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Novel self-aggregates of chitosan oligosaccharide grafted stearic acid: preparation, characterization and protein association

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Received November 15, 2004, accepted March 23, 2005

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Pharmazie 61: 194–198 (2006)

A novel hydrophobically modified chitosan oligosaccharide (CSO) containing 5.4 stearic acid (SA) groups per 100 anhydroglucose units was synthesized by an 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC)-mediated coupling reaction. The purified copolymer was structurally characterized by IR spectroscopy. Characteristics of self-aggregates of the amphiphilic copolymers were studied by fluorescence spectroscopy and dynamic light scattering. The critical aggregation concentration (cac) value of the self-aggregates in aqueous solution was determined by measuring the fluorescence intensity of pyrene as a fluorescent probe. Mean diameter of self-aggregates in pH 7.0 PBS was 25.0 ± 14.7 nm with a unimodal size distribution. The diameter, as well as the zeta potential of self-aggregates increased when the pH value of dispersion medium decreased. Bovine serum albumin (BSA) was further enveloped in the interface of different single self-aggregate and formed nanoparticles. The size of BSA-loaded stearic acid modified CSO nanoparticles depended on the pH values of the dispersed aqueous vehicle, and the size diminished when the pH values of the dispersed aqueous vehicle decreased, whilst, the BSA encapsulation efficiency enhanced. The nanoparticles were characterized by Transmission Electron Microscopy (TEM). BSA release from stearic acid modified CSO nanoparticles decreased when the pH values of the delivery media decreased, in the range from 7.2 to 5.8.

1. Introduction

Over the past several decades, many studies have focused on particulate carrier systems. Particulate carrier systems have received much attention due to various advantages in the versatility of particulate size and surface properties. Polymers in particulate carrier system are employed for various purposes (Yoo et al. 2001). Among them, self-aggregates of hydrophobically modified polymers can influence particulate characteristics such as size distribution, surface charge, and hydrophilic or hydrophobic properties of the surface (Kang et al. 2002). Hence, these polymers are widely employed in the field of pharmaceuticals (Li et al. 2004).

Chitosan has already been widely used both in pharmaceutical research and in industry as a carrier for drug delivery (Mao et al. 2001). Recent reports indicate that chitosan exhibits a dose-dependent blood compatibility and cell viability (Kojima et al. 2001). Such drawbacks of chitosan seem to relate to chemical and physical characteristics such as the deacetylation degree and molecular weight (M_w) (Lee et al. 1995). Thus, chitosan with low molecular weight, namely chitosan oligosaccharide (CSO), has become the focus of investigation (Simon et al. 1999). As reported by other groups (Youjin et al. 2000), CSO is soluble in water and can be obtained by enzymatic degradation of chitosan and ultrafiltration separation. On the other hand, CSO has both reactive amino and hydroxyl groups, thus, many CSO derivatives for pharmaceutical application can be obtained by chemically altering its properties under mild reaction conditions (Jeon and Holl 2003).

In particular, stearic acid (SA), an endogenous long-chain saturated fatty acid (Qiang et al. 2000), is available for pharmaceutical use. As a main composition of fat, stearic acid is biocompatible with low toxicity. In order to choose a better carrier material, a new kind of hydrophobically modified CSO can be prepared by an 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC)-mediated coupling reaction of SA and CSO backbones. Stearic acid modified CSO can yield self-aggregates in aqueous media. Under an acidic environment, stearic acid modified CSO self-aggregates are protonated by the residual amine groups of CSO. The electrostatic interaction drives the formation of nanoparticles between self-aggregates and electronegative macromolecular drugs.

The purposes of the current study are to prepare stearic acid modified CSO and evaluate the feasibility of hydrophobically modified CSO as macromolecular drug carriers. Bovine serum albumin (BSA) was chosen as a model protein. The influences of initial concentration of BSA and pH values of dispersion medium on particle size, zeta potential and encapsulation efficiency were investigated. Moreover, the effect of pH values of release media on the release behavior *in vitro* of BSA from nanoparticles is reported here.

2. Investigations, results and discussion

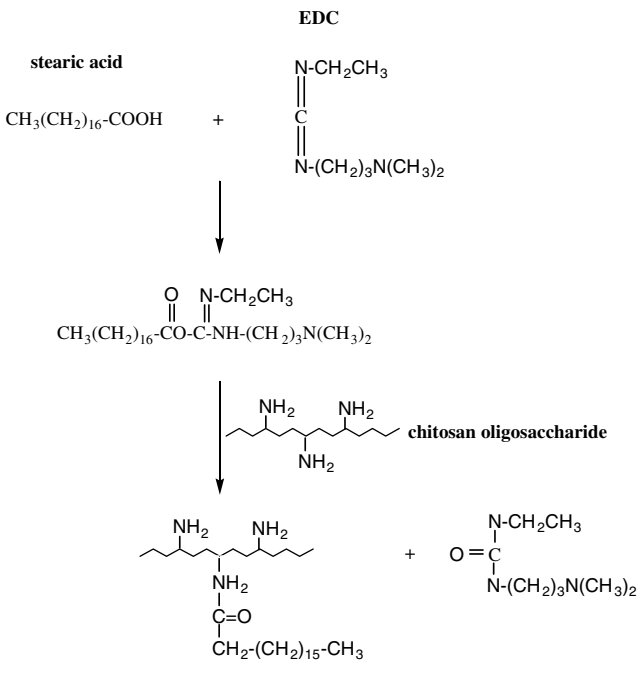
2.1. Molecular weight of CSO

With the elution curves as a control, the CSO molecular weight was obtained by determination of gel permeation chromatography, after hydrolysis and ultrafiltration treatment. The M_w (weight average molecular weight) measured was 22.4 Kda. In this extent of molecular weight, CSO can easily dissolve in the aqueous phase at pH values below 7.4.

2.2. Synthesis of stearic acid modified CSO and IR analysis

The water-soluble EDC is called 'zero-length' cross-linker because the amide linkages are formed without leaving a spacer molecule (Pieper et al. 2000). The Scheme shows the mechanism for this synthesis and the chemical structure of the final graft polymer. EDC reacts with the carboxyl group of stearic acid to form an active ester intermediate, which can further react with a primary amine of CSO to form an amide bond. The by-product, isourea, is easily removed by dialysis with water (Lee et al. 1998).
The formation of an amide bond between stearic acid and CSO is confirmed from the IR spectrum. The amide I band at 1620 cm^{-1} (ν_{CO}) and the amide II band at 1510 cm^{-1} (ν_{CONH}) produced by residual acetamide groups could be observed clearly from the IR spectrum of pure CSO (data not shown). At the sample of stearic acid modified CSO, intensities of amide bands mentioned above dramatically decreased, meanwhile, two new absorption bands at 1640 cm^{-1} (ν_{CO}) and 1560 cm^{-1} (ν_{CONH}) appeared (data not shown), which can be assigned to the absorption peaks of a new amide band from the formation between stearic acid and CSO. The degree of substitution was 5.4 from the determination by 2,4,6-trinitrobenzene sulfonic acid method.

Scheme



stearic acid modified chitosan oligosaccharide

Table 1: Influence of various pH values of dispersion medium on particle size and zeta potential of stearic acid modified chitosan oligosaccharide self-aggregates with a concentration of 1.0 mg/ml

pH of dispersed phase	Size (nm)		Zeta potential (mV)	
	Mean	Width	Mean	Width
2.0	190.8	99.8	46.4	4.1
5.0	45.7	27.2	36.8	1.7
7.0	25.0	14.7	18.7	4.0

(pH value of dispersion medium = 2.0, 5.0, 7.0, T = 25 °C)

2.3. Characteristics of stearic acid modified CSO self-aggregates

The aggregation behavior of stearic acid modified CSO in aqueous media was investigated by fluorometry with pyrene as a fluorescent probe (Morimoto et al. 2003). At a certain concentration, stearic acid modified CSO self-assembled to form a hydrophilic outer shell and a hydrophobic inner core. This concentration is defined as a critical aggregation concentration (cac) meaning the threshold concentration of self-aggregation of amphiphilic polymer, at which the total emission fluorescence intensity increases, especially the intensity of the third highest vibrational band. After measuring the intensity ratio (I_1/I_3) of the first (I_1, 377 nm) and the third (I_3, 395 nm) highest energy band in the emission spectra of pyrene, the cac value can be determined by the intercept of two straight lines in the low concentration region. The cac value of stearic acid modified CSO in distilled water is 6.0×10^{-2} mg/ml, which is lower than the cac of other low molecular weight surfactants in water (Riess et al. 2003).
The size of self-aggregates and their distribution in various PBS solutions were measured by dynamic light scattering. Table 1 shows the influence of pH values on average particle size and zeta potential of stearic acid modified CSO self-aggregates. The mean diameter of stearic acid modified CSO self-aggregates was 25.0 ± 14.7 nm in pH 7.0 PBS and rose to 190.8 ± 99.8 nm in pH 2.0 PBS. The charge density of stearic acid modified CSO increased significantly by the enhancement of the concentration of hydrogen ion. Consequently, the inter- and intra- molecular electrostatic repulsive forces increased, the chain stretched further and the twist of stearic acid modified CSO decreased. Thus, stearic acid modified CSO self-aggregates would form a much looser hydrophobic core. Finally, an increase in the mean size of self-aggregates was observed (Park et al. 2001).
As shown in Table 1, the positive charge of stearic acid modified CSO self-aggregates was in the same range as reported for other chitosan nanoparticles because of the cationic characteristic of chitosan (Cui et al. 2001). Zeta potential of self-aggregates was 18.7 ± 4.0 mV in pH 7.0 PBS and reached 46.4 ± 4.1 mV when the pH value of PBS was declined to 2.0. This phenomenon is explained as the stronger ionization of the free amino groups of stearic acid modified CSO at lower pH values condition.

2.4. Characteristics of BSA-loaded nanoparticles

Table 2 shows the influence of BSA initial concentration on characteristics of BSA-loaded nanoparticles after preparation in distilled water. In distilled water (pH 5.7), the stearic acid modified CSO self-aggregate without BSA has the mean diameter of 31.4 ± 8.7 nm and shows a positive zeta potential (33.4 ± 4.1 mV). The size of BSA loaded

Table 2: Influence of BSA initial concentration on particle size, zeta potential and BSA encapsulation efficiency of BSA-loaded nanoparticles in distilled water

Concentration of BSA	Size (nm)		Zeta potential (mV)		Encapsulation efficiency (%)	
(mg/ml)	Mean	Width	Mean	Width	average	SD (n = 3)
0	31.4	8.7	33.4	4.1	–	–
0.05	136.7	42.7	31.4	4.1	16.87	2.83
0.1	143.7	32.6	29.1	7.9	33.74	3.21
0.2	229.6	62.5	17.8	4.0	40.87	2.58

(pH value of dispersion medium = 5.7, CSO self-aggregates with a concentration of 1.0 mg/ml, T = 25 °C)

nanoparticles increased with the amount of BSA. The average size of BSA-loaded nanoparticles was 136.7 ± 42.7 nm in distilled water when the concentration of BSA was at 0.05 mg/ml in this dispersion system, and reached 229.6 ± 62.5 nm when the initial concentration of BSA rose to 0.2 mg/ml. The zeta potential of nanoparticles decreased from 31.4 ± 4.1 mV to 17.8 ± 4.0 mV when BSA concentration increased from 0.05 mg/ml to 0.2 mg/ml. BSA molecule is an anionic protein when it dissolved in distilled water or in pH 5.8, 6.5, 7.2 PBS due to the isoelectric point value of BSA is pH 4.7. When BSA is added into the dispersion of stearic acid modified CSO self-aggregates, it can interact strongly with $[NH_3]^+$ groups of stearic acid modified CSO self-aggregates through static electricity conjugation. When different amounts of BSA are mixed with stearic acid modified CSO self-aggregates, the electrostatic interaction drives the formation of complexes and the size is in the nanometer range, so the complexes may be called "nanoparticles". If one stearic acid modified CSO self-aggregate integrates one BSA molecule by static electricity conjugation, the size of BSA-loaded particles would not increase so rapidly. As the zeta potential shows a positive value there are more BSA molecules integrating to the surface of one stearic acid modified CSO self-aggregate, and forming multi-layer BSA molecules around the stearic acid modified CSO self-aggregate with the enhanced the BSA concentration in the system.

Accompanying the enhancement of BSA concentration in stearic acid modified CSO self-aggregates, BSA encapsulation efficiency shows a distinct increase, while the size of nanoparticles also increased. When the initial BSA concentration increased from 0.05 to 0.2 mg/ml, the BSA encapsulation efficiency was enhanced significantly from 16.87 ± 2.83% to 40.87 ± 2.58%.

With the low concentration, BSA molecules may only be adsorbed on the surface of single self-aggregates by electrostatic interaction. At this time, the BSA loaded self-aggregate is already larger compared with the BSA-free self-aggregate. The number of BSA molecules that can be loaded on each self-aggregate is limited by the dimension of the interface and relative large volume of BSA compared with the self-aggregate itself. When the BSA concentration is increased continuously, more and more single BSA-loaded self-aggregates may assemble by the negative charge of BSA and positive charge of self-aggregate, forming much larger congregated nanoparticles, and more BSA molecules are enveloped in the interface of different single self-aggregate. These different single self-aggregates are forming the final nanoparticles. Sequentially, the BSA encapsulation efficiency and particle size of nanoparticles are enhanced at the same time.

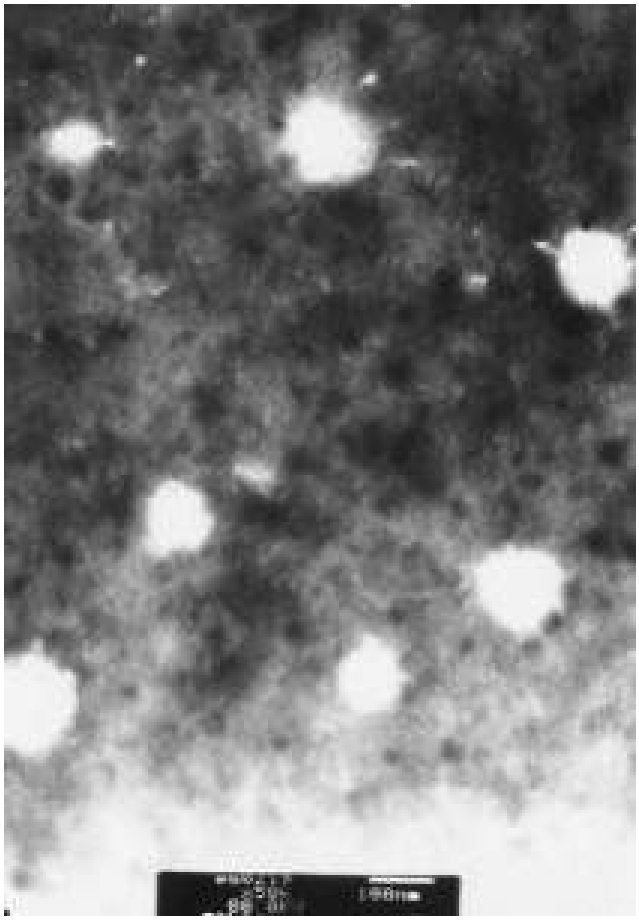


Fig. 1: Transmission electron microscopic photograph of BSA-loaded stearic acid modified CSO nanoparticles in distilled water with BSA initial concentration of 0.1mg/ml (× 50,000, bar: 100 nm)

When the BSA concentration was up to 0.2 mg/ml, the congregation of nanoparticles was observed 0.5 h after preparation under room temperature and a turbid suspension was formed. Therefore, the initial concentration of BSA was selected at 0.1 mg/ml in the latter study.

2.5. Transmission electron microscopy (TEM) of BSA-loaded nanoparticles

Fig. 1 shows transmission electron microscopy of BSA-loaded nanoparticles prepared in distilled water with BSA initial concentration 0.1 mg/ml. The nanoparticles have near spherical shape and own smooth surface. The particle sizes determined by TEM was similar to the results from a zetasizer.

2.6. In vitro release

The effect of pH values of dispersion medium on stearic acid modified CSO nanoparticles loaded with BSA during preparation is shown in Table 3. When pH values of the dispersion medium declined from 7.2 to 5.8, the size of nanoparticles decreased, while the zeta potential and BSA encapsulation efficiency increased.

The BSA-loaded nanoparticles differ from the BSA-free self-aggregates in size distribution under different pH values of dispersion medium. BSA-loaded nanoparticles, formed by the congregation of different single self-aggregates, with a relatively low zeta potential, are easily affected by the degree of protonation of the amide groups

Table 3: Effect of various pH values of dispersion medium on particle size, zeta potential and BSA encapsulation efficiency with a BSA initial concentration of 0.1 mg/ml

pH	Size (nm)		Zeta potential (mV)		Encapsulation efficiency (%)	
	Mean	Width	Mean	Width	average	SD (n = 3)
7.2	220.7	127.4	7.8	1.6	24.27	3.69
6.5	181.7	111.0	16.6	4.0	37.33	3.07
5.8	163.0	70.3	23.6	3.0	39.20	1.88

(CSO self-aggregates with a concentration of 1.0 mg/ml, T = 25 °C)

under the various pH values of the dispersion medium. Decreasing the pH values of the dispersion medium, means increasing the protonation degree of amide groups, and the formation of BSA-loaded nanoparticles will be tighter. Therefore, smaller nanoparticles are produced, and zeta potentials of the system as well as BSA encapsulation efficiency are increased.

The *in vitro* BSA release behavior of re-suspended BSA-loaded nanoparticles, which were originally prepared in distilled water with a BSA initial concentration 0.1 mg/ml in release media at different pH values (pH 7.2, 6.5, 5.8 PBS), are shown in Fig. 2. A burst release was observed at the initial 0.5 h and released nearly 30% of the BSA from the nanoparticles. After that, a slow release at various pH values of release media with different rates was obtained. The release rate was delayed when the pH values of release media was decreased from 7.2 to 5.8. Thus, the release rate varied from a relatively rapid rate to a relatively slow rate correspondingly.

The burst release of BSA from stearic acid modified CSO self-aggregate nanoparticles demonstrate that the partial of BSA molecules may envelop in the interface of different single BSA-loaded self-aggregates. Later, the sustained release of BSA from stearic acid modified CSO self-aggregate nanoparticles may be due to the partial of BSA molecules conjugated on the surface of the stearic acid modified CSO self-aggregates.

As shown in Table 3, under an acidic condition such as pH 5.8, a significant enhancement of the charge density of $[H]^+$ induces increasing electrostatic repulsive forces between $[H]^+$ and $[NH_3]^+$. More $[NH_3]^+$ facilitates the electrostatic interaction of BSA molecules and stearic acid modified CSO self-aggregates, leading to a small size of BSA-loaded nanoparticles and a slow release profile of BSA from nanoparticles.

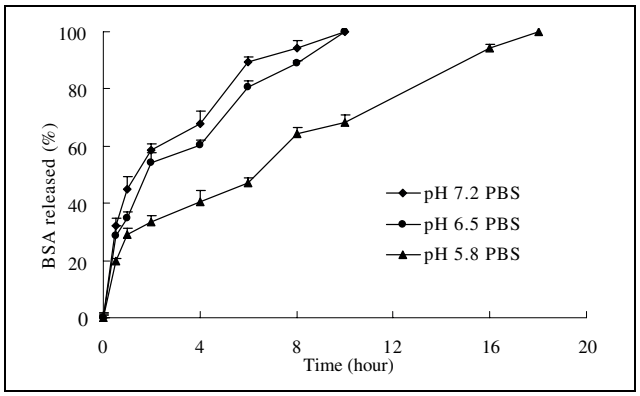


Fig. 2: Effect of pH of release media on BSA release profiles in vitro (pH value of release media = 7.2, 6.5, 5.8, T = 37 °C)

3. Experimental

3.1. Materials

Biomedical grade chitosan ($M_w = 4.5 \times 10^5$ Kda, deacetylation degree = 95%) was obtained from Yuhuan Marine biochemistry Co., Ltd, China. Chitosanase was purchased from Chemical Industries Co., Ltd, Japan. Stearic acid was supplied from Shanghai Chemical Reagent Co., Ltd, China. Polysaccharide (Part Number: 2090-0100) with different molecular weight (M_w = 5.9, 11.8, 22.8, 47.3, 112, 212 Kda) was purchased from Polymer Laboratories Co., Ltd, USA. 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) was purchased from Sigma (St. Louis, MO USA). Bovine serum albumin ($M_w = 6.8 \times 10^4$ Kda) was supplied from Huamei biochemistry Co., Ltd, China. Coomassie brilliant blue (G250) was obtained from Huadong medicine Co., Ltd, China. Ethanol and other chemicals were of analytical grade and used as purchased without further purification.

3.2. Enzymatic degradation of chitosan and determination of molecular weight

3.2.1. Enzymatic degradation of chitosan

A 3% chitosan solution was prepared by dispersing 15 g of chitosan in 500 ml of distilled water, dissolving it by stirring after adding 6.25 ml of 36.5% (w/v) hydrochloric acid. The temperature for hydrolysis of chitosan was controlled at 50 °C in a batch reactor. The reaction mixture containing 1 U/ml chitosanase was incubated for an appropriate time at this temperature. After enzymatic hydrolysis, the reaction mixture was centrifuged for 10 min at 4000 r/min. The supernatant was filtered through a 0.45 μm filter, and the filtrate was ultrafiltrated using two different molecular weight cut off (NMWCO, 10 and 50 Kda) ultrafiltration membranes (Millipore Labscale TFF system, Millipore Co., USA). Then, the ultrafiltrate was lyophilized.

3.2.2. Determination of molecular weight of CSO

The molecular weight of CSO was determined by gel permeation chromatography with a TSK-gel column (G3000SW, 7.5 mm I.D. × 30 cm) at 25 °C. The weighed lyophilized powder of CSO was dissolved in ultrafiltrated water to a final concentration of 1.0 mg/ml. Then, 10 μl of the sample was chromatographed using 0.1 M NaAc (pH6.0) as the elution buffer with a flow rate of 0.8 ml/min. The CSO were detected by monitoring the refractive index.

Master samples of polysaccharide with different molecular weight (M_w = 5.9, 11.8, 22.8, 47.3, 112, 212 Kda) were dissolved in ultrafiltrated water, and their final concentration was 0.5 mg/ml. Calibration was performed by means of polysaccharide samples using the integral molecular weight distribution method.

3.3. Synthesis and purification of stearic acid modified CSO and preparation of self-aggregates

EDC (10 mol/mol SA) was added to a 2% CSO solution under mechanical stirring at room temperature. The mixture was heated to 90 °C under vigorous stirring followed by the dropwise addition of 0.5% SA (10 mol/mol CSO) alcohol solution. After 5 h at 90 °C, the reaction mixture was cooled to room temperature and reaction proceeded for further 24 h under stirring. After copolymerization, the obtained copolymers were dried in a vacuum oven at 50 °C. The copolymers were then subjected to an additional purification process. For this purpose, the copolymers were precipitated by dispersing in 20 ml ethyl alcohol. The suspensions were filtered with 0.22 μm millipore filter and washed with 10 ml ethyl alcohol twice. It was shown in a pre-experiment that stearic acid modified CSO could be dissolved in pH = 12 aqueous vehicle, while CSO could not. Thus, the precipitate was further purified by dissolving in 20 ml aqueous vehicle (pH = 12, adjusted by 0.1 M sodium hydroxide solution) and followed by removing the remaining CSO with a 0.22 μm millipore filter. The purified copolymer was extensively dialyzed (MWCO: 6 ~ 10 Kda, Spectrum Laboratories, Laguna Hills, CA) against distilled water for 24 h with successive exchange of fresh distilled water in order to remove by-products. The aqueous product solution was lyophilized. The degree of substitution, defined as the number of stearic acid groups per 100 anhydroglucose units of CSO, was determined by the 2,4,6-trinitrobenzene sulfonic acid method (Kaya et al. 2001).

IR spectra were measured by a 460-type spectrometer (Shimudza Co., Japan) under dry air at room temperature to determine the chemical interaction between CSO and stearic acid. The stearic acid modified CSO powder was dispersed in PBS with different pH values (pH 2.0, 5.0, 7.0) or distilled water (pH 5.7) under gentle shaking at 37 °C for 24 h, followed by sonication using a probe-type sonicator (Scientz Biotechnology Co., Ltd, China, JY92-II) at 100 W for 20 times (active every 1 second for a 2 second duration in ice-bath). The initial concentration of stearic acid modified CSO solution was fixed at 1.0 mg/ml.

3.4. Determination of critical aggregation concentration (cac) of stearic acid modified CSO

An aqueous solution of stearic acid modified CSO self-aggregates which contains 6.0×10^{-7} M of pyrene was placed in a 1.0×1.0 cm square cell and the fluorescence spectrum was obtained with a HITACHI F-4000 fluorimeter (HITACHI Co., Japan). The concentration of sample solutions varied from 2.5×10^{-4} to 2.5 mg/ml. The slit openings were set at 5 nm (excitation) and 5 nm (emission). The excitation wavelength (λ_{ex}) was 336 nm and the spectra were accumulated with an integration time of 5 s/nm. The intensity ratio was calculated from the first (377 nm) and the third (395 nm) highest energy bands in the pyrene emission spectra.

3.5. Preparation and characteristics of BSA-loaded nanoparticles

The BSA-loaded nanoparticles were formed by dispersing BSA (BSA initial concentrations of 0.05, 0.1, 0.2 mg/ml) in stearic acid modified CSO self-aggregates aqueous solution followed by incubation at 37 °C for 30 min. Nanoparticles were also prepared as described above by dispersing BSA in PBS (pH = 7.2, 6.5, 5.8) with a BSA initial concentration of 0.1 mg/ml.

The mean diameter and zeta potential of stearic acid modified CSO self-aggregates and its BSA-loaded nanoparticles were determined with a zetasizer (3000HS, Malvern Instruments Ltd, UK). Samples are first treated with sonication by a probe-type sonicator (Scientz Biotechnology Co., Ltd, China, JY92-II) at 100 W for 20 times (active every 1 second for a 2 second duration in ice-bath) and diluted 20 times with the original dispersion medium to get the appropriate sample concentration.

3.6. Transmission electron microscopy (TEM) of BSA-loaded nanoparticles

About 5 μl BSA-loaded nanoparticles prepared in distilled water with a BSA concentration 0.1 mg/ml were placed on a copper grid and negatively stained by phosphotungstic acid. TEM observation was carried out on a Phillips TECNA10 (TECNA10, PHILIPS Co., Holland) operated at an accelerating voltage of 80 kV.

3.7. Determination of BSA encapsulation efficiency

BSA-loaded nanoparticle suspension was placed in a 10 ml ultracentrifugation tube and centrifuged for 30 min at 25,000 × g (3K30, SIGMA Laborzentrifugen GmbH, Germany). The unloaded BSA in supernatant was determined by UV spectrophotometry at 595 nm using Coomassie brilliant blue G250 (Cao et al. 2002). The BSA encapsulation efficiency in the nanoparticles was calculated from Eq. (1).

BSA encapsulation efficiency (%) = (weight of BSA added in system − analyzed weight of BSA in supernatant) × 100%/weight of BSA added to the system (1)

3.8. BSA release from the nanoparticles in vitro

The original precipitate of BSA-loaded nanoparticles originally prepared in distilled water with BSA initial concentration 0.1 mg/ml was re-suspended in 30 ml PBS (pH = 7.2, 6.5, 5.8, in 50 ml appropriate glass test-tube) and sonicated (Sonic Purger CQ250, Academy of Shanghai Shipping Electric Instrument) in water bath at room temperature for 5 min and then shaken horizontally (Incubator Shaker HZ-88125, Hualida Laboratory Equipment Company, China) at 37 °C and 60 strokes per min. One milliliter of the suspension was withdrawn from the system at each time interval and centrifuged for 30 min at 25,000 × g (3K30, SIGMA Labrorzentrifugen GmbH, Germany). The unloaded BSA in the supernatant was determined by UV spectrophotometry as described above.

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