Drug Delivery

Designed Synthesis of Lipid-Coated Polyacrylic Acid/Calcium Phosphate Nanoparticles as Dual pH-Responsive Drug-Delivery Vehicles for Cancer Chemotherapy


Abstract: Herein, we report a facile strategy to prepare supported lipid-bilayer-coated polyacrylic acid/calcium phosphate nanoparticles (designated as PAA/CaP@SLBNPs) as a new dual pH-responsive drug-delivery platform for cancer chemotherapy. The synthesized PAA/CaP NPs exhibited both a high payload of doxorubicin (DOX) and dual pH-responsive drug-release properties. Additionally, the coated lipid bilayer had the ability to enhance the cellular uptake of PAA/CaP NPs without affecting the pH-responsive drug release. Moreover, the blank PAA/CaP@SLBNPs exhibited excellent biocompatibility and the DOX-loaded PAA/CaP@SLBNPs markedly increased the cellular accumulation of DOX and its cytotoxic effects on HepG-2 cells. Furthermore, when used to evaluate the in vivo therapeutic efficacy in mice with the hepatocarcinoma cell line (H-22), the DOX-loaded PAA/CaP@SLBNPs exhibited superior inhibition of tumor growth compared with the free DOX group. Thus, PAA/CaP@SLBNPs are a promising drug-delivery vehicle to increase the therapeutic efficacy of anticancer drugs.

Introduction

Chemotherapy plays an important role in cancer treatment, but the low therapeutic effect and severe toxic side-effects significantly limit its clinical applications.[1–3] In recent decades, the incorporation of a chemotherapeutic agent into nanoscale drug carriers has attracted increased interest in terms of biomedical applications because this approach can improve therapeutic efficacy and reduce side-effects by increasing the accumulation of the chemotherapeutic agent in tumors through enhanced permeability and retention (EPR) effects and by reducing unwanted release in normal tissues.[4–8] However, problems owing to poor cellular uptake by tumor cells and inadequate intracellular drug release in tumor tissues still remain to be resolved.[9,10]

Based on the weakly acidic environment of tumor tissues compared with normal physiological conditions,[11–13] various pH-responsive drug carriers have been developed to produce high intracellular drug release, such as liposomes, polymeric micelles, polymers, dendrimers, and organic/inorganic NPs.[14–20] Of these pH-responsive drug carriers, the development of CaP-based nanocarriers has shown that they are potential candidates for intracellular drug delivery because CaP remains stable at physiological pH, but dissolves rapidly in an acidic endosomal (pH 5.0) or lysosomal (pH 4.5) environment, which leads to fast release of anticancer drugs.[21] Moreover, as a major component of bones and teeth, CaP exhibits extraordinary biocompatibility, superior biodegradability, and is nontoxic,[22,23] which makes it suitable for drug delivery. To date, researches have reported the use of organic amphiphilic polymers, dendrimers, or adenosine 5'-triphosphate as templates for CaP-based nanocarriers[24–28] to avoid the problems associated with the direct synthesis CaP NPs. However, the above approaches have suffered from some limitations, such as a complicated synthesis process, the use of toxic organic solvents, large diameters, and a very low drug capacity. To address the above challenges, and inspired by our previous study,[29] we used PAA, a pH-responsive material, as a template to prepare PAA/CaP NPs with dual pH-responsiveness and a high drug-loading capacity.

In addition, helped by the lipid bilayer that characterizes the unique properties of biological membranes, lipid-coated hybrid NPs have emerged as a novel type of nanocarrier that combines the advantages of liposomes and NPs.[30] The NPs play an important role in increasing the drug-loading capacity and/or exhibit stimuli-responsive properties, whereas the lipid contributes to an improvement in stability, which prolongs the circulation time in blood, increases the cellular uptake of the NPs, and controls the drug-release profiles.[31–36]
Herein, we adopted a facile approach, controlled solvent-exchange deposition, to prepare supported-lipid-bilayer (SLB)-coated PAA/CaP NPs. In these nanocomposites, the PAA/CaP NPs have a high payload of doxorubicin (DOX) and serve as the drug reservoir and the source of pH-responsive material, whereas the lipid bilayer acts as a “gatekeeper” to control drug release and improve the cellular uptake. In addition, the in vitro drug release, cytotoxicity, cellular uptake, and in vivo anticancer efficacy of PAA/CaP@SLB NPs were systematically investigated and these PAA/CaP@SLB NPs proved to be effective drug-delivery carriers to improve cancer chemotherapy.

Results and Discussion

The preparation process of PAA/CaP@SLB NPs is shown in Scheme 1. PAA was used as the template in the first step of the synthesis of PAA/CaP NPs. Initially, a PAA-Ca aqueous solution was formed based on an acid–base neutralization reaction between aqueous PAA and calcium hydroxide (Ca(OH)\(_2\)). Then, the PAA-Ca NPs (Figure 1A) were obtained by the dropwise addition of isopropyl alcohol (IPA) because the PAA-Ca NPs are insoluble in the presence of IPA, as confirmed by the change in color from colorless and clear to a milk-white liquid (see the Supporting Information, Figure S1). Finally, disodium hydrogen phosphate dodecahydrate ((Na\(_2\)HPO\(_4\)) was used as the phosphate anion source to form PAA/CaP NPs. A hydrothermal treatment was performed to improve the stability of PAA/CaP NPs. Transmission electron microscopy (TEM) and scanning electron microscopy (SEM) images (Figure 1B and C) showed that the PAA/CaP NPs had a uniform spherical shape with an average diameter of (165 ± 10) nm. The SEM image showed that the PAA/CaP NPs had a rough surface, which confirmed the random distribution of small CaP NPs on the PAA template. The energy-dispersive X-ray (EDX) spectrum demonstrated that the PAA/CaP NPs were composed of Ca, O, C, and P (Figure S2). The Ca\(^{2+}\) and P contents were shown to be 18.5 and 4.3 wt\%, respectively, by using inductively coupled plasma atomic emission spectroscopy (ICP-AES). The X-ray diffraction (XRD) patterns of PAA/CaP NPs (Figure S3) displayed a characteristic peak at around 2\(\theta\) = 30°, which confirmed the presence of amorphous CaP and showed the more compatible and bio-
The degradable nature of the PAA/CaP NPs. The nitrogen adsorption–desorption isothermal curve and pore-size distribution of CaP NPs are shown in Figure S4. The total surface area and pore volume of the PAA/CaP NPs were 226.379 m²/g and 0.554 cm³/g, respectively. In addition, the PAA/CaP NPs had a wide pore-size distribution, which gave them a higher drug-loading capacity.

In the next step, 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC, Figure S5) was selected to be deposited on the as-prepared “empty” or drug-loaded PAA/CaP NPs in accordance with the principle of the controlled solvent-exchange deposition method. DOPC was dissolved in a mixture of water and alcohol to form a monolayer, in which the as-prepared empty or drug-loaded PAA/CaP NPs were subsequently dispersed, and a micelle-to-bilayer transition occurred as the water content was gradually increased. Finally, the lipid bilayer was deposited on the surface of the PAA/CaP NPs. PAA/CaP@SLB NPs with a diameter of about (180 ± 5) nm can be seen in the high-resolution TEM (HRTEM) image (Figure 1E and Figure S6). Compared with PAA/CaP NPs (Figure 1D), they appeared to be surrounded by a thin layer about 8 nm thick and the surface was smooth, which confirmed that DOPC had been successfully deposited on the PAA/CaP NPs. The elemental mapping of an individual PAA/CaP@SLB NP (Figure 1F–I) clearly illustrated the presence of Ca (purple), O (blue), P (green), and N (red). The N was associated with the DOPC, which suggested the successful synthesis of PAA/CaP@SLB NPs.

The hydrodynamic size of PAA/CaP@SLB NPs (Figure 2A and Figure S7) increased to 211.3 nm, whereas the size of PAA/CaP NPs was 183.6 nm, and the change in particle size further confirmed the above result, which is consistent with previous reports. The relatively larger particle size compared with that observed by using TEM is due to the fact that the NPs are highly hydrated in water. Fourier-transform infrared (FTIR) spectra were used to characterize the PAA/CaP NPs and PAA/CaP@SLB NPs (Figure 2B). The characteristic peaks located at 1558 and 1414 cm⁻¹ can be attributed to the carbonyl (C=O) group, which originates from the carboxylic acid of PAA. The absorption peaks at 551 and 1062 cm⁻¹ are the characteristic peaks of PO₄³⁻. Also, compared with PAA/CaP NPs, there was an absorption peak at 1740 cm⁻¹ due to the C=O stretching vibration of the lipid chain, which demonstrated the presence of a lipid bilayer on PAA/CaP NPs. X-ray photoelectron spectroscopy (XPS) analysis was conducted to explore the sur-

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**Figure 2.** A) The particle size of PAA/CaP NPs (a) and PAA/CaP@SLB NPs (b). B) FTIR spectra of PAA/CaP NPs (a) and PAA/CaP@SLB NPs (b). C–E) XPS spectrum of PAA/CaP NPs, PAA/CaP@SLB NPs, and N1s.
To study the kinetics of DOX release from these carriers, zero-order kinetics, first-order kinetics, and Higuchi models were applied to fit the accumulative release data. The results are listed in Table S1. The resulting correlation that indicated the release profiles was described by using the Higuchi model (\(Q = Kt^{1/2}\)), in which the drug released from the carriers is proportional to the square root of time. \(Q\) is the amount of drug released in time \(t\), and \(K_t\) is the Higuchi rate constant. According to the equation, we concluded that the release mechanism of DOX from these particles was governed by a diffusion process.[32]

To examine the cellular cytotoxicity of blank PAA/CaP NPs, PAA/CaP@SLB NPs, free DOX, DOX-loaded PAA/CaP NPs, and DOX-loaded PAA/CaP@SLB NPs in HepG-2 cells, a 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay was performed. As shown in Figure 3C, blank PAA/CaP NPs and PAA/CaP@SLB NPs over a wide concentration range (0.1–100 \(\mu\)g mL\(^{-1}\)) had no obvious effect on HepG-2 cell viability.

Figure 3. A) UV/Vis absorption spectra; inset: images of DOX solutions before (a) and after interaction (b) with PAA/CaP NPs. B) In vitro DOX release profiles for PAA/CaP NPs and PAA/CaP@SLB NPs at pH 7.4 and 5.0 in PBS. C) The cell viability of free DOX, DOX-loaded PAA/CaP NPs, and PAA/CaP@SLB NPs and the corresponding amount of blank NPs against HepG-2 cells at different concentrations.
which confirmed the good biocompatibility of PAA/CaP NPs and PAA/CaP@SLB NPs when used for drug delivery. The DOX-loaded PAA/CaP@SLB NPs showed a slightly higher cytotoxicity than DOX-loaded PAA/CaP NPs at both tested concentrations because the better affinity of PAA/CaP@SLB NPs for cell membranes could enhance cellular cytotoxicity through improved cell uptake. DOX-loaded NPs showed slightly weaker cytotoxicity than free DOX because DOX is an amphiphilic small molecule that can be easily delivered into cells by passive diffusion.

To compare the extent of cellular uptake and intracellular DOX distribution after free DOX, DOX-loaded PAA/CaP NPs, and DOX-loaded PAA/CaP@SLB NPs were incubated with HepG-2 cells for 3 and 6 h, confocal laser-scanning microscopy (CLSM) and flow cytometry (FCM) were used to examine the results qualitatively and quantitatively. As shown by the CLSM results (Figure 4A), images were obtained for the red fluorescence of DOX and the blue fluorescence of 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI) after the nuclei were stained. The DOX fluorescence of DOX-loaded PAA/CaP NPs
and DOX-loaded PAA/CaP@SLB was concentrated in the nuclear regions, which suggested that both DOX-loaded NPs could facilitate cellular internalization and final delivery of DOX to the cell nuclei. All free DOX and DOX-loaded NPs groups exhibited time-dependent DOX accumulation in the nuclei of the HepG-2 cells. The DOX fluorescence of DOX-loaded PAA/CaP@SLB was significantly stronger than that of DOX-loaded PAA/CaP NPs for both incubation periods of 3 and 6 h. The reason for this is that PAA/CaP@SLB can produce a higher accumulation of DOX in the HepG-2 cells by improving the cellular uptake instead of influencing intracellular DOX release.\cite{32}

Notably, the DOX fluorescence between DOX-loaded PAA/CaP@SLB and free DOX were very different after incubation for 3 and 6 h. The fluorescence intensity of DOX-loaded PAA/CaP@SLB NPs after incubation for 3 h was slightly weaker than that of free DOX, whereas the fluorescence of DOX-loaded PAA/CaP@SLB NPs after incubation for 6 h was similar to that of free DOX. It followed that DOX-loaded PAA/CaP@SLB NPs and free DOX show different intracellular fates for the free DOX and NPs after a short time.\cite{42,43} After incubation for 3 h, free DOX was faster than DOX-loaded PAA/CaP@SLB NPs because free DOX can be easily delivered into cells by passive diffusion, whereas PAA/CaP@SLB takes time to enter the cells by endocytosis. When the incubation period was extended to 6 h, there was enough time for the maximum amount of DOX-loaded PAA/CaP@SLB NPs to become internalized in the cells, which led to a similar fluorescence intensity for both DOX-loaded PAA/CaP@SLB NPs and free DOX. The results obtained from FCM (Figure 4B and C) agreed well with the CLSM results. These results suggest that DOX-loaded PAA/CaP@SLB could effectively deliver DOX into the nuclei.

To investigate the safety of PAA/CaP@SLB NPs for in vivo applications, a hemolysis assay was conducted by using rabbit red blood cells (RBCs) from rabbit blood obtained from Laboratory Animal Center of Jilin University. Saline and deionized (DI) water were used as positive and negative controls, respectively. As presented in Figure 5, PAA/CaP@SLB NPs exhibited a negligible percentage of hemolysis of RBCs at various concentrations from 15.63 to 1000 \( \mu \text{g mL}^{-1} \). The result revealed that the PAA/CaP@SLB NPs have remarkable compatibility and may become a potential drug carrier for vein injection.

To investigate the potential of free DOX and DOX-loaded PAA/CaP@SLB NPs for the inhibition of tumor growth, an in vivo therapeutic efficacy study was carried out by using BALB/c mice with H-22 liver cancer. The body weight and tumor volume were measured over the whole treatment period of 11 d. As shown in Figure 6A, the average tumor volume in the control group (treated with saline) increased rapidly. Compared with the control group, the increase in tumor volume in the animals treated with free DOX and DOX-loaded PAA/CaP@SLB NPs was significantly reduced. DOX-loaded PAA/CaP@SLB NPs exhibited higher antitumor activity than free DOX, which showed that the improved cellular uptake with the lipid coating, the high accumulation at tumor sites through the EPR effect, and the pH-sustained DOX release of PAA/CaP@SLB NPs all contributed to the above result. As shown in Figure 6B, the body weight of the group treated with free DOX was significantly reduced whereas the group treated with DOX-loaded PAA/CaP@SLB NPs showed a smaller weight loss, which indicated that DOX incorporated in PAA/CaP@SLB NPs can reduce the toxicity of free DOX. After treatment for 11 d, the mice tumors were excised and weighed. As shown in Figure 6C and Figure S10, the tumor-inhibition rate of free DOX and DOX-loaded PAA/CaP@SLB NPs was 41.46 and 77.72, respectively, which was consistent with the tumor volume results. The result revealed that DOX-loaded PAA/CaP@SLB NPs resulted in higher inhibition than free DOX. According to the previous report,\cite{65} the inhibition rate of DOX-loaded AuNCs-A@PAA/CaP NPs and free DOX were 68.0 and 42.0%, respectively, with the H-22 cancer model. Compared with Au nanocluster assemblies@PAA/CaP NPs, the DOX-loaded PAA/CaP@SLB NPs also exhibited higher tumor-inhibition efficiency. This can be explained by the better cellular uptake and sustained DOX release after lipid coating. To evaluate the potential toxicity of synthetic PAA/CaP@SLB NPs in treated mice, histological analysis of major organs (heart, liver, spleen, kidneys) was performed. As shown in Figure 6D, no apparent changes were detected after treatment with PAA/CaP@SLB NPs in the hematoxylin and eosin (H&E)-stained sections (heart, liver, spleen, kidneys; see Figure 6D, images E–H) in comparison with the control group (Figure 6D, images A–D). These results suggest that PAA/CaP@SLB NPs have good biocompatibility for in vivo applications. Therefore, PAA/CaP@SLB NPs are a promising nanocarrier for effective intracellular DOX delivery, sustained release, and fewer side-effects in cancer therapy.

### Conclusions

In summary, the use of PAA as a template to prepare CaP NPs has a number of important merits, such as a simple and scalable synthetic route, a high DOX payload, and dual pH-responsive drug release. The use of lipids to engineer the surface of PAA/CaP NPs through a controlled solvent-exchange deposition is a simple and effective way to modify the surface of PAA/CaP NPs. The in vitro release experiment showed that the lipid bilayer could reduce DOX release in a physiological environment and had no effect on the pH-responsive drug-release profile. Also, the improved cellular uptake of PAA/CaP@SLB NPs was confirmed by the CLSM and FCM results. More importantly, when compared with free DOX, PAA/CaP@SLB NPs ex-
hibited excellent tumor growth inhibition in the in vivo antitu-
mor study, with fewer side-effects. These results reveal that the
PAA/CaP@SLB NPs are an ideal drugs nanocarrier for cancer
chemotherapy.

Experimental Section

Materials

Polyacrylic acid (PAA, $M_w \approx 1800$), the anticancer drug doxorubicin hydrochloride (DOX), Dulbecco’s modified Eagle’s medium (DMEM), fetal bovine serum (FBS), 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), and 4',6-diamidino-2-phenylindole di-
hydrochloride (DAPi) were obtained from Sigma (USA); 1,2-dioleyl-

3-glycerol-3-phosphocholine (DOPC) was obtained from Shanghai Advanced Vehicle Technology Pharmaceutical, whereas calcium
hydroxide (Ca(OH)2), isopropyl alcohol (IPA), alcohol, and disodium hydrogen phosphate dodecahydrate (Na2HPO4·12H2O) were sup-
plied by Sinopharm Chemical Reagent Beijing Co. Deionized (DI) water was used in all experiments.

Synthesis of PAA/CaP NPs
The synthesis method was a modification of our previous work.29 In brief, PAA (1 mL, 0.2 g mL−1) was dispersed in DI water (100 mL), in which Ca(OH)2 (60.0 mg) was subsequently dissolved. After com-
plete dissolution, IPA (200 mL) was introduced to the flask with magnetic stirring. Subsequently, Na2HPO4·12H2O (194.0 mg) was added to the flask under stirring for 2 h at RT. Finally, the resulting solution was transferred into a Teflon tube, sealed in a stainless steel auto-
clave and heated for 30 min at 120 °C. The obtained PAA/CaP NPs were washed three times with DI water and then re-dispersed in DI water for further use.

Preparation of PAA/CaP@SLB NPs
A controlled solvent-exchange deposition method was used to form PAA/CaP@SLB NPs. Briefly, empty or DOX-loaded PAA/CaP NPs (1 mg) was dispersed in a solution of DOPC (200 μL, 2.5 mg mL−1) in water/alcohol (1:1 v/v) in a flask. Then, water (1800 μL) was grad-
ually added to the flask. The lipid bilayer was deposited on the sur-
face of the PAA/CaP NPs, then the samples were washed three times to remove any residual solvent and remaining vesicles.

Physicochemical characterization
Transmission electron microscope (TEM) images were obtained by using an H-7500 transmission electron microscope at an accelerat-
ing voltage of 80 kV (Hitachi, Japan). Scanning electron microscopy (SEM) images and the energy-dispersive X-ray (EDX) spectrum were taken by using a HITACHI SU8010 field-emission scanning electron microscope (JEOL, Japan). High-resolution TEM (HRTEM) was car-
rried out by using a JEOLFETEM-2100F at an accelerating voltage of 200 kV (JEOL, Japan). X-ray diffraction (XRD) images were acquired by using a D8 Focus diffractometer with CuKα radiation (Brucker, Germany). The particle size was determined by using a Zetasizer Nano instrument (Malvern Instruments, UK) whereas FTIR spectra were recorded by using a Nicolet 6700 FTIR spectrometer (Nicolet, USA). X-ray photoelectron spectroscopy (XPS) analysis was carried out by using an ESCALAB 250Xi XPS (TFS, USA) with an AlKα X-ray source at 15 kV. Inductively coupled plasma atomic emission spectroscopy (ICP-AES) was measured with a Leeman ICP-AES Prodigy instrument. Nitrogen adsorption/desorption analysis was per-
fomed to measure the specific surface area and the pore size of original and supernatant DOX was analyzed by using UV/Vis at a wavelength of λ = 480 nm. The DOX loading efficiency (LE) was calculated by using Equation (1):

\[
LE = \frac{|m_{\text{original DOX}} - m_{\text{DOX in supernatant}}|}{m_{\text{NP}}} \times 100 \%
\]  

(1)

Release of DOX from PAA/CaP NPs and PAA/CaP@SLB NPs
The release profiles of DOX from PAA/CaP NPs and PAA/CaP@SLB NPs were investigated by using the semipermeable dialysis bag dif-
fusion method at pH 7.4 and 5.0 in PBS at 37 °C. PAA/CaP NPs and
PAA/CaP@SLB NPs that contained an equal amount of DOX were dispersed in PBS (1 mL, pH 7.4 and 5.0) separately and then trans-
ferred into the pretreated dialysis bags. Subsequently, the dialysis bags were placed in the corresponding PBS solution (5 mL) at 37 °C. At predetermined intervals, the amount of DOX released from the DOX-loaded NPs into the outer dialysis bags was quanti-
fied by using a UV/Vis spectrophotometer at a wavelength of λ = 480 nm. PAA/CaP NPs (1 mg) were dispersed into PBS (pH 5.0, 4 mL). The supernatants were collected at predetermined time intervals by using centrifugation, and tested by using ICP-AES to measure the Ca2+ content.

Cell viability assessment
MTT assays were carried out to evaluate the in vitro cytotoxicity of blank PAA/CaP NPs, PAA/CaP@SLB NPs, free DOX, DOX-loaded PAA/CaP NPs, and DOX-loaded PAA/CaP@SLB NPs by using HepG-2 cancer cells. For this, cells were seeded in 96-well plates (2.5 × 104 cells per well) and incubated for 24 h in DMEM with 10% FBS at 37 °C under 5% CO2 to allow attachment. Then, fresh media that contained a range of concentrations of blank PAA/CaP NPs, PAA/
CaP@SLB NPs, free DOX, DOX-loaded PAA/CaP NPs, and DOX-
loaded PAA/CaP@SLB NPs (100 μL) were added. One row of the 96-
well plate was a blank control that contained only culture medium. After incubation for an additional 24 h, the cells were washed three times with PBS, then MTT solution (20 μL, 5 mg mL−1 in PBS solution) was added to each well and cultured for a further 4 h. Then, the medium was replaced with DMSO (150 μL) to dissolve the MTT formazan crystals and the absorbance of each well at λ = 490 nm was measured by using a microplate reader. The cell viabil-
ity was determined by using Equation (2):

\[
\text{Cell viability} = \frac{\text{Abs(test cells)}}{\text{Abs(reference cells)}} \times 100 \%
\]  

(2)

In vitro intracellular uptake
FCM and CLSM were used to investigate the cellular internalization and intracellular release of free DOX, DOX-loaded PAA/CaP NPs, and DOX-loaded PAA/CaP@SLB NPs. For FCM, cells were seeded in a six-well plate (5 × 104 cells per well) and grown for 24 h. After cell attachment, the medium was re-
placed with serum-free culture medium that contained free DOX, DOX-loaded PAA/CaP NPs, or DOX-loaded PAA/CaP@SLB NPs (the concentration of DOX was 5 μg mL−1) and incubated for 3 or 6 h. The cells were then washed three times with PBS, harvested by using trypsinization, and collected by using centrifugation at 1000 rpm for 5 min, and suspended in PBS medium (400 μL) for FCM analysis.
For CLSM, cells were seeded onto glass cover slips on a 24-well plate (1 x 10^4 cells per well) and cultured overnight; then the cells were treated with free DOX, DOX-loaded PAA/CaP NPs, or DOX-loaded PAA/CaP@SLB NPs (5 μg mL⁻¹) for 3 and 6 h. Next, the cells were washed three times with PBS, fixed with paraformaldehyde (4% weight) for 20 min, and counterstained with DAPI for the cell nuclei. The glass cover slips were examined by using CLSM.

In vitro hemolysis assay

RBCs were obtained by using centrifugation to remove the serum, and carefully washed three times with physiological saline. Subsequently, the diluted RBC suspension (0.3 mL, 10%) was added to PAA/CaP@SLB NPs to make a series of NP concentrations (1.5 mL, 15.63, 31.25, 62.5, 125, 250, 500, 1000 μg mL⁻¹). The mixtures were vortexed and incubated at 37 °C for 2 h, then the mixtures were centrifuged at 3000 rpm for 5 min and the absorbance values of the supernatant were measured at λ = 570 nm by using a UV/Vis spectrophotometer. Saline and DI water were used as positive and negative controls, respectively. The hemolysis percentage was calculated by using Equation (3):

\[
\text{Hemolysis} \% = \frac{(A_{\text{sample}} - A_{\text{negative}})}{(A_{\text{positive}} - A_{\text{negative}})} \times 100 \%
\]  

in which \(A_{\text{sample}}\), \(A_{\text{positive}}\), and \(A_{\text{negative}}\) represent the absorbency of the sample and the positive and negative controls.

In vivo antitumor efficacy

All animal experiments were performed in accordance with the guidelines of the Institutional Animal Use Committee. The in vivo anticancer efficacy of DOX-loaded PAA/CaP@SLB NPs was assessed by using a model that involved mice with H-22 BALB/c. When the tumor reached 100–150 mm³, the mice were randomly assigned to three groups (n = 6), and were given an intravenous injection of physiological saline into the tail, free DOX, or DOX-loaded PAA/CaP@SLB NPs (dose: 5 mg DOX per kg body weight) once a day for 5 d. The tumor volumes and body weights were recorded every other day after treatment and the tumor volume was calculated by using Equation (4):

\[
\text{Volume} = a \times b \times \frac{c}{2}
\]  

in which \(a\) and \(b\) are the length and width of the tumor, as measured by calipers. After treatment for 11 d, all the mice were sacrificed, and their tumors were excised and weighed, and the tumor growth inhibition rate was calculated according to Equation (5):

\[
\text{Inhibition} = \frac{(C - T)}{C} \times 100 \%
\]  

in which \(C\) is the average tumor weight of the control group and \(T\) is the average tumor weight of each of the treated groups.

Histology examination

Eight healthy mice were randomly assigned to two groups (n = 4 per group) and given an intravenous injection of physiological saline or PAA/CaP@SLB NPs into the tail. 11 d after treatment, the major organs (heart, liver, spleen, and kidney) were excised from the sacrificed mice, fixed with paraformaldehyde solution (4% weight), and sectioned (4 μm thick) routinely. All the tissue sections were stained with H&E for histological analysis.

Statistics analysis

All experiments were performed at least three times and expressed as mean SD. Data were analyzed statistically by using the Student’s independent sample t-test and expressed as a one-way p-value; \(p < 0.01\) was considered to be statistically significant.

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Conflict of interest

The authors declare no conflict of interest.

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