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Monodisperse pH-Sensitive Protamine Hollow Microspheres as Carriers for Drug Delivery

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ABSTRACT: Novel pH-sensitive protamine sulfate (PS) hollow microspheres with controllable structure were fabricated and can be used as carriers for drug delivery and medical diagnostics. The carboxyl group-functionalized polystyrene microspheres prepared by soap-free emulsion polymerization were used as the templates. The self-assembled PS microspheres were prepared via electrostatic attraction between PS and carboxyl group-functionalized polystyrene. The single-layer PS was self-assembled and subsequently cross-linked with glutaraldehyde (GA). Then, the PS hollow microspheres (PSHMs) were obtained after the templates were removed. It was found that the pH of the external environment played an important role on the particle size of the PSHMs. To estimate the feasibility as novel carriers, an antitumor model drug 5 fluorouracil (5 Fu) and gold nanoclusters (Au NCs) were incorporated into hollow microspheres. The antitumor activity of the 5Fu/Au NCs-loaded PSHMs against cancer HepG2 was evaluated by measuring the body weight change and tumor volume of tumor bearing mice. The gold nanoclusters kept their fluorescent stability during the whole study. The 5 Fu/Au NCs-loaded PSHMs showed comparable anticancer efficacy with the free drug. © 2012 Wiley Periodicals, Inc. J. Appl. Polym. Sci. 129: 568–576, 2013

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INTRODUCTION

Recently, the design and synthesis of the hollow spheres with nano/micrometer dimensions, unique properties, and environmental response have attracted increasing interest because of their application in drug delivery and controlled release systems.[1,2] Among these hollow materials, the hollow polymer capsules are of high interest for their biocompatibility, low density, and high thermal and mechanical stability.[3] The template synthesis is commonly used as an effective way for preparing hollow polymer capsules, and is assisted by layer-by-layer (LbL) nanoassembly. This method is based on the alternating adsorption of oppositely charged components, such as synthetic and natural polyelectrolytes, proteins, and nanoparticles, which saturate the available surface.[4] Hollow polymer capsules are achieved by removal of the hard cores such as silica or polystyrene latexes either by dissolution or calcination at high temperature.[5] The empty capsules can be loaded with drugs or proteins through pH-dependent opening capsule wall pores.[4]

More and more research about stimuli-sensitive polymer microspheres has been reported for their special physical–chemical properties. Temperature, pH, and light are the most commonly used external stimuli. *N*-isopropylacrylamide (NIPAAm), carboxylic acid monomers which can exhibit reversible changes in response of their environment.[6,7] These kinds of smart microspheres can be used as stimuli in many fields such as target-drug delivery, cell cultivation, enzymatic immobilization, clinical diagnosis and so on.[8,9] pH sensitive capsules have been extensively investigated due to their potential applications in controlled release systems.[10] It is well known that polyacrylic acid (PAA) is an important polyelectrolyte and pH-sensitive material used for preparing pH-sensitive membranes and is characterized by its ionizable hydrophilic properties. Its reversible swelling–shrinking behavior is caused by the transformation between the deionized form (COOH group) and the ionized form (COO^- group) at pH values near a pK_a of about 4.7.[11]

Fluorescent labeling techniques have been used extensively in both biological research and clinical diagnosis due to their sensitivity, simplicity, and diversity. Metal nanoclusters (such as Au and Ag) are being extensively pursued in nanoscience research because of their interesting optical and electronic

Table I. Recipes and Results of the Soap-Free Emulsion Polymerization ($n = 3$)

Sample	Styrene (m L^{-1})	Acrylic acid (m L^{-1})	Ammonium persulfate (g)	Water (m L^{-1})	Mean diameter (nm)	Polydispersity index	Carboxyl group content ($\times 10^{-5}$ mol g^{-1})
1	10	0.5	0.054	100	226.5 ± 3.5	0.142 ± 0.021	7.06 ± 1.2
2	10	1.0	0.054	100	150.3.1 ± 1.0	0.125 ± 0.015	8.01 ± 1.5
3	10	2.0	0.054	100	165.3 ± 2.1	0.159 ± 0.044	12.1 ± 1.9

properties as well as their wide range of applications. The nanoclusters (NCs) for Au are synthesized by reducing the precursor $HAuCl_4$ in the presence of templates or protecting species such as dendrimers,[12] phosphines,[13] and thiols.[14] Thiol-protected Au NCs that consist of several to a hundred atoms (<1.2 nm) have been synthesized previously via chemical reduction (e.g., with sodium borohydride, $NaBH_4$ of Au precursors in the presence of BSA, the most abundant plasma protein widely used in applications such as sensing, self-assembly, and imaging, was selected as the model protein for the synthesis of Au NCs.[15,16]

Protamines are small, arginine-rich, nuclear proteins that replace histones late in the haploid phase of spermatogenesis and are believed essential for sperm head condensation and DNA stabilization.[17,18] Protamine sulfate (PS) is an arginine-rich peptide which can efficiently condense negatively charged DNA at low mass ratio by electrostatic interaction.[19] PS has been widely used for biomedical and biotechnological applications because it is biodegradable.[20] PS is a highly cationic peptide and the only FDA approved agent for the reversal of heparin anticoagulation. It binds to heparin to form a stable ion pair which does not have anticoagulant activity. On its own, PS has a weak anticoagulant effect. PS is usually administered to reverse the large dose of heparin administered during certain surgeries, especially heart surgery. Thus, whether the PS could be better utilized as the drug carriers are a moot point and an interesting change.

By far, construction of biocompatible and biodegradable protamine sulfate (PS) hollow microspheres with pH-sensitive rarely reported.[21] To combine the advantage of PS and at the same time maintain the desirable properties of hollow microspheres and Au NCs such as excellent biocompatibility, higher drug-loaded capacity and bio-detected property. In this study, we demonstrate that novel monodisperse pH-sensitive protamine sulfate hollow microspheres (PSHMs) with well controlled size and higher drug content can be fabricated by the assembly of the cationic PS with PAA modified polystyrene microspheres. Then etched polystyrene cores after the PS shells were crosslinked with glutaraldehyde were achieved. The physicochemical properties of the PSHMs were characterized and antitumor drug 5 Fu[22,23] and Au NCs incorporated into the PSHMs. We evaluated the efficacy of this system for drug delivery applications. The 5 Fu/Au NCs-loaded PSHMs could keep their fluorescent stability. The release of 5 Fu from the 5 Fu/Au NCs-loaded PSHMs is pH-dependent. Moreover, the potential of these PSHMs as efficient 5 Fu carriers was evaluated by examining their anti-tumor activity *in vivo*. PSHMs have the potential applications for targeted delivery of antitumor drug and in bio-imaging.

EXPERIMENTAL

Materials

Acrylic acid (AA) was purchase from Tianjin Chemical, (China) and was distilled under vacuum before use. Ammonium persulfate, glutaraldehyde (GA), and DMF were all of analytical reagent grade from Tianjin Chemical, (China) and used without further purification. The 5 Fluorouracil (5 Fu), styrene, bovine serum albumin (BSA), and protamine sulfate (PS) were obtained from Sigma Chemical (St Louis, USA). Ultrapure water (18.2 MΩ) was used in all experiments. Male BALB/c nude mice (initially weighing 18–22 g was purchased from Beijing Vital River Company) were used for investigating the antitumor efficacy *in vivo*.

Preparation of PAA-Polystyrene Microspheres

PAA-polystyrene was synthesized according to the literature.[13] The recipes for the soap-free emulsion polymerization of styrene with acrylic acid are listed in Table I. Briefly, styrene (10 mL), acrylic acid (0.5–2.0 mL) and ammonium persulfate (0.054 g) were dissolved in 100 mL of distilled water then added in a 250 mL three-necked reaction flask equipped with a mechanical stirrer, a condenser, and a gas inlet. After the mixture was deoxygenated by bubbling nitrogen gas at room temperature for 30 min, the flask was placed in a 70°C oil bath and stirred mechanically at 300 rpm. The reaction was continued for 12 h to insure a complete reaction. The raw product was purified by repeating cycle of centrifugation and washing with ethanol. The white fine powder (PAA-polystyrene) microspheres were finally obtained after being dried in a vacuum oven at 50°C.

The carboxyl group content of PAA-polystyrene microspheres was determined by conductometric titration method. The conductometric analyses were performed using conductometer (DDS-11C, HongYi Instruments and Meters, ShangHai, China). Briefly, 20 g of PAA-polystyrene microspheres was titrated using has been calibrated sodium hydroxide (0.1838 mol L^{-1}). A change of conductivity was used to determine the end point of titration. The carboxyl group contents (X) were calculated from the following equation:

$$X(\text{mol/g}) = 0.1838 \times V \times 10^{-3}/M$$

V is the consumption volume of sodium hydroxide of the titration microspheres and the unit is expressed in milliliters. M is the weight of the microspheres and the unit is expressed in grams. Each sample was assayed in triplicate.

Preparation of PS Hollow Microspheres

PS (150 mg) was dissolved in 100 mL of water at 40°C. PAA-polystyrene microspheres (0.5 mg mL^{-1}) were dissolved in 20

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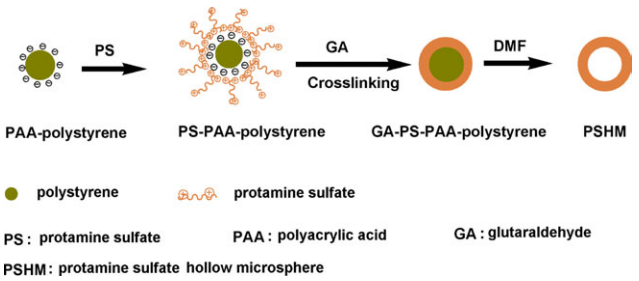

Scheme 1. Schematic representation of the preparation process of the PS hollow microspheres (PSHMs). (PS: protamine sulfate; PAA: polyacrylic acid; GA: glutaraldehyde; PSHM; protamine sulfate hollow microsphere). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

mL of water (at weight ratios of 15/1, 10/1, 5/1, 2/1, and 1/1 PS/PAA-polystyrene). Then PAA-polystyrene solution was dropped into the PS solution. After stirring for 12 h, the PS-PAA-polystyrene microspheres were centrifuged at 12,000 rpm at room temperature for 10 min. The centrifuged microspheres were redispersed in distilled water with sonication and centrifuged for three cycles to remove the dissociated PS, which was not adsorbed on the surface of the microspheres. The purified PS-PAA-polystyrene microspheres were dispersed into 100 mL of water and crosslinked with 5 mL of 2.0% GA for 48 h at 40°C. After washing with water, the GA-PS-PAA-polystyrene microspheres were dialyzed against DMF using a dialysis bag with a molecular weight cut-off of 3000 Da with stirring at room temperature for 1 week to remove the polystyrene core templates. The absence of the templates was confirmed by mixing the final dialysate with three times volume of water, ensuring the absence of any precipitate. Then the obtained products were collected by centrifugation at 12,000 rpm for 10 min at room temperature, and then washed several times by centrifuging/resuspending in DMF to remove all the dissociated polystyrene. The resulting purified PS hollow microspheres (PSHMs) were dried at 25°C for 2 days in a vacuum oven to get a white powder. The self-assembly behavior of PSHMs was schematically illustrated in Scheme 1.

Preparation of Au NCs

$HAuCl_4$ (5 mL; 10 mmol L^{-1}), 5 mL of BSA (50 mg mL^{-1}), and 0.5 mL of 1 mol L^{-1} NaOH were mixed and shaken at 37°C for 24 h, and then the red Au-NCs were formed. The Au-NCs were purified by repeating the cycle of centrifugation at 20,000 rpm at 4°C for 20 min and washed with distilled water.

Preparation of 5 Fu/Au NCs-Loaded PS Hollow Microspheres

To evaluate the drug loading and release properties, 5 Fu/Au NCs-loaded PSHMs were prepared by incubation technique.[24] Briefly, 5 Fu was dissolved in distilled water to produce 4 mg mL^{-1} solutions. An appropriate volume of the 5 Fu and 400 μL of Au NCs solution were pipetted into a test tube containing 20 mg of freeze-dried PSHMs. The volume of the 5 Fu solution was calculated to obtain a 5 Fu : PSHMs ratio of 1 : 1–1 : 50 (w/w). The PSHMs were completely immersed in the 5 Fu and Au NCs solution for 24 h at room temperature to allow an equilibrium loading. Then the dispersion was centrifuged at 15,000 rpm for 15 min to separate the loaded PSHMs. The amount of 5 Fu loaded in the PSHMs was determined by measuring the amount of 5 Fu in the loading solution and in the supernatant using a UV spectrophotometer (Perkin Elmer Lambda850 [USA]) at 266 nm. The optical density of the Au NCs was measured at 365 nm by a fluorometer. The encapsulation efficiency (EE) of 5 Fu was calculated from the following equation:

$$EE(\%) = W_0 - W_t/W_0 \times 100\%$$

W_0 and W_t are the weight of the initial 5 Fu and that of the amount of 5 Fu detected in supernatant, respectively. Each sample was assayed in triplicate.

Characterization of PS Hollow Microspheres

Fourier transform infrared spectra (FT-IR) were determined on a Perkin Elmer FT-IR spectrometer. Samples were pressed with KBr for measurement.

The morphologies of the PAA-polystyrene microspheres, the PS-PAA-polystyrene microspheres, the GA-PS-PAA-polystyrene microspheres, and the PSHMs were characterized with a Hitachi H-600 transmission electron microscope operating at 250 kV. They were dispersed in water in an ultrasonic bath for 30 min and then deposited on a copper grid covered with a perforated carbon film, dried at room temperature and were stained with uranyl acetate when necessary. The mean hydrodynamic diameter and size distribution of PAA-polystyrene microspheres, PS-PAA-polystyrene microspheres, GA-PS-PAA-polystyrene microspheres, and the PSHMs were determined by dynamic light scattering (DLS) using a ZetaSizer Nano series Nano-ZS (Malvern Instruments, Malvern, UK). Determinations were performed at 633 nm with a constant angle of 90° at 25°C after samples were appropriately diluted in distilled water. Each sample was assayed in triplicate.

Fluorescence spectra of 5Fu/Au NCs-loaded PSHMs were recorded on a fluorometer (Pekin Elmer Instruments, LS-55). A solution of samples was placed in a quartz absorption cell and the fluorescence spectrum was obtained with a fluorometer. The concentrations of sample solution were varied from 1.0×10^{-7} to 10^{-3} mg mL^{-1}. For all fluorescence measurements, excitation wavelength was focused to 470 nm. Emission intensity was collected at 670 nm. The excitation and emission bandwidths were set at 5 and 10 nm, respectively.

In Vitro Release Experiment

Nearly 10 mg of 5 Fu/Au NCs-loaded PSHMs were suspended in a polyethylene tubes containing 15 mL of PBS (pH 5.0 and 7.4) at 37°C under horizontal shaking (300 rpm, Thermomixer, Eppendorf). At predetermined time intervals, 3 mL of samples were withdrawn and centrifuged at 15,000 rpm for 25 min and washed twice with water. Finally, the free 5 Fu in solution was further for purified in an ultrafiltration tube (cut off M_w 3 kDa; MicroconYM-3 tube, Ultracel YM-3 membrane, Millipore). The purified supernatant was assayed for 5 Fu release and replaced by 3 mL of fresh release medium. The amount of 5 Fu in the release medium was determined by UV–vis at 266 nm. Each PSHM batch was analyzed in triplicate.

Antitumor Activity

The 2×10^6 HepG2 cells were resuspended in 100 μL of phosphate buffered saline, and inoculated subcutaneously in the flank of male BALB/c nude mice. Mice were randomly divided into five groups, each group had six mice. At 10 days post inoculation the average tumor volume had reached 60 mm^3. The inoculation consisted of PBS, free PS hollow microspheres (PSHMs), 5 Fu in solution (10 mg kg^{-1}), 5 Fu-loaded PSHMs (10 mg kg^{-1}), 5 Fu-loaded PSHMs (15 mg kg^{-1}) groups. Drugs were administered by intravenous injection in the tail vein of mice twice a week for 3 weeks for all the groups. Tumor volume and body weights of mice were measured every other day during treatment for 21 days. Measurement of tumor size was performed with a caliper in two dimensions, and tumor volume was calculated using the following formula: $V = \pi/6 \times m^2 \times n$ (where "m" and "n" represent the shortest and the longest diameter, respectively of a given tumor). All of the animal experiments were conducted under approved protocols of the Institutional Animal Care and Use Committee at the Institute of Tumor in Chinese Academy of Medical Science.

Statistical Analysis

Experimental data were compared using the Student's t test. Results were expressed as mean or mean $\pm$SD (number of experiments) and considered to be statistically significant when $P < 0.05$. All experiments were repeated three times unless otherwise indicated.

RESULTS AND DISCUSSION

Preparation and Characterization of PS Hollow Microspheres

In this work, we developed a facile strategy for the fabrication of monodisperse PS hollow microspheres (PSHMs) with controllable structures by the assembly of the highly cationic peptide PS with decorated polystyrene microspheres to encapsulate the 5 Fu and Au NCs, etching the polystyrene cores after the PS shells were crosslinked with glutaraldehyde (GA), as illustrated in Scheme 1. Herein, we also investigated the physicochemical properties of PSHMs and 5 Fu/Au NCs-loaded microspheres, including the microsphere size, morphology, fluorescent stability, and drug release profiles. Finally, we also evaluated the potential of these hollow microspheres as efficient 5 Fu/Au NCs carriers by examining their antitumor effect.

A series of the PAA-polystyrene microspheres with different particle sizes and surface carboxyl group contents were prepared by the soap-free emulsion polymerization method. The surface carboxyl group content of the PAA-polystyrene microspheres determined by the titration method is also shown in Table I. It increased from 7.06×10^{-5} to 12.1×10^{-5} mol g^{-1} with increasing the AA amount from 0.5 to 2.0 mL into this soap-less emulsion polymerization. The results showed that the surface carboxyl group content of the PAA-polystyrene microspheres increased with increasing the AA feeding ratio in the soap-less emulsion polymerization. As shown in Table I, the microspheres prepared at styrene 10 mL, acrylic acid 1.0 mL, ammonium persulfate 0.054 g and water 100 mL, which displayed the smallest size among all studied groups, were chosen as the templates for further studies. After the PAA-polystyrene microspheres were used as templates to absorb PS onto their surface via the electrostatic interactions as the driving force between the surface carboxyl groups of the polystyrene spheres and the amino groups of PS, the PS-adsorbed composite (PS-PAA-polystyrene) microspheres were treated with GA for 48 h at 40°C to achieve the crosslinking of the peripheral PS shell (GA-PS-PAA-polystyrene microspheres). Finally, the GA-PS-PAA-polystyrene microspheres were treated with *N,N*-dimethylformamide (DMF) to remove the sacrificial cores to obtain the PS hollow microspheres (PSHMs).

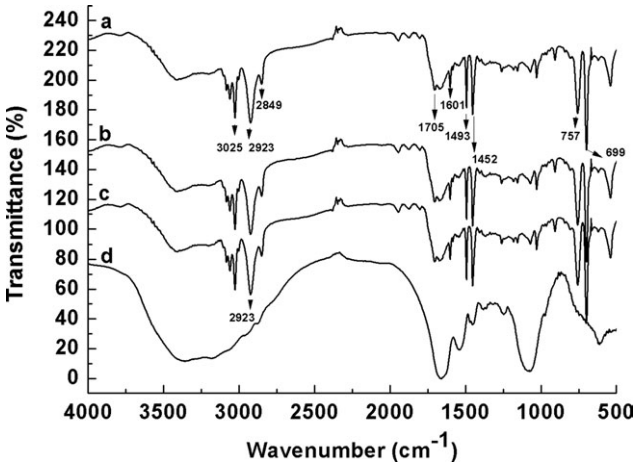

Figure 1. FT-IR spectra of PAA-polystyrene (a), PS-PAA-polystyrene (b), GA-PS-PAA-polystyrene (c), and protamine sulfate (d).

Figure 1 shows the FT-IR spectra of protamine sulfate, PAA-polystyrene microspheres, PS-PAA-polystyrene microspheres and GA-PS-PAA-polystyrene microspheres. The typical absorbance peaks of the phenyl groups from the FT-IR spectra of the PAA-polystyrene microspheres (Figure 1) were at 3025, 1601, 1493, 1452, 757, and 699 cm^{-1}. The absorbance bands at 2923 and 2849 cm^{-1} were ascribed to the methylene and methenyl groups, respectively. The absorbance at 1705 cm^{-1} assigned to the C=O stretching vibration of the carboxylic groups was also found in the spectrum of the PAA-polystyrene microspheres. It indicated that acrylic acid (AA) had acted as the surfmer in the soapless emulsion polymerization of styrene.[25] The obtained PS-PAA-polystyrene shows stronger peaks around 2923 cm^{-1} which is attributed to the saturated C—H group, compared with the original PS [Figure 1(d)]. The peak is overlapped with the C—H group of PAA-polystyrene microspheres. The results prove the conjugation of protamine onto PAA-polystyrene.[26]

Figure 2 shows the FT-IR spectra of hollow microspheres (PSHMs) and 5 Fu/BSA-Au NCs coated PSHMs. Au NCs present the characteristic vibration peaks of the protein BSA which stabilized the gold nanoclusters. The protein amide I in the region 1660 cm^{-1} (mainly CO stretch) has a relationship with the secondary structure of the protein. The amide I band is more sensitive to the change of protein secondary structure than amide II.[27,28] Peaks located at 3354 and 2903 cm^{-1} correspond to primary amines and C—H vibration. The 5 Fu/Au NCs-loaded PSHMs exhibit a strong peak at 1089 cm^{-1} which is assigned to the C—O—C asymmetric stretch. In addition, the characteristic bands at 890 cm^{-1} are assigned to the C—O—C symmetric stretch. A broad band centered at 3430 cm^{-1}

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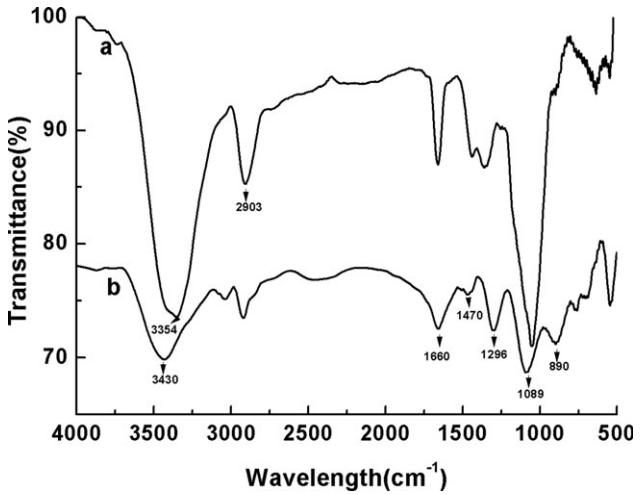

Figure 2. FT-IR spectra of PSHMs (a) and 5Fu/Au NCs-loaded PSHMs (b).

corresponds to the OH stretch originating from alcohol group. The vibration modes of amide II, the bending of the C—H bond and the stretching of the carboxyl groups are located at 1660, 1470, and 1296 cm^{-1}, respectively. The intense peak at 1660 cm^{-1} could not be only attributed to the amide I due to the overlap of the bending mode of H—O—H adsorbed at the surface. Therefore, FT-IR spectroscopy confirms the presence of Au NCs and 5 Fu in the hollow microspheres.

The changes of these characteristic peaks became more pronounced with the 5 Fu loading, confirming the formation of hydrogen bonds between the 5 Fu and the PSHMs matrix. With 5 Fu loading, the characteristic peak of carbonyl stretching of PSHMs at 1660 cm^{-1} became visible, suggesting the existence of free 5 Fu within the hollow microspheres.[3,29]

The morphology of the PAA-polystyrene microspheres was investigated by transmission electron microscopy (TEM) [Figure 3(a)]. It could be observed that the PAA-polystyrene

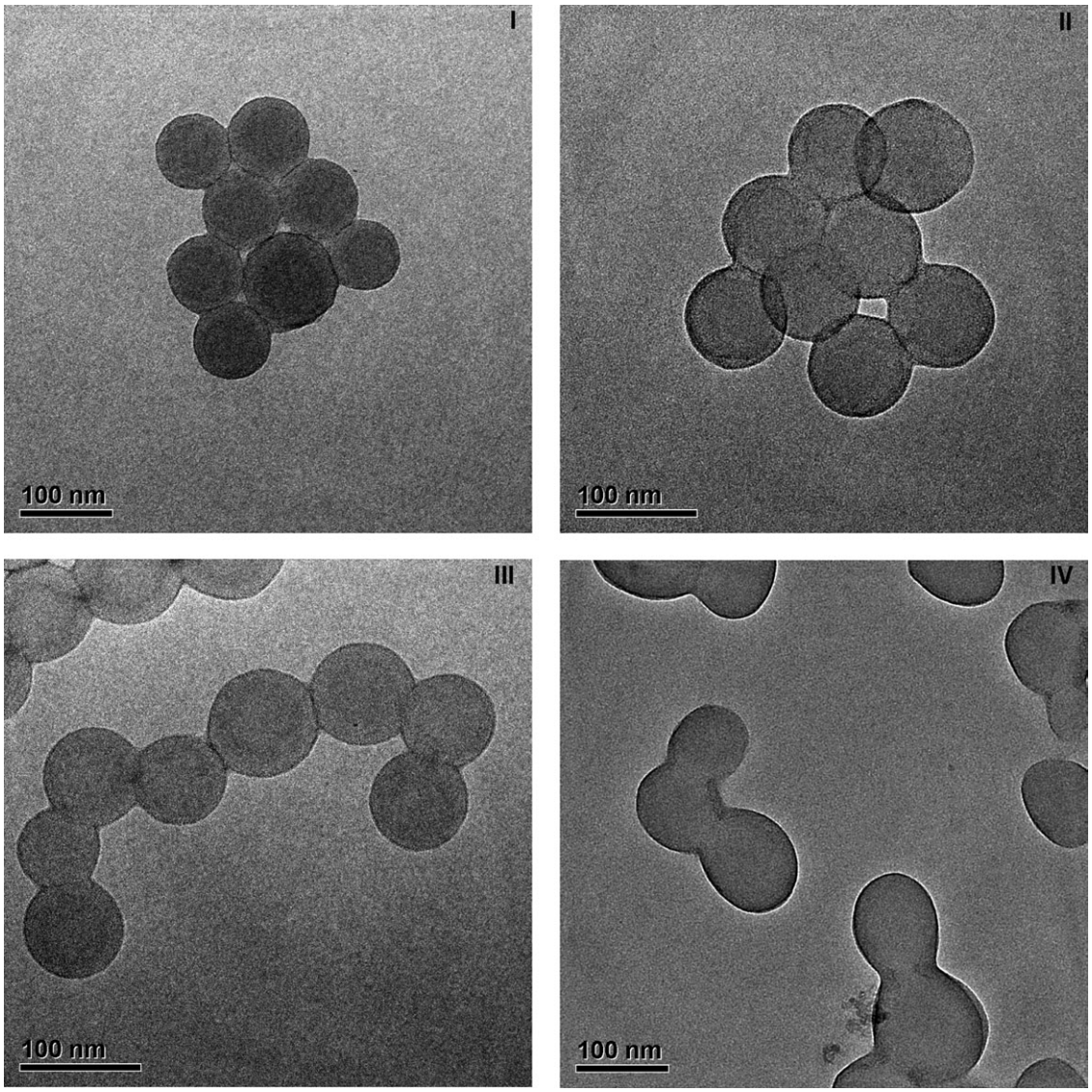

Figure 3. TEM images of PAA-polystyrene microspheres (a), PS-PAA-polystyrene microspheres (b), GA-PS-PAA- polystyrene microspheres (c), and PSHM (IV).

Table II. The Size and Distribution of Various Microspheres ($n = 3$)

Sample	Mean diameter (nm)	Polydispersity index
PAA-polystyrene microspheres	150.3 ± 1.0	0.125 ± 0.015
PS-PAA-polystyrene microspheres	300.1 ± 3.6	0.137 ± 0.042
GA-PS-PAA-polystyrene microspheres	166.0 ± 2.7	0.171 ± 0.037
PSHMs	125.4 ± 4.1	0.163 ± 0.041

microspheres were uniformly spherical, and have relatively narrow size distribution. The PAA-polystyrene microspheres were then used as templates to absorb PS onto their surface via the electrostatic interactions as the driving force between the surface carboxyl groups of the PAA-polystyrene microspheres and the amino groups of PS [Figure 3(b)]. The PS-adsorbed PAA-polystyrene microspheres (PS-PAA-polystyrene) were treated with GA for 48 h at 40°C to achieve the crosslinking of the peripheral PS shell. Figure 3(c) showed the typical morphologies of the crosslinked PS encapsulated PAA-polystyrene microspheres (GA-PS-PAA-polystyrene) and the microspheres became compact compared with the PS-PAA-polystyrene microspheres. Finally, the GA-PS-PAA-polystyrene microspheres were treated with *N*,*N*-dimethylformamide (DMF) to remove the polystyrene cores to obtain PS hollow microspheres (PSHMs). Figure 3(d) showed the TEM images of the PSHMs and obviously the microspheres exhibited the hollow structure. It was found that the inner diameters of the obtained hollow microspheres were smaller than the corresponding PSHMs microspheres before the removal of the template cores. This resulted from the shrinking of the PS shells to some degree during the gradual drying process of preparation TEM samples.

The mean particle sizes and distribution were determined by dynamic light scattering (DLS), and the results are shown in Table II. The average size of PAA-polystyrene microspheres, PS-PAA-polystyrene microspheres, GA-PS-PAA-polystyrene microspheres and PSHMs were ~ 150, 300, 166, and 125 nm in water, respectively. All these microspheres showed a narrow size distribution (PDI < 0.3). The DLS histograms of PAA-polystyrene microspheres, PS-PAA-polystyrene microspheres, GA-PS-PAA-polystyrene microspheres and PSHMs were shown in Figure 4(a–d), respectively.

The influence of ratio of PS to PAA-polystyrene on size, size distribution, and zeta potential was studied. The zeta potential of PAA-polystyrene is negative, while the pure PS has a positive charge. As shown in Table III, the PSHMs also present a positive value. The positive value of the zeta potential goes down as the content of PAA-polystyrene increases. The result strongly indicates that an electrostatic interaction between PS and PAA-polystyrene has taken place.

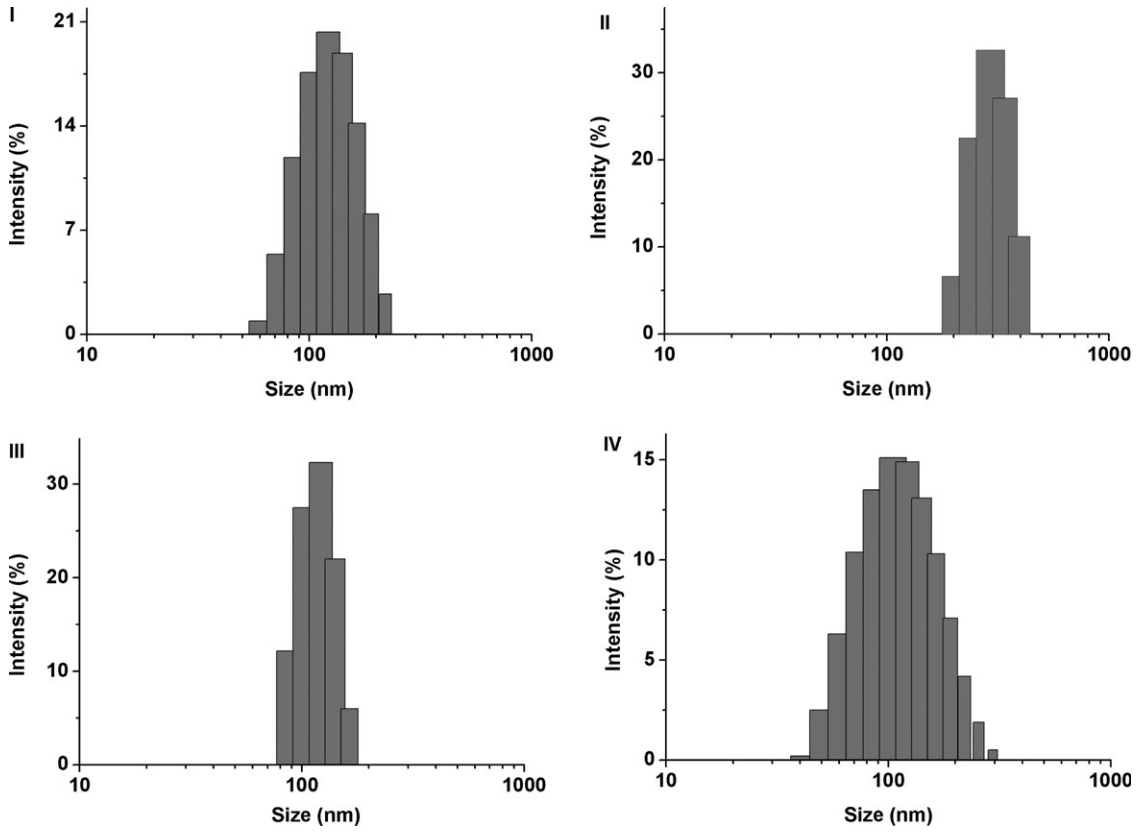

Figure 4. DLS histograms PAA-polystyrene microspheres (a), PS-PAA-polystyrene microspheres (b), GA-PS-PAA- polystyrene microspheres (c), and PSHM (d).

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Table III. Physicochemical Properties of Selected PSHMs Prepared by Addition of Different Weight Ratios of PAA-Polystyrene to PS ($n = 3$)

Ratio PS/PAA-polystyrene	Mean diameter (nm)	Polydispersity index	Zeta potential (mV)
15 : 1	185.6 ± 2.3	0.173 ± 0.033	23.0 ± 0.8
10 : 1	210.3 ± 4.5	0.168 ± 0.025	21.3 ± 1.1
5 : 1	235.6 ± 3.1	0.21 ± 0.031	17.6 ± 1.4
2 : 1	276.4 ± 2.1	0.192 ± 0.045	12.1 ± 1.0
1 : 1	300.1 ± 1.6	0.25 ± 0.036	9.8 ± 1.3

The influence of pH on the mean diameter of the PSHMs was investigated by DLS after the hollow microspheres had been immersed into the aqueous solutions with different pH values for 24 h. It could be seen from Figure 5 that the size of the PSHMs decreased in the range of pH value from 3 to 9. The size of the hollow microspheres reached their minimum at pH 9.0 and then increased at pH value 10 and 11. A low pH environment such as at pH 3.0, PS was fully ionized as $-NH_3^+$. However, because of the crosslinking shell and the stabilization of positively charged PS chains in the shells, the hollow microsphere structure was not destroyed. The size of the hollow microspheres (PSHMs) decreased with the increase in the pH values because of the shrinkage of the cross-linked shells, which resulted from the deprotonation of the $-NH_3^+$ groups. However, a marked increase in the hydrated diameter was observed when the pH was in the range of 9–11, which could be attributed to the disintegration of the cross linked PS shells. At high pH (>9) the deprotonation of the $-NH_3^+$ groups, the free PS chains entangled with each other led to the increase in the size of hollow microspheres.

Preparation of 5 Fu/Au NCs-Loaded PS Hollow Microspheres

The drug encapsulation efficiency is an important factor for the drug-loaded PSHMs. To investigate the drug-loading capacity of such PSHMs, the Au NCs and antitumor drug 5 Fu were loaded into the hollow microspheres. The major interaction between the PSHMs matrix and 5 Fu (or Au NCs) is attributed to hydrogen bonding and it is confirmed by FT-IR spectra (Figure 2).

Table IV. The Influence of the Ratio of Copolymer to Drug on Size, Size Distribution, and Encapsulation Efficiency (EE) of 5 Fu-Loaded PSHMs (10 : 1 of PS/PAA-Polystyrene) Copolymer ($n = 3$)

PSHMs/5Fu weight ratio	EE (%)	Mean diameter (nm)	Polydispersity index
50 : 1	78.8 ± 0.7	220.7 ± 2.2	0.172 ± 0.020
20 : 1	73.4 ± 1.1	235.6 ± 2.0	0.181 ± 0.025
10 : 1	70.3 ± 0.5	256.2 ± 3.4	0.202 ± 0.031
5 : 1	65.1 ± 0.8	267.8 ± 3.6	0.216 ± 0.039
1 : 1	60.4 ± 0.9	281.2 ± 2.7	0.195 ± 0.052

The influence of ratio of PSHMs to 5 Fu on EE and mean diameter was investigated. As shown in Table IV, as the weight ratio of 5Fu/copolymer increases, the EE showed the decreased from 78.8 to 60.4%. The results were similar to the previous reports.[30] The size and size distribution of PSHMs prepared with various weight ratios of PSHMs/5 Fu were examined. As illustrated in Table IV, the higher the mass ratio of PSHMs to 5 Fu, the smaller the mean diameter of 5 Fu-loaded PSHMs. This can be attributed to the increase of the interaction between the copolymer and 5 Fu with the increase of the weight ratio of PSHMs to 5 Fu and it can form a more compact structure, so the size of the 5 Fu-loaded PSHMs decreased.

Fluorescent gold nanoclusters (Au NCs) were coated inside the hollow microspheres prepared by an incubation technique. The 5 Fu/Au NCs-loaded PSHMs presented a bright reddish color under UV irradiation (365 nm) (as shown in Figure 6) and maintained fluorescent stability at pH 3–9. The fluorescent stability of 5 Fu/Au NCs-loaded PSHMs may facilitate applications of the hollow microspheres in bio-imaging.

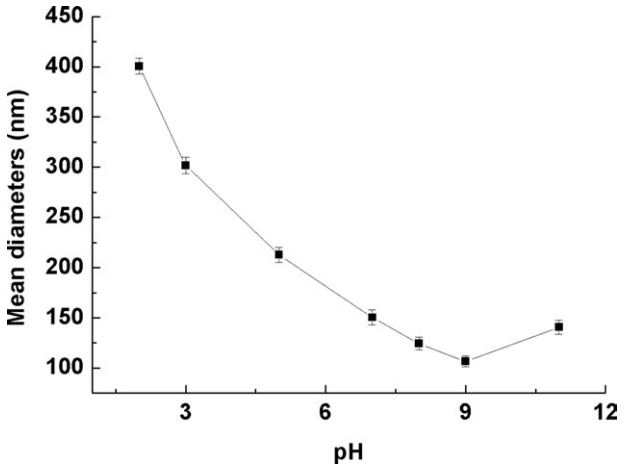

Figure 5. Mean diameters of the PS hollow microspheres (PSHMs) in different pH conditions. Values are the mean ± standard deviation (SD), $n = 3$.

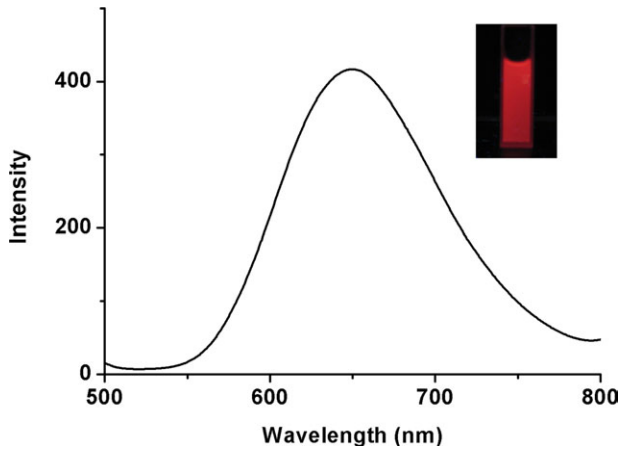

Figure 6. Photoemission ($\lambda_{ex} = 470$ nm) spectra of aqueous solution of 5Fu/Au NCs-loaded PSHMs. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

To investigate the *in vitro* release experiment and anti-tumor activity, the 5 Fu/Au NCs-loaded PSHMs with the encapsulation efficiencies of 5 Fu was 78.8%, Au NCs was 63.5%, respectively were used for the follow experiment.

In Vitro Release Experiment

The appropriate release rate of 5 Fu from the 5 Fu/Au NCs-loaded PSHMs is important for the prolonged and sustained use of the PSHMs as drug carriers. *In vitro* release of 5Fu into PBS solution was evaluated for free 5 Fu and 5 Fu/Au NCs-loaded PSHMs at different pH. The release profiles are shown in Figure 7. Free 5Fu released rapidly from the tube 0.0230, 0.0208 mol L^{-1} within the first 0.5 h and the release of 5 Fu was 0.0246, 0.0231 mol L^{-1} by 8 h for pH 5.0 and 7.4, respectively. The release profiles of the 5 Fu from the 5 Fu/Au NCs-loaded PSHMs were much slower compared to free 5 Fu. The initial release rate of 5 Fu from the 5 Fu/Au NCs-loaded PSHMs was 0.0039, 0.0034 mol L^{-1} within the first 0.5 h and the release rate of 5 Fu from the 5 Fu/Au NCs-loaded PSHMs was 0.0111, 0.0089 mol L^{-1} by 8 h for pH 5.0 and 7.4, respectively. The constant release rates of 5 Fu from the 5 Fu/Au NCs-loaded PSHMs were 0.0172 and 0.0153 mol L^{-1} by 16 days for pH 5.0 and 7.4, respectively. For the cross-linked PS shell, 5Fu released slowly from the PSHMs, and the reason might be due to the formation of denser film on the surface of the PSHMs. Solid tumors have a weakly acidic extracellular pH (pH < 7), and cancer cells have an even more acidic pH in endosomes and lysosomes (pH 4–6).[31] Figure 6 also shows the release of 5 Fu from the PSHMs in pH 7.4 and 5.0. The 5 Fu-loaded PSHMs exhibited faster release in pH 5.0 buffer than in pH 7.4 buffer. This phenomenon could be explained by the fact that the protonated 5 Fu had a higher solubility. Also, as the hydrophobic interactions decreased, the electrostatic repulsion increased between the PSHMs and 5 Fu at pH 5.0. The pH-sensitive properties of the PSHMs may benefit from the 5 Fu release in tumor cells, whose pH is lower than that of normal cell.[32–34]

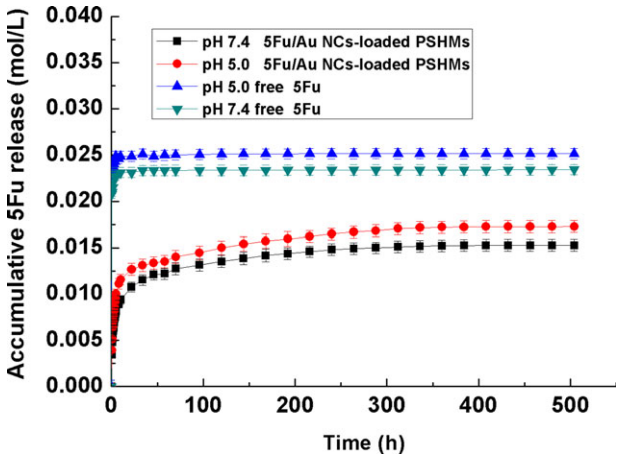

Figure 7. Release profiles of free 5Fu and 5Fu/Au NCs-loaded PSHMs in PBS at pH 5.0 and 7.4. Values are the mean ± standard deviation (SD), $n = 3$. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

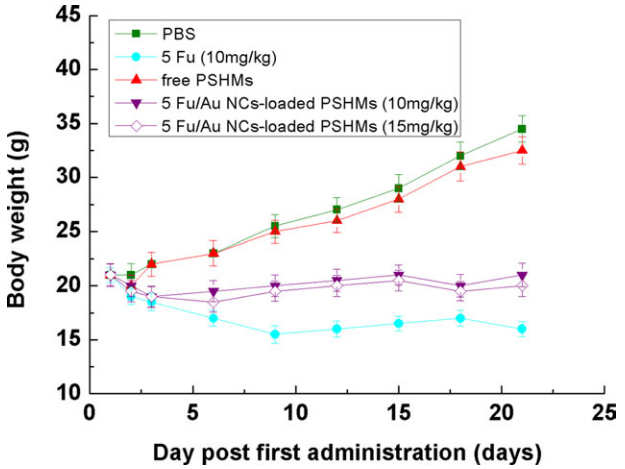

Figure 8. The body weight changes of tumor bearing mice were measured after injected free 5Fu, 5Fu/Au NCs-loaded PSHMs, free PSHMs and PBS groups. Values are the mean ± standard deviation (SD), $n = 3$. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Antitumor Activity

In vivo studies in mice, a tumor was induced by the subcutaneous injection of HepG2 cells and the drugs were injected at 10 days after tumor inoculation. Compared with the initial body weights of tumor-bearing mice, a gradual increase in the body weight was found for the mice PBS (phosphate buffered saline) buffer solution and treated with PSHMs groups, whereas a gradual decrease in the body weight was observed for the free 5 Fu-treated mice group (Figure 8), suggesting systemic toxicity of free 5 Fu. Irrespective of the dose we used, the intravenous injection of 5 Fu/Au NCs-loaded PSHMs, resulted in a gradual increase in the body weight, although the increasing rate was lower than that of the untreated mice. This may indicate that 5 Fu/Au NCs-loaded PSHMs exhibit mild toxicity. With regard to the change in the tumor volume, it was evident that 5 Fu/Au

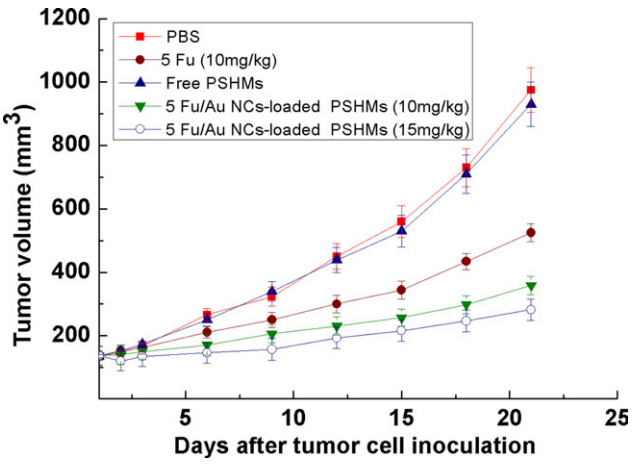

Figure 9. The changes of the tumor volume of tumor bearing mice were measured after injected free 5Fu, 5Fu/Au NCs-loaded PSHMs, free PSHMs and PBS group. Values are the mean ± standard deviation (SD), $n = 3$. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

NCs-loaded PSHMs and 5Fu treatments effectively suppressed the tumor growth (Figure 9). After intravenous injection, tumor volumes of the mice treated with 5 Fu/Au NCs-loaded PSHMs and 5 Fu were significantly smaller than those of untreated mice. The tumor volumes of the mice treated with 5 Fu/Au NCs-loaded PSHMs were significantly smaller than those treated with the free 5 Fu. By comparing the tumor volumes of the mice treated with 5 Fu/Au NCs-loaded PSHMs and free 5 Fu, there were indications that the 5 Fu/Au NCs-loaded PSHMs administration was more effective than the free 5Fu.

CONCLUSIONS

In summary, the novel pH-sensitive protamine sulfate hollow microspheres (PSHMs) were fabricated by the crosslinked PS shells to encapsulate polystyrene microspheres were proposed after removing the polystyrene core templates. Compare with published protamine sulfate paper,[17,35,36] in the current study, PSHMs exhibited multiple functional properties. The 5 Fu and Au NCs can be efficiently encapsulated into the hollow microspheres and 5Fu/Au NCs-loaded PSHMs can keep the fluorescent stability. The hollow structure of the microspheres can enhance volume phase transition compared to solid gel particles, thus favoring drug loading and release. In addition, *in vitro* release experiments of the 5Fu/Au NCs-loaded PSHMs exhibited that faster release at pH 5.0 than their release at pH 7.4 buffer. *In vivo* antitumor activity of the 5Fu/Au NCs-loaded PSHMs was assessed following systemic administration via the tail vein of the tumor-bearing mice. The 5Fu/Au NCs-loaded PSHMs showed good antitumor activity against HepG2 tumor. These results indicate that PSHMs might be applied as a carrier in targeted cancer chemotherapy. This study can be considered as the first step for further studies on the application of PSHMs for possible targeted delivery and potential applications in cellular staining and bio-imaging.

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