

MULTILAYERS AND POLY(ALLYLAMINE HYDROCHLORIDE)-GRAFT-POLY(ETHYLENE GLYCOL) MODIFIED BOVINE SERUM ALBUMIN NANOPARTICLES: IMPROVED STABILITY AND pH-RESPONSIVE DRUG DELIVERY*

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Abstract To improve the colloidal stability of bovine serum albumin (BSA) nanoparticles (NPs) in diverse mediums, poly(allylamine hydrochloride) (PAH)/sodium poly(4-styrene sulfonate) (PSS) multilayers and poly(allylamine hydrochloride)-graft-poly(ethylene glycol) (PAH-g-PEG) coating were coated on the surface of BSA NPs. Stabilities of the BSA NPs in diverse mediums with different surfaces were detected by dynamic light scattering (DLS). Multilayers and PAH-g-PEG coated BSA NPs can be well dispersed in various mediums with a narrow polydispersity index (PDI). The BSA NPs with the highest surface density of PEG show the best stability. The multilayers and PAH-g-PEG coating do not deter the pH-dependent loading and release property of BSA NPs. At pH 9, the encapsulation efficiency of doxorubicin reaches almost 99%, and the release rate at pH 5.5 is significantly higher than that at pH 7.4.

Keywords: BSA nanoparticle; PEG; LBL; Drug delivery; pH-responsive.

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INTRODUCTION

Albumin, especially bovine serum albumin (BSA) and human serum albumin (HSA), is universally employed as a vehicle of molecular probe^[1, 2] and drug carrier^[3, 4] for cancer diagnostics^[1, 5] and therapy^[6], due to its excellent biocompatibility and biodegradability. The first commercial product based on albumin NPs in oncology is the 130 nm albumin-bound paclitaxel approved by the Food and Drug Administration (FDA) of USA in 2005^[7]. The NPs are prepared by high-pressure homogenization of paclitaxel in the presence of HSA^[8], but this method is incapable of loading hydrophilic drugs. Since 2000, desolvation procedure has been systematically characterized and optimized to provide a rational basis and a simple method for producing albumin NPs^[9, 10]. In the presence of drugs^[3, 11] or other polymers^[4], addition of poor solvent such as ethanol causes gradual precipitation of albumin, resulting in stable drug loading albumin NPs after crosslinking. However, the encapsulation efficiency (EE) of this method is limited by preparation conditions and the interaction between drug and albumin. The suspension stability of albumin NPs is also affected by the presence of drug. Moreover, the particles lack functions, such as stealth function and specific targeting ability, which can be fulfilled by

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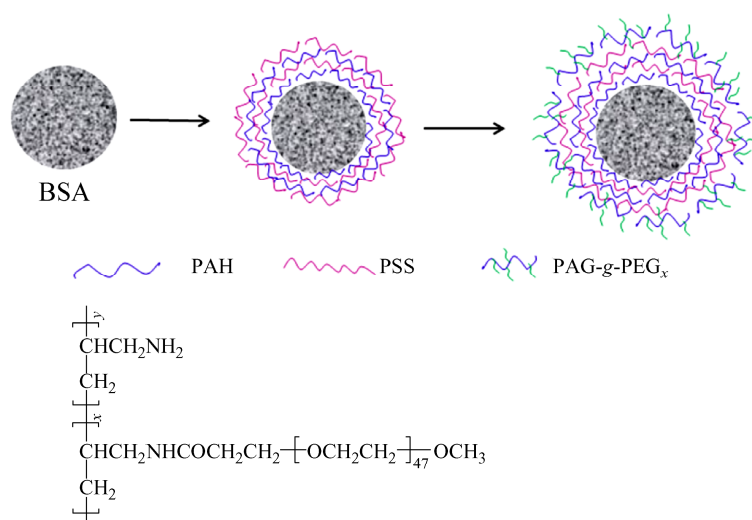
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successive surface modification.

Layer-by-layer (LBL) assembly, a technology introduced by Decher *et al.* in 1991^[12], is prevalent in surface modification^[13, 14] by offering an endless variety of materials and hence the preparation of tailor-made surfaces. Recently, many reports have shown that polyelectrolytes can be assembled in a LBL manner on various NPs such as gold NPs^[15, 16] and PLGA NPs^[17] to endow them with multi-properties such as uniform surface charge density, numerous active groups and targeting property. Poly(ethylene glycol) (PEG), with a highly flexible hydrous structure^[18], can enhance the stability and biocompatibility of substance, such as metal and metal oxide^[19–21], micelles^[22], microcapsules^[23] and hydrogels^[24] by forming a protective hydrophilic layer on the surface. These encouraging results confirm that the coating of polyelectrolyte multilayers and PEG represents a promising platform for a drug delivery system, which can simultaneously provide multiple functions and excellent stability.

In this work, BSA NPs are fabricated by a desolvation and crosslinking method and further coated with poly(allylamine hydrochloride) (PAH)/sodium poly(4-styrene sulfonate) (PSS) multilayers, onto which a layer of poly(allylamine hydrochloride)-graft-poly(ethylene glycol) (PAH-g-PEG) is adsorbed to increase the stability of BSA NPs in diverse mediums (Scheme 1). Through the electrostatic interaction positively charged doxorubicin (DOX) is loaded into the NPs and the encapsulation efficiency (EE) and release behavior of DOX are measured at different pH. The modification process is monitored by ζ -potential measurements, and the stability of NPs with different outmost layers in diverse mediums is investigated by dynamic light scattering (DLS).



Scheme 1 BSA NPs modified with multilayers and PAH-g-PEG_x and molecular structure of the PAH-g-PEG_x copolymer (shown in its uncharged form)
 x and y represent the allylamine units either conjugate with PEG or not, respectively.

EXPERIMENTAL

Materials

Bovine serum albumin (BSA, 98%), sodium poly(4-styrene sulfonate) (PSS, M_w 70 kDa) and poly(allylamine hydrochloride) (PAH, M_w 15 kDa) were purchased from Sigma-Aldrich. Glutaraldehyde (GA, 25% water solution) was obtained from Shanghai Chemical Reagent Co., Ltd. Doxorubicin hydrochloride for injection use (DOX·HCl, 10 mg per dose) was obtained from Zhejiang Hisun Pharmaceutical Co., Ltd. Monomethoxy poly(ethylene glycol) succinimido carbonate (mPEG-SC) with a molecular weight of 2 kDa was purchased from PegBio Co., Ltd., Suzhou, China. Other chemicals were locally serviced. All the chemicals were used without further purification.

Synthesis of Poly(allylamine hydrochloride)-graft-Poly(ethylene glycol) (PAH-g-PEG)

In this study, the abbreviation of PAH-g-PEG_x represents PAH (M_w 15 kDa) grafted with PEG (M_w 2 kDa) with a grafting percentage of $x\%$ (number of allylamine grafted with PEG chain/number of allylamine unit $\times 100\%$). The PAH-g-PEG_x was synthesized according to a method reported previously^[25]. Briefly, 40 mg PAH was dissolved in a sodium tetraborate buffer solution (50 mmol/L, pH 8.5) to reach a concentration of 100 mmol/L allylamine monomer unit. After 45 mg mPEG-SC was added, the mixture solution was stirred for 6 h at room temperature. To label PAH-g-PEG, 1.6 mg fluorescein isothiocyanate (FITC, mole ratio of PAH to FITC was 1:1.5) was optionally added. The reaction mixture was dialyzed in a bag (Spectra-Por, cutoff molecular weight 8–12 kDa, Spectrum Laboratories, Inc., USA) for 4 days against Milli-Q water by changing the water every 24 h. The product was freeze-dried and stored at room temperature before use. Characterization was performed by ¹H-NMR spectroscopy, using D₂O as a solvent on a 500 MHz Bruker instrument. The characteristic peaks were found at $\delta = 3.4\text{--}3.8$ ($-\text{CH}_2\text{CH}_2\text{O}-$ of PEG) and $\delta = 2.6\text{--}3.2$ ($-\text{CH}_2-\text{N}-$ of PAH). The area of the PEG peaks (60.6) was compared with the area of the allylamine side-chain peak (7.2) to determine the graft ratio of the comb copolymer (Fig. S1). The grafting ratio of 9% was determined from the NMR spectra^[25]. Thus the polymer is named as PAH-g-PEG₉. Similarly, PAH-g-PEG_{15.4} and PAH-g-PEG_{25.6} were synthesized through the same protocol by adding 91 and 182 mg mPEG-SC in the PAH solution, respectively (Table 1).

Table 1. Grafting ratio of the PEG determined by ¹H-NMR and the adsorption amount of PAH-g-PEG_x on BSA quantified by fluorescence spectroscopy ($n = 3$)

Grafting ratio	Weight of PAH (mg)	Weight of PEG-SC (mg)	Adsorption amount of PAH-g-PEG _x (μg/mg)	Grafting density of PEG on NPs surface (μg/mg)
PAH-g-PEG ₉	40	45	1.9 ± 0.3	1.0 ± 0.2
PAH-g-PEG _{15.4}	40	91	3.9 ± 0.7	2.6 ± 0.5
PAH-g-PEG _{25.6}	40	182	5.7 ± 2.41	4.4 ± 2.1

Preparation of BSA NPs

BSA nanoparticles (NPs) were prepared by a desolvation technique as described previously^[10]. Briefly, 200 mg BSA was dissolved in 2.0 mL Milli-Q water, and the pH was adjusted to 7.4 with 0.01 mol/L NaOH. Under constant stirring at 500 r/min, 8.0 mL ethanol was continuously added with a rate of 0.5 mL/min using a peristaltic pump. 200 μL of 8% glutaraldehyde solution was added gradually to crosslink the formed BSA particles during desolvation. After 24 h incubation at 20°C under constant stirring, the NPs were purified by repeated centrifugation at 10000 r/min and re-dispersed in Milli-Q water under the assistance of ultrasonication. The BSA NPs were finally freeze-dried and stored at 4°C before use.

BSA NPs Coated with Multilayers

The BSA NPs were coated with PAH/PSS multilayers to activate their surface. The polyelectrolytes (PEs) were dissolved in 1 mol/L NaCl solution to reach a final concentration of 2 mg/mL. 0.5 mL PAH solution was added into 1 mL BSA NPs suspension (5 mg/mL). After being sonicated for 30 s and followed by incubation for 20 min, the suspension was centrifuged at 10000 r/min for 10 min. 3 washings were carried out before adsorption of PSS. The assembly was repeated for 2 cycles to obtain the BSA@(PAH/PSS)₂ NPs. To adsorb the PAH-g-PEG (or FITC labeled PAH-g-PEG), 0.5 mL of 1 mg/mL PAH-g-PEG (or FITC labeled PAH-g-PEG) solution was added into 1 mL of 1 mg/mL BSA@(PAH/PSS)₂ NPs suspension. Following the same incubation and washing protocols, the PEG modified NPs were obtained.

Characterizations of BSA NPs

The particle size and zeta potential were measured by a zeta potential and particle size analyzer (Beckman Coulter DelsaTM Nano Z). The NPs were dispersed in water at room temperature in triplicates for test. To study the colloidal stability, the particles with different surface chemistries were also dispersed in diverse mediums. Transmission electron microscopy (TEM) images were recorded on a Philips CM 120 electron microscope at an accelerating voltage of 80 kV. Samples were prepared by dropping the particles suspension onto a carbon-coated

copper grid, followed by removal of excess solution by blotting the grid with a filter paper. Amount of PAH-g-PEG adsorbed was quantified by fluorescence spectroscopy (LS55, PerkinElmer, USA) by comparing intensity of the FITC labeled PAH-g-PEG solution before and after adsorption.

Drug Loading and Release

5 mg BSA NPs or multilayers modified BSA NPs were dispersed in 1 mL buffer solution at different pH, and mixed with 1 mL 0.5 mg/mL DOX solution at room temperature. After overnight incubation, the solution was centrifuged at 10000 r/min for 10 min, followed by rinsing twice to remove the free DOX. The supernatant was used to quantify the free DOX by UV-Vis spectroscopy (UV2550, Shimadzu) at 480 nm according to a calibration curve.

The DOX drug loading percentage (LR) and encapsulation efficiency (EE) were calculated according to:

$$LR = \frac{w_{\text{total DOX}} - w_{\text{free DOX}}}{w_{\text{DOX-loaded BSA nanoparticle}}} \times 100\%, \quad EE = \frac{w_{\text{total DOX}} - w_{\text{free DOX}}}{w_{\text{total DOX}}} \times 100\%$$

where w is the weight of corresponding substance.

The release of DOX from different particles was studied at 37°C in PBS of different pH values. In a typical experiment, 5 mg of DOX containing NPs were dispersed in 0.5 mL PBS. Then the solution was transferred to a dialysis bag with a cutoff Mw of 8–14 kDa, which was immersed in 50 mL of pH 5.5 or pH 7.4 PBS at 37°C. At desired time intervals, 1 mL of the incubation medium was taken out for UV-Vis measurement, and 1 mL fresh PBS was supplemented. The results were averaged from triplicate experiments.

RESULTS AND DISCUSSION

Preparation and Surface Modification of BSA NPs

BSA NPs were prepared by a desolvation and crosslinking method^[10], the NPs size was influenced by many factors, such as the ratio of ethanol to BSA, the adding rate of ethanol, pH values of the HSA solution and so on. Figure 1(a) shows that BSA NPs had a spherical shape and a size of 160–180 nm. The multilayers modified BSA nanoparticles had a similar size as the unmodified ones (Fig. 1b). The magnified images (insets) show no obvious difference in surface morphology after surface modification. The LBL modification process was monitored by ζ -potential measurements. As expected, with different electrolyte as the top layer, the surface ζ -potential of BSA nanoparticles changed alternately: bare BSA was negatively charged as -36 mV, PAH adsorbed NPs was positively charged as $+50$ mV, and PSS adsorbed NPs was negatively charged as -40 mV (Fig. 2).

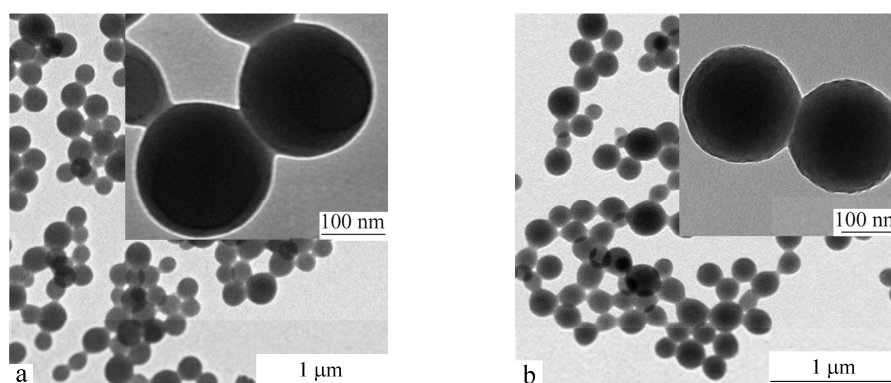


Fig. 1 TEM images of BSA nanoparticles (a) and BSA NPs coated with (PAH/PSS)₂ multilayers (b). The insets are the corresponding magnified images.

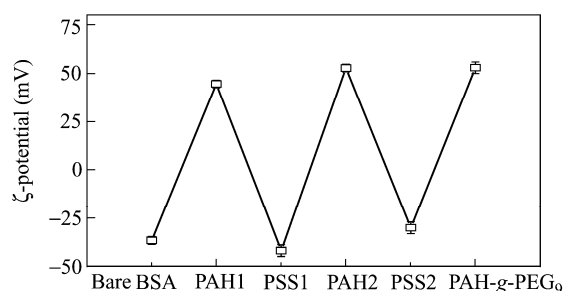


Fig. 2 Changes of ζ -potential during the assembly of multilayers on BSA nanoparticles ($n = 3$)
The last point corresponds to the layer of PAH-g-PEG with grafting density $x = 9$.

With PEG (PAH-g-PEG_x)s of different grafting ratios as the top layer, the surface ζ -potentials were similar to that of the particles with PAH as the outmost layer. But the amount of adsorbed substance increases with the increase of PEG grafting ratio, resulting in the increased PEG density on the surface (Table 1). The PAH grafted with a higher density of PEG had a lower positive charge density. Therefore, more molecules are needed to compensate the negatively charged PSS surface, leading to the increase of adsorbed mass.

Stability of NPs in Diverse Mediums

With different polymers as the top layer, the BSA NPs show different stabilities in different mediums such as H₂O, PBS and growth medium RPMI 1640/10% FBS (Table 2). In pure water, all kinds of NPs are well dispersed with a narrow polydispersity index (PDI) except those with PAH as the outmost layer. Due to the weak polyelectrolyte and relatively stronger hydrophobicity natures of PAH, aggregation of NPs with PAH as the outermost layer should easily occur and thereby induces the increase of the hydrodynamic diameter. The hydrodynamic diameters of BSA NPs with PAH-g-PEG as the top layer were at least 50 nm larger than NPs with PSS as the outermost layer. Along with increase of the grafting density of PEG, the diameter of NPs becomes larger, likely due to the highly hydrated PEG chains. To some degree, the ionic strength of PBS is strong enough to destroy the charge balance of BSA NPs and PAH/PSS multilayers coated BSA NPs, leading to the aggregation of these NPs. Similar to PBS, in physiological saline, except the BSA NPs with PAH-g-PEG as the outmost layer, the NPs aggregated to various degrees. The NPs with PAH-g-PEG as the outmost layer exhibited excellent suspension stability in different mediums, since the flexible PEG chains outside can repel the particle aggregation^[18] to some extent due to steric effect. The PAH-g-PEG_{25.6} modified NPs show the best colloidal stability in PBS, whose size is comparable with that in water. The PEG grafting ratio of PAH-g-PEG_{25.6} is 3 times as that of PAH-g-PEG₉, conveying a same grafting ratio of PEG on the NPs surface (Table 1). The higher grafting ratio of PEG on the surface is, the more stable the NPs will be^[19].

Table 2. Size of BSA NPs with different top layers in different mediums determined by DLS

Top layer	Particle size (nm) [*]		
	H ₂ O	PBS	RPMI1640/10% FBS
BSA	214 (0.06)	634 (0.26)	247 (0.18)
PSS	222 (0.10)	Agglomeration	487 (0.21)
PAH	699 (0.19)	Agglomeration	443 (0.24)
PAH-g-PEG ₉	267 (0.18)	435 (0.19)	267 (0.19)
PAH-g-PEG _{15.4}	280 (0.11)	314 (0.08)	273 (0.18)
PAH-g-PEG _{25.6}	290 (0.11)	284 (0.08)	253 (0.23)

^{*}Data in bracket are polydispersity index (PDI).

Loading and Release of DOX

BSA has many carboxylic groups, which can entrap hydrophilic positive drugs, such as doxorubicin (DOX) through electrostatic interaction. The optimized conditions for the loading of DOX were investigated. At a fixed ionic strength (IS) (50 mmol/L NaCl) and weight ratio of BSA to DOX (10:1), the DOX encapsulation

efficiency (EE) and loading percentage (LR) of BSA NPs and multilayers coated BSA NPs increase along with the increase of medium pH (Fig. 3). At pH 5.5, the NPs with PAH as the outmost layer lose the ability of DOX encapsulation, while the BSA NPs with PAH-g-PEG_{25.6} as the outmost layer sustained the encapsulating capacity as BSA NPs. At pH 9, the DOX EE and LR of all the NPs reached 99% and 9%, respectively.

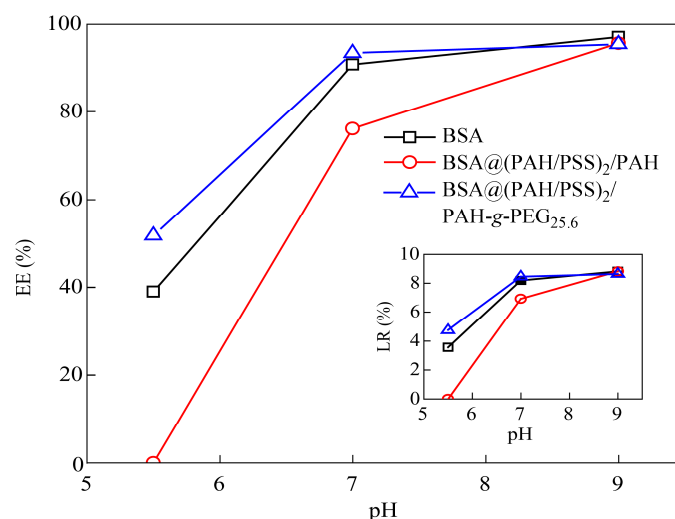


Fig. 3 DOX encapsulation efficiency (EE) and loading percentage (LR) of BSA, BSA@((PAH/PSS)₂)/PAH and BSA@((PAH/PSS)₂)/PAH-g-PEG_{25.6} nanoparticles at different pH. Weight ratio of BSA to DOX = 10:1; IS = 50 mmol/L.

The charging degrees of carboxylic groups and amino groups are greatly influenced by pH. At pH 5.5, the PAH surface possesses high positive charge and can repel absorption of positive DOX into the BSA NPs. By contrast, PEG grafting on the PAH decreases this repulsion of PAH. And the PAH-g-PEG_{25.6} may form more loose structure with PSS compared with PAH, which can facilitate the diffusion of DOX into BSA. Thus DOX can be encapsulated in BSA NPs with PAH-g-PEG_{25.6} as the outmost layer. However, many carboxylic groups in BSA NPs are protonated at pH 5.5, weakening the EE and LR of DOX. With the increase of pH, more negative charges are created in the BSA NPs while the repulsion between DOX and surface positive charges decreases, resulting in enhanced EE and LR. At the optimized condition (pH = 9), the surface modification has negligible influence on the EE and LR, which are close to the theoretical maximal values.

The loaded DOX can be sustainably released as shown in Fig. 4. Resulting from the large drug concentration difference between the particle interiors and the bulk solution at the initial stage, DOX was released fast. The release was leveled off after about 20 h. Compared with the influence of the solution pH, different surface coatings do not affect BSA NPs release behaviors significantly. For example, at pH 5.5 the released amount reached more than 50% within 6 h, and eventually reached to 60%–80% after 48 h, depending on the surface coating. By contrast, at pH 7.4 only 10% DOX was released within 6 h, and the equilibrium amount was about 25%. This pH-triggered drug release has been diversely reported before^[26], which is caused by a larger degree of protonation of the carboxylic groups at lower pH, weakening the interaction of DOX and BSA. Nonetheless, this performance is beneficial for controlled release or faster release of drugs at tumor site or inside cells, because the pH values of extracellular matrix of tumors, lysosomes and endosomes are lower than that of serum (*i.e.* 7.4)^[26]. Although the multilayers and PAH-g-PEG_{25.6} coating does not influence significantly the drug release patterns, it does improve the colloidal stability and dispersity of the BSA NPs for further study *in vivo*.

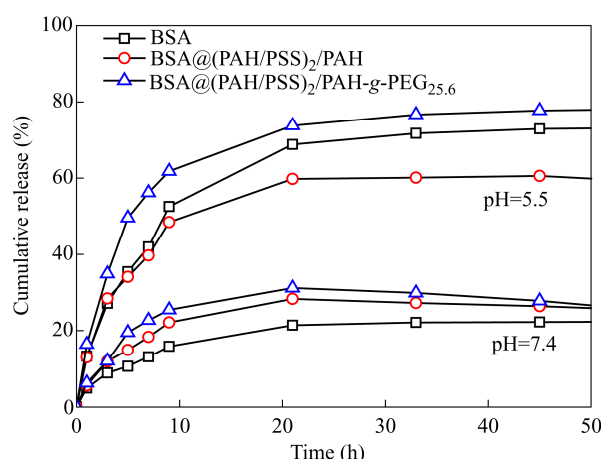


Fig. 4 Release profiles of DOX from BSA and BSA@((PAH/PSS)₂/PAH) and BSA@((PAH/PSS)₂/PAH-g-PEG_{25.6}) nanoparticles in PBS at pH 5.5 and 7.4, respectively

CONCLUSIONS

PAH/PSS multilayers and PAH-g-PEG were successfully adsorbed on BSA NPs surface to enhance the stability of NPs and sustain the pH-dependent loading and release properties. The higher density of PEG on NPs surface resulted in better stability of BSA NPs. Since the multilayers can endow the NPs with more functional groups, the multilayers and PAH-g-PEG coated BSA NPs are promising to be used as multifunctional drug delivery system.

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