weight, mass ratio of polyelectrolytes (PEs), and salt concentration. The smallest nanoparticles had a diameter of 212 ± 11 nm which was achieved with LMW-PDL dissolved with 0.1 M NaCl at pH 7 and a mass ratio of 2.0 (BSA: PDL). SEM images showed that coacervate nanoparticles of LMW-PDL are relatively spherical in shape, while nanoparticles of HMW-PDL were irregular. Crosslinking of the protein/polypeptide with glutaraldehyde had variable impact on the stability and particle size over 21 days at 4 and 25 °C. The encapsulation efficiency (EE) for curcumin to BSA molar ratio of 0.5 was 47%. The EE increased to 60% when the curcumin to BSA molar ratio was 10 with a loading capacity of 22 μg of curcumin per mg of coacervate nanoparticles. The average particle size of the loaded colloidal dispersions increased as the curcumin concentration was increased. For the colloidal dispersions with 0.5 molar ratio of curcumin to BSA, the particle size was around 204 ± 14 nm at day 1, while the nanoparticles with molar ratio of 10 showed a particle size around 316 ± 43 nm. The curcumin loaded BSA:LMW-PDL nanoparticles were pretty stable over a period of 21 days.

1. Introduction

Coacervation is widely used for food, cosmetics and pharmaceutical applications as well as in other fields [1–5]. More recently, complex coacervation has been used for the microencapsulation of nutraceutical bioactives including sweet orange oil, lycopene, turmeric oleoresin and garlic oil making it a technology suitable for the microencapsulation of bioactive compounds [2,5–7]. Coacervation gives rise to a complex between two previously soluble oppositely charged polyelectrolytes (PEs) to form a colloidal dispersion in which the dispersed phase is the coacervate. The most predominant interactions are strong electrostatic interactions [1,2]. Other types of interactions such as hydrophobic interactions, hydrogen bonds, and van der Waals forces can complement electrostatic interactions [3,4].

Two major steps control the formation of complex coacervates: (1) the kinetics of diffusion and entanglement of PEs, which happens at short times and is affected by molar size differences, and (2) thermodynamic reorganization of the previously created aggregates due to conformational changes and disentanglement which happen at relatively long times and causes instability of the coacervates. The second step is a consequence of immiscibility of the polyelectrolytes in one another resulting in phase separation [12]. The polyelectrolyte complex (PEC) forms in less than 5 μs as shown by stop flow measurements, which corresponds to the diffusion and controlled collision of polyion coils [13]. Formation of coacervates is driven by entropy changes which drive the mixing of components as shown by ITC data [14] while enthalpy does not contribute to the molecular interactions during the mixing of polyelectrolytes [15].

Three different types of PEC result in 1) soluble PECs (this occurs when small and soluble polymers are present in a homogeneous system); 2) turbid colloidal dispersion (they are larger polymer molecules mutually insoluble and represent the transition to phase separation); and 3) two phase systems including liquid and precipitated PECs (strong aggregation leads to large non-colloidal particles which phase separate and sediment and therefore is not desirable) [16].

Size, size distribution, shape and morphology of coacervate nanoparticles can be affected by different factors such as zeta potential of the polymers, ionic strength (salt concentration), polymers concentrations and their ratio, molecular conformation and weight, temperature, pH, etc. [8,9,15,17–22,28]. Several studies have shown that increasing ionic strength can improve the dissolution of each polymer and affect the binding affin-

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http://dx.doi.org/10.1016/j.colsurfb.2017.08.047
0927-7765/

ity of PEs [23–26]. Molecular weights (MW) of the PEs can also affect the formation of PECs as well as their stability, since the MW can enhance or suppress efficient ion pairing [24,27]. To evaluate the effect of polyelectrolyte structure and concentration, Müller et al. successfully prepared needle-like and spherical nanoparticles from poly-L-lysine (PLL) and poly (maleic acid-co-propylene), 2) PLL and poly (maleic acid-co-R-methylstyrene), 3) poly-(diallyldimethylammonium chloride) and poly (maleic acid-co-R-methylstyrene), respectively. They reported that stable PEC nanoparticles were formed when the solution concentration was between cPOL = 0.001 and 0.01 mol/L at molar mixing ratio of $n-/n+ = 0.6$. In all PE combinations, increasing the concentrations of polymers (cPOL) led to increase in the hydrodynamic radius and turbidity, suggesting that many layers of PEs were being formed leading to larger aggregates since the collision probability of PEs during coacervation increased.

Coacervate nano- and microparticles can be used to improve the bioavailability and stability of poorly water-soluble bioactive compounds [2,6,28]. PEs can be proteins, carbohydrates or any other organic polymer that can form a colloidal system [25,27,29–31]. An important bioactive compound from food is curcumin (*curcuma longa*) which is found in the herb turmeric and most commonly in curry spice [32]. Curcumin has very low solubility in water at acidic and neutral pH [33,34] because of its hydrophobicity. It has high antioxidant, anti-cancer and anti-inflammatory properties and it has been found to be effective in the treatment of several diseases such as Alzheimer, cancer, and heart failure [33,35,36]. Several approaches have been used to improve its solubility in aqueous solutions including encapsulation in nanoparticles [37,38], coacervates [7,39], liposomes [40,41] and micelles [42,43].

The majority of coacervation literature focused on polysaccharide-protein [2,7,18,26,44], polysaccharide-polysaccharide [20,45–47], protein-protein [48], polypeptide-polyacid [49], protein-polyionic synthetic polymer [11,19,50,51], polysaccharide-polypeptide [52], and polypeptide-polypeptide [53–55] complexation, while there is no published work on the coacervation of protein and polypeptide. Most of the coacervate studies in the literature deal with insoluble coacervates in the micron scale [2,4,6,7,52,56,57]; however there are limited published studies reporting colloidal nanoscale particles mostly fabricated by protein-polysaccharide interactions [12,17,18,58]. Positively charged polypeptides such as poly-D-lysine are protonated in a wide range of pH and they promote cell adhesion properties which can be useful for designing effective delivery systems [59]. Due to D-form peptide bonds, PDL can resist against tryptic proteases. However, it can be hydrolyzed by pancreatic extract to the extent of about 90% (after 5 h incubation) [60], and it may improve the stability of the coacervates in the stomach against trypsin. Singh et al. (2010) also reported that BSA nanoparticles coated by poly-L-lysine can be more resistant to *in vitro* enzymatic digestion.

This research focuses on the interaction of BSA and PDL to form soluble coacervates, and specifically on the effects of MW of PDL, salt concentration, addition of cross-linking agent, and mass/molar ratio of BSA to PDL on the particle size (PS), polydispersity index (PDI), zeta potential (ZP) and morphology of soluble colloidal coacervate nanoparticles, which has never been done before for this particular system. At the same time, the stability of the coacervates is studied for a period of 21 days under room (25 °C) and refrigeration (4 °C) temperature conditions. Most of the coacervates studied in the literature did not report the stability of their particles over extended periods of time. Our studies focused on using dynamic light scattering (DLS) and scanning electron microscopy (SEM) for colloidal particles obtained in the size range of 100–500 nm. In particular, we have identified conditions that favor the formation of coacervate nanoparticles around and below 200 nm. Our motivation to focus on this range is due to the fact that particles can stay in the circulatory system for longer times [61,62]. At the optimal fabrication conditions, where we obtained the smallest coacervate nanoparticle size, we evaluated the encapsulation efficiency and loading capacity of BSA:LMW-PDL coacervates using curcumin as a hydrophobic model at three curcumin to BSA molar ratios.

2. Materials and methods

2.1. Materials

Bovine serum albumin (BSA, lyophilized powder, purity ~98%, ~66 kDa), Poly-D-Lysine hydrobromide (PDL, Mw ≥ 70–150 kDa), Poly-D-Lysine hydrobromide (PDL, Mw ≥ 300 kDa), curcumin from *Curcuma longa* (Turmeric) and Bradford assay reagent were purchased from Sigma-Aldrich. Milli-Q water (18.2 MΩcm) was used in this study. Ethanol, NaCl, NaOH, HCl, Phosphate buffer saline, and glutaraldehyde were analytical grade and used without further purification.

2.2. Size exclusion chromatography (SEC)

The MW distribution for PDLs was determined using Size Exclusion Chromatography (SEC) with a Superdex 200 10/300 GL column (GE Healthcare) in an ÄKTA FPLC system (Amersham Biosciences) at 6 °C. SEC elution was performed with 10 mM PBS, pH 7.15, 100 mM NaCl at a flow rate of 0.2 mL/min and a wavelength of 220 nm. The column was calibrated using protein standards (MWGF1000) from Sigma-Aldrich and covering a MW range from 29 to 669 kDa [63]. A standard curve was generated using the elution times of 6 proteins with known molecular weights. The Log MWs of these 6 standard proteins was plotted against their respective elution time to determine a regression equation that was used to estimate the MW for both PDLs.

2.3. Preparation of polyelectrolyte solutions

The solutions of BSA (1 mg mL^{-1}), low molecular weight (LMW) and high molecular weight (HMW) PDL (0.5 mg mL^{-1}) were prepared in PB (pH 7.0, 10 mM, without NaCl) or PBS (pH 7.0, 10 mM, [NaCl] = 0.1 M) as solvents. BSA was used as the anionic polyelectrolyte and PDL was used as the cationic polyelectrolyte. BSA and PDL solutions were stirred for 1 h at 1200 rpm. The solutions were then stored overnight at 4 °C for completion of biomolecules hydration. To remove any possible large aggregates, before coacervation, PDL and BSA solutions were filtered through 0.45 μm low protein binding syringe filter (Whatman, Maidstone, UK). The protein concentration before and after filtration was determined according to Bradford assay with slight modifications (0.26 ± 0.03% variation) [64,10,11]. A standard curve ($R^2 = 0.9996$) was prepared to determine unknown concentrations of BSA. Briefly, 0.1 mL of the protein solution was mixed with 3 mL of Bradford reagent, vortexed and incubated at ambient temperature for 5 min and then absorbance was measured at 595 nm in a microplate reader (Synergy H1, Biotek, Winooski, VT, USA).

2.4. Fabrication of coacervate nanoparticles

A constant mass of PDL (LMW or HMW) in solution was mixed with different volume ratios of BSA. The starting solution was 1 mL PDL (0.5 mg mL^{-1}), and BSA solution (1.0 mg mL^{-1}) was added dropwise with a flow rate of 0.5 mL min^{-1} using a syringe pump (NE-300, New Era Pump System, Inc., NY, USA). BSA aqueous solution was added in the amounts of 0.15, 0.25, 0.5, 0.75, 1.0, 1.25, or 1.5 mL in order to vary the mass and molar ratios of the two polyelectrolytes. The mass ratio (BSA: PDL) for the mixtures was