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ARTICLE

Reverse micelle-in-microspheres with sustained release of water-soluble combretastatin A4 phosphate for S180 tumor treatment

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Combretastatin A4 phosphate (CA4P), the water-soluble phosphate pro-drug of combretastatin A4 (CA4), acts as a novel vascular disrupting agent (VDA). It strongly inhibits tubulin polymerization and selectively exerts action against tumor blood vessels. However, the quite short half-life (~30 min) results in short action time and consequently limits its application. Therefore, it is beneficial to develop a sustained-release preparation of CA4P for relatively long action duration. On the other hand, conventional PLGA microspheres display poor encapsulation capability for water-soluble small molecules due to rapid diffusion outside. Hence, we developed a CA4P-loaded microsphere (CA4P-MS) composed of methoxy poly(ethylene glycol)-b-polylactide (PELA) reverse micelle and PLGA with nano-in-micro sea-island structure. The CA4P encapsulation efficiency of CA4P-MS reached 92.88%. More interestingly, sustained drug release was achieved, which contributed to the prolonged inhibition effect on S180 tumor during a 30-day period with one-time subcutaneous administration. In conclusion, our study established a biodegradable polymer based platform to construct microspheres for water-soluble small molecular compound with sustained release and long-lasting action.

Introduction

Combretastatin A4 phosphate (CA4P) is under investigation as a vascular disrupting agent (VDA) in Phase III clinical trials.[1,2] Post intravenous injection, CA4P gets hydrolyzed into active combratastin A4 (CA4) by nonspecific endogenous phosphatases in plasma, which then strongly inhibits tubulin polymerization and selectively acts against tumor blood vessels.[3] So far the action of CA4P has been confirmed in the treatment of anaplastic thyroid,[4] non-small-cell lung[5] and ovarian cancers.[6] However, the short half-life (~30 min)[7] of CA4P results in quite transient effective reaction time, frequent dosing and negative patient compliance, which significantly limits its application.[8] Therefore, it is beneficial to develop a sustained release preparation of CA4P with relatively long duration of action and sustained activity.

Microspheres, a kind of well-known drug microcarriers, have displayed high potential for long-acting therapy with sustained drug release.[9] Biodegradable polymer microspheres, in particular, provide controlled drug release kinetics by regulating polymer chemical structure with appropriate degradation characteristics.[10] In addition they are hydrolyzed *in vivo* to generate products that could be easily reabsorbed or eliminated.[11] Poly(D,L-lactic-co-glycolic acid) (PLGA) is one of the few polymers approved by the US FDA for medical application for its biodegradability and biocompatibility.[12] PLGA has gained reputation for delivery of various proteins, peptides and also small molecule hydrophobic drugs. Several products related to peptides were commercialized, including leuprolide acetate for prostate cancer with 1, 3 or 4 months release period, triptorelin for LHRH agonist with 1 month release period, octreotide acetate for acromegaly with 1-4 months release period.[13] However, conventional PLGA microspheres display poor encapsulation for water-soluble small molecules due to their quick diffusion outside. For instance, the encapsulation efficiency (EE) of water-soluble etanidazole was only 39.6% with PLGA

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polymer alone for microsphere preparation.[14] Similarly, the EE of CA4P in PLGA was determined as low as 48.73% in this study.

Therefore, we established a novel method to prepare CA4P-loaded microspheres (CA4P-MS) by the combination of methoxy poly(ethylene glycol)-b-polylactide (PELA) reverse micelles and the PLGA microspheres. Amphiphilic copolymers are composed of hydrophilic blocks and hydrophobic blocks. The ratio of hydrophilic blocks to hydrophobic blocks determines the water solubility and application of copolymers. Taking PELA as an example, those with excellent water solubility were widely explored as self-assembled micelles for cancer target therapy.[15] As for water insoluble PELAs, they are attractive to be microsphere matrix to improve the stability of peptides, proteins and genes due to their better compatibility compared with PLGA.[16] Furthermore, PELA was supposed to be compatible with PLGA due to their similar chemical structure. Therefore, by regulating the weight ratio of methoxy poly(ethylene glycol) (PEG) block to polylactide (PLA) block, PELA herein was hypothesized to solubilize water soluble CA4P in the organic phase of PLGA as a macromolecular surfactant distributed in the PLGA base with the aim to improve CA4P loading in PLGA microspheres.[17] In addition, this resultant microsphere with the unique nano-in-micro structure was also expected to sustain the release and action of CA4P.

Experimental

Materials

Combretastatin A4 phosphate (CA4P) was purchased from Great-forest Biology (Hangzhou, China). Poly(D,L-lactic-co-glycolic acid) (PLGA, molecular weight (MW: 20 k, LA:GA=50:50) were purchased from Dai Gang Biology (Shandong, China). Methoxy poly(ethylene glycol) (PEG, MW: 5k) was purchased from Sigma-Aldrich (St. Louis, MO, USA). D,L-lactide (LA) was purchased from Shandong Institute of Medical Instruments (Shandong, China). Dimethyl carbonate and poly(vinyl alcohol) (MW 44.05, alcoholysis: 87.0~89.0%, mol/mol) were purchased from Aladdin Chemistry Co. Ltd (Shanghai, China). Ethyl acetate, Tween-80 and chloroform were purchased from Sinopharm Chemical Reagent Co. Ltd (Shanghai, China). All chemicals were used as received without purification.

Synthesis and characterization of amphiphilic copolymer PELA

Methoxy poly(ethylene glycol)-b-polylactide (PELA) was synthesized by ring-opening polymerization of LA using PEG as a macromolecular initiator at the weight ratio of 15:85 (LA:PEG) and 0.5 wt% Sn(Oct)2 as a catalyst. PEG, LA and Sn(Oct)2 were dissolved in distilled toluene in a reaction vessel which was incubated at 130 °C for 24 h under stirring. After cooling, the reaction product was dissolved in dichloromethane (DCM) and precipitated in cold diethylether. Subsequently, the copolymer was filtered and dried under vacuum. The PELA structure was characterized by 1H NMR spectroscopy using CDCl3 as the solvent. The molecular weight (MW) of PELA polymer was determined by gel permeation chromatography (GPC D40, TSK Co., Japan) analysis using dimethylformamide (DMF) as the mobile phase and monodispersed polystyrenes as standards.

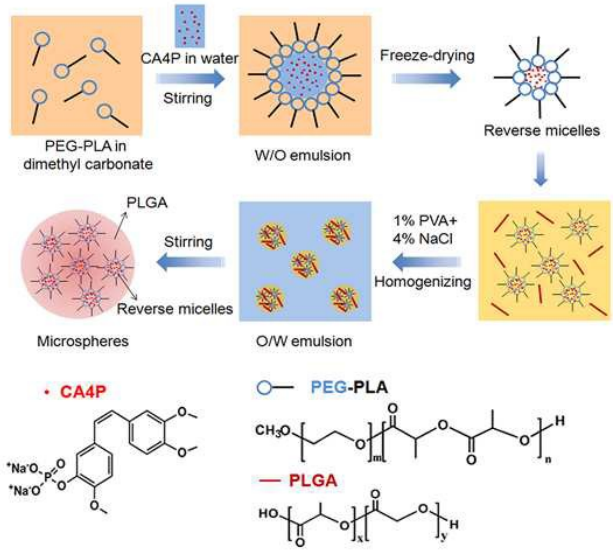
Solubilization of water-soluble CA4P in chloroform phase

To validate the solubilization effect of PELA on CA4P in an organic phase, the drug extraction test was carried out. 1 mL PELA chloroform solution at various concentrations was mixed with 1 mL aqueous solution of CA4P (10 μg/mL). Then the mixture was shaken for 3 min. After centrifugation at 4000 rpm for 5 min, the concentration of CA4P in both aqueous and organic phases was determined by HPLC (Shimadzu, LC-20AT, Japan) at flow rate of 1.0 mL/min using a mobile phase consisting of acetonitril/methanol/20 mM ammonium acetate buffer (10:40:50, v/v), and detected at 301 nm.

The preparation method for the CA4P-MS

The preparation method of the novel microsphere CA4P-MS is outlined in **Sheme.1**. The typical procedure is as followed. Firstly, we added an aqueous solution of CA4P (20 mg/mL, 100 μL) drop by drop to a dimethyl carbonate solution (DMC) of PELA (100 mg/mL, 1 mL). The resultant mixture was sonicated at 400 w for 20 s (Scientz, JY92-2D, China) and then was lyophilized to produce solid CA4P-loaded reverse micelles (CA4P-RM). Secondly, the quantitative CA4P-RM (102 mg) and PLGA (100 mg) were dissolved in ethyl acetate (1 mL). This organic phase was added dropwise to 30 mL external aqueous phase containing 1% w/v poly(vinyl alcohol) (PVA) and 4% w/v NaCl, which was homogenized for 3 min. Afterward the CA4P-MS suspension was stirred for 4 h and collected by centrifugation. The resulting CA4P-MS was washed by

deionized water for three times and lyophilized for 24 h. The blank microsphere was prepared by the same method just without CA4P.



Scheme 1 Experimental procedures for preparing reverse micelles-in-microspheres and the structure illustration of microspheres.

Characterization of CA4P-RM and CA4P-MS

Differential scanning calorimetry (DSC) was used to examine the compatibility of PELA and PLGA. Three samples including PELA, PLGA, and the blank PELA/PLGA microspheres were weighted (about 7 mg) in an aluminium pan and analyzed (Q100, TA Znotruments, USA). The samples were cooled to -50 ℃, followed by heating to 150 ℃ at a constant rate of 10 ℃/min.

The morphology of CA4P-RM was examined by the transmission electron microscopy (TEM, NEC, JEM-1200EX, Japan). Briefly, CA4P-RM was dispersed in ethyl acetate. Then one drop of this solution was incubated on carbon coated copper grid and then was fixed onto the sample holder, placed in the vacuum chamber of TEM and observed under high vacuum.

SEM observation of CA4P-MS was performed on a Hitachi S-3000 microscope at an acceleration voltage of 1.5 kV. The samples were coated with a thin layer of Pt in a vacuum, and then transferred to the SEM instrument for measurement.

To visualize the structure of CA4P-MS more clearly, we prepared Rhodamine-B loaded microspheres (Rhodamine-B-MS) by the same method as CA4P-MS. Its constructure was observed by confocal laser scanning microscopy (CLSM, LSM-510, ZEISS, Germany).

The particle size and size distribution of CA4P-RM were determined by dynamic light scattering (DLS, Malvern, Nano-S90, UK) and that of CA4P-MS were measured by Laser Particles Size Analyzer (Winner, 2005A, China).

X-ray diffraction (XRD) analysis was performed to examine the crystalline properties of CA4P encapsulated in reverse micelles and microspheres. XRD spectra of free CA4P, PELA, PLGA, CA4P-RM and CA4P-MS were determined using an X-ray diffractometer (Ultima IV, Rigaku, Japan).

The amount of CA4P was determined by dissolving about 10 mg of the freeze-dried microspheres in the mixture of 1 mL distilled water and 3 mL acetonitrile, filtered (0.45 μm) and then performed by HPLC (Shimadzu, LC-20AT, Japan) at flow rate of 1.0 mL/min using a mobile phase consisting of acetonitril/methanol/20 mM ammonium acetate buffer (10:40:50, v/v), and detected at 301 nm. The CA4P loading content (LC) and EE was calculated by the following equations, respectively: LC% = (Mass of drug in microspheres/Mass of microspheres) × 100%, EE% = (Mass of drug in microspheres /mass of drug feed) × 100%. All analyses were conducted in triplicate (n=3) and the means ± SD are reported.[18]

***In vitro* drug release from microspheres**

To examine drug release profiles, around 10 mg CA4P-MS of the optimal formulation was suspended in 4 mL of the release buffer consisting of 0.02% (v/v) Tween-80 and PBS (pH7.4). The samples were placed in an incubator, and shaken at 100 rpm, 37 °C. At the predetermined time point (1, 5, 10, 15, 20, 25, and 30 d), the samples were centrifuged at 4000 rpm for 8 min and replaced with fresh buffer of equal volume. The concentration of CA4P in the supernatant was measured using HPLC at a wavelength of 301 nm. All samples were run in triplicate and data points are shown as mean ± standard deviation.

***In vitro* degradation of microspheres**

In order to track the structure deformation of the microspheres during the drug release, we performed the SEM analysis on the samples. About 10 mg CA4P-MS were incubated in 2 mL of release buffer consisting of 0.02% (v/v) Tween-80 and PBS (pH7.4) at 37 ℃

for a period of 1 month and the samples were removed periodically and freeze-dried to examine the changes of the physical characters by SEM.

***In vivo* antitumor effect and pharmacokinetics studies**

S180 tumor-bearing ICR mice (18~20 g) were used in the pharmacodynamics experiment. The mice were implanted with mouse S180 ascites sarcoma subcutaneously in the right flank, and when the tumor volume reached 100-300 mm^3, fifteen mice were randomly divided into 3 groups (*n*=5 per group) for subcutaneous injection in the left flank with different formulations. The administration plan was carried out as follows: saline once for control group, a CA4P solution at 5 mg/kg dose once daily for 6 days , and a CA4P-MS suspension once at a dose of 30 mg/kg CA4P. Tumor diameters and mice body weight were measured every 2 days and tumor volumes (mm^3) were calculated by the formula: tumor volume = (length×$width^2$)/2. At the end of the treatment, the tumors were excised for immunohistochemical examination. The tumors were fixed in 4% formaldehyde overnight, embedded and prepared into serial sections (4 μm thick). a CD31 antibody was used for microvessel staining for analysis of tumor vasculature and a Ki67 antibody was used for cell proliferation analysis.[8]

In the pharmacokinetics experiment, CA4P-MS group, at the predetermined time points of 1, 2, 4, 10, 12, 16, 18, 21, 26, and 28 d post-injection, 0.8 mL of blood samples were collected into EDTA tubes and then centrifuged at 6000 rpm for 5 min to obtain plasma. For CA4P solution group, the time points for blood collection were set as 20, 30, 60, 90 min after each administration and this performance was repeated 6 times. 100 μL samples of plasma were added to 100 μL ethyl acetate and vortexed for 5 min, followed by centrifugation at 3000 rpm for 10 min. Then the supernatant was obtained and the precipitation was re-extracting once according to like the above. All the supernatant were mixed, blow-dryed through pure nitrogen and then added 100 μL acetonitrile to dissolve the drug. The samples were measured using HPLC to determine concentration of CA4 because CA4P had been hydrolyzed by the phosphodiesterase to the active pharmaceutical ingredients of CA4. Based on the data of CA4 concentration in plasma, a series of pharmacokinetics parameters (half-life ($t_{1/2}$), area under drug plasma concentration - time curve (AUC), total body clearance (CL), mean residence time (MRT), etc) were automatically calculated using pharmacokinetic software (Kinetica 4.4).

The above animal studies were performed in accordance with the guidelines of the Institutional Animal Care and Use Committee of Zhejiang University.

Results and discussion

Synthesis and characterization of PELA

PELA was synthesized by ring-opening polymerization of LA. The chemical structure was confirmed using ^{1}H NMR. In the ^{1}H NMR spectrum (**Fig.1**), the characteristic signals of PLA were at 1.57 ppm (-CH_3) and 5.18 ppm (-CH), and those of PEG were at 3.38 ppm (-OCH_3) and 3.65 ppm (-CH_2-CH_2-). The MW of PLA segments was calculated as 26548 according to the MW of PEG (5000) and the integral values of peak area of -CH (δ 5.18) and -OCH_3 (δ 3.38), so the experimental value of PELA MW was about 31548. According to the GPC measurements, the average molecular weight of PELA was 31233 with a polydispersity index (PDI) of 1.66, which was consistent with the ^{1}H NMR analysis result.

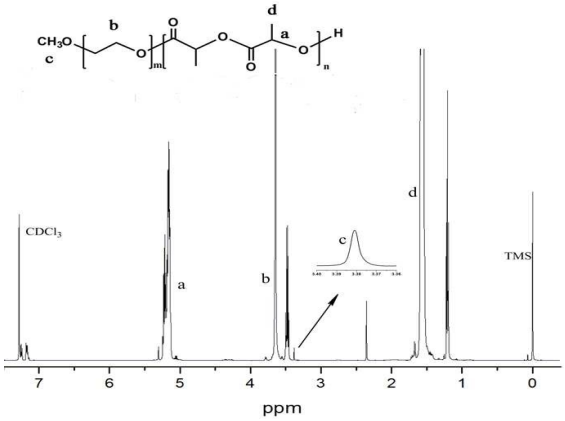


Fig.1 The ^{1}H NMR spectrum of PELA.

Solubilization of water-soluble CA4P in organic phase

The PELA could only be dispersed in water but is easily dissolved in chloroform owing to the relatively long hydrophobic PLA segments. Therefore, according to the self-assemble characteristics of amphiphilic copolymers, we assumed PELA could form reverse micelles with PEG cores and PLA shells in an organic solvent.

To confirm the above assumption and investigate the solubilization effect of PELA on CA4P in organic solvents, the concentration of CA4P in the oil phase and water phase was

measured after extraction. As displayed in **Fig.2**, no solubilization of CA4P in the chloroform was observed in the absence of PELA. This illustrates that CA4P is completely chloroform-insoluble compound. However, a significant decrease and increase in CA4P concentration was observed in water and chloroform, respectively, after PELA addition. Furthermore, the drug concentration change in both phases was closely related to the increase of PELA amount. These results suggest that amphiphilic PELA could provide an isolated aqueous microenvironment for water soluble CA4P in an organic phase in form of self-assembled reversed micelles.[19]

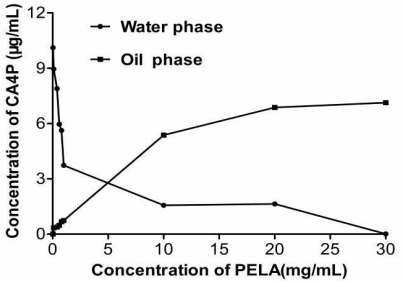


Fig. 2 CA4P concentration in water phase and oil phase before and after extraction with PELA solutions in chloroform.

Preparation and characterization of microspheres

The CA4P-MS was prepared by a novel method of two steps (**Scheme 1**). In the first step, we prepared CA4P-RM by emulsification and lyophilization. Based on the solubilization effect of PELA on CA4P in an organic solvent, CA4P loading and self-assembly of PELA reversed micelles in DMC was simultaneously accomplished with the addition of CA4P solution. After freeze-drying, the solid CA4P-RM was collected for the subsequent step. DMC is considered to be a green reagent with much lower toxicity compared to dichloromethane in common use during the conventional microspheres preparation. Moreover DMC can freeze at the same temperature as water and Koyamatsu[20] used it to produce solid nanoparticles by lyophilization. TEM images in **Fig. 3a and 3b** displayed the discrete and spherical morphology of CA4P-RM with a narrow size distribution (Z-average 213.8 nm, PDI 0.063). The inserted photograph in **Fig.3b** indicated CA4P-RM was suspended but not dissoloved in water.

In the second step, the quantitative PLGA and CA4P-RM were dissolved in ethyl acetate to form the organic phase. This organic phase was added dropwise to 30 mL external aqueous phase containing 1% w/v PVA and 4% w/v NaCl to form CA4P-MS. The addition of NaCl in the external phase could increase osmotic pressure and slow polymer precipitation. This led to the formation of nonporous microspheres with increased encapsulation efficiency.[21] Besides, an appropriate PVA concentration increased the viscosity of external phase to prevent emulsion droplets from coalescence, which enhanced the stability and reduced particle size of emulsion droplets.[22] Drug encapsulation and release profile could be adapted by choosing PLGA with appropriate properties[23]. The MW of PLGA used for sustained release are often relatively less than 50k and the LA:GA ratio ranges from 50:50 to 100:0. Relatively low MW, low LA:GA ratio and un-capped polymer end groups made the PLGA polymer less hydrophobic with increased speed for water absorption, hydrolysis and matrix erosion.[24,25] So we selected PLGA (MW: 20 k, LA:GA=50:50) for the second step encapsulating CA4P-RM. The CA4P-MS was prepared by the solvent evaporation method and the resultant lyophilized CA4P-MS was loose white powder (**Fig. 3d**). The images of CA4P-MS taken by SEM were displayed in **Fig. 3c** with spherical outline and smooth and intense surface devoid of any obvious defects.

Since the compatibility of PELA and PLGA was the premise to construct composite microspheres, we performed DSC measurement of PELA, PLGA and the blank microspheres. In the DSC curves (**Fig. 3e**), PELA or PLGA displayed a crystallization peak at about 48.9 ℃ and 40.5 ℃, respectively, while the crystallization peak of blank microspheres was about 47.0 ℃, between those of PLEA and PLGA. This result indicated that PELA could be compatible with PLGA well. Therefore, the nano-in-micro sea-island structure of microspheres was presumed, where hydrophilic PEG blocks of PELA formed nanoscaled aqueous area while the hydrophobic PLA segments of PELA fully stretched out and homogeneously mixed with hydrophobic PLGA base.

This assumption was further confirmed by the following experiment. Since Rhodamine-B is a water soluble fluorescent substance, it was ready to be encapsulated in the aqueous lumen of PELA reverse micelles during the microsphere preparation process and was thus used to label PELA reverse micelles in the microspheres. As shown in **Fig. 3f**, the dots with red flurorensence were distributed wildly throughout the whole microsphere matrix. This result confirmed that PLGA could encapsulate PELA reverse

micelles successfully with a unique nano-in-micro sea-island structure.

According to the results of XRD spectra, the characteristic XRD peaks of CA4P disappeared in the spectrum of CA4P-RM and CA4P-MS (**Fig.4**), thus implying that CA4P was encapsulated into the reverse micelles and then into the microspheres in an amorphous state.

We also investigated the effect of PLGA and PELA feed mass on the CA4P EE. With the regulation of PLGA and PELA amount, the mean diameters of the resultant microspheres were changed in the range of 16.54~20.41 μm (**Table 1**). Since the polymers PELA and PLGA were the main skeleton materials, increasing the concentration of PELA and PLGA could apparently enhance the viscosity of the oil phase, thus decreasing the leakage of CA4P. As a result, the drug LC and EE of CA4P was improved from 0.64% to 0.92% and from 48.73% to 92.88%, respectively, with the increase of polymers PELA and PLGA amount. Thus, Formulation 5 of 2 mg CA4P, 100 mg PELA and 100 mg PLGA was optimized for the further research, in which CA4P EE achieved 92.88% and particle size of 20.41 μm with a narrow size distribution of 0.85 μm (**Fig. 3d**).

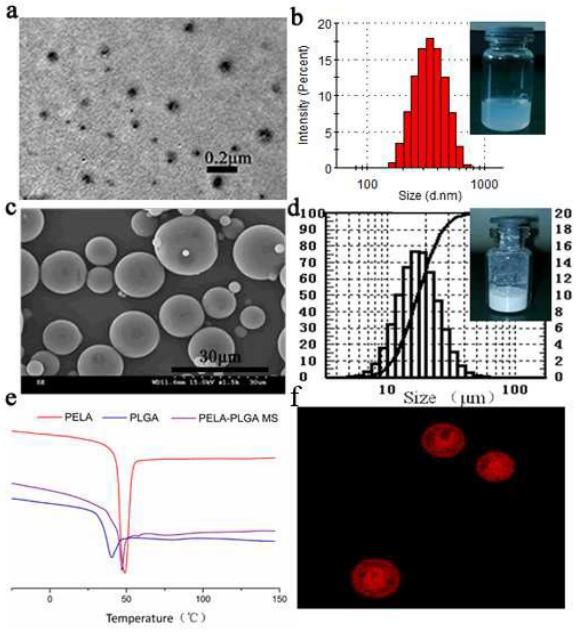


Fig. 3 (a) TEM image of CA4P-RM. Scale bar=200 nm. (b) Particle size distribution and the photograph of CA4P-RM dispersed in ethyl acetate . (c) SEM image (scale bar=30 μm) of CA4P-MS. (d) Particle size distribution and the photograph of CA4P-MS powders. (e) DSC thermogram of PELA, PLGA, and the blank microspheres. (f) Confocal image of Rhodamine-B-MS.

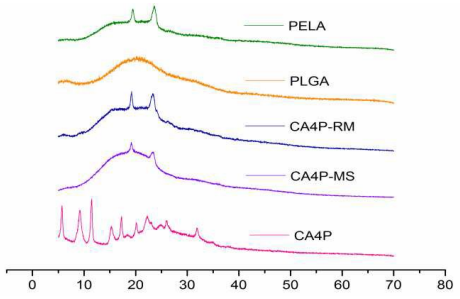


Fig. 4 XRD analysis of PELA, PLGA, CA4P-RM, CA4P-MS, and CA4P.

Table 1 Particle size, drug loading (LC) and encapsulation efficiency (EE) of various formulation parameters.

Formulation	CA4P (mg)	PELA (mg)	PLGA (mg)	Size (μm)(span)	LC (%)±SD	EE (%)±SD
1	2	50	100	16.54 (0.90)	0.64±1.6	48.73±2.6
2	2	100	50	19.89 (1.02)	0.74±1.4	56.06±3.5
3	2	75	100	17.52 (0.78)	0.78±2.2	69.35±2.1
4	2	100	75	17.27 (0.96)	0.85±3.0	75.47±2.4
5	2	100	100	20.41 (0.85)	0.92±2.6	92.88±1.8

***In vitro* drug release from microspheres**

The drug release profile at 37 °C was shown in **Fig. 5**. A typical triphasic release profile was observed with: 1) an initial burst release phase mainly during the 24 h where more than 20% of the drug was released; 2) a lag phase for about 30% of drug release in the next 5-15 days; and 3) a sustained release phase at the following 10 days.[26] It was validated that the drug release of polymer microspheres is simultaneously controlled by particle size, polymer degradation rates, and pore formation during degradation.[27] The main mechanisms connected with drug release from PLGA-based microspheres refer to drug diffusion and polymer degradation. It is accepted to be diffusion-controlled initially, degradation-controlled in the lag phase, and diffusion and degradation-controlled during the sustained drug release period.[28] In our study, the high initial release rate could be attributed to the high water-solubility of CA4P and mainly involved the drug adsorbed or adhered on the surface of the particles.[29] During the lag phase, the inner structure of microspheres was still relatively dense due to the slow bulk degradation of PLGA, which limited the migration of CA4P from the core position toward exterior of the

microspheres. Transport through water-filled pores is the most general way of drug release, as the encapsulated CA4P is too hydrophilic to be transported through the hydrophobic polymer matrix phase. As for our nano-in-micro sea-island structure, the PEG segments of CA4P-RM facilitated the CA4P loading and also formed individual hydrophilic areas. And these hydrophilic pores could transport water inside to dissolve CA4P and cause hydrolysis to PLGA. These hydrophilic pores were too small for drug transport during the early release period, but as the number and size of water-filled pores in the polymer matrix increased, a porous connected network allowing drug diffusion was formed[30]. Furthermore the polymer degradation was accelerated in the last stage followed by the accumulation of much more acidic oligomers, which also accelerated drug release.[12] As a result, the CA4P released fully from CA4P-MS when the physical structure of microspheres no longer existed due to the complete degradation by a month.

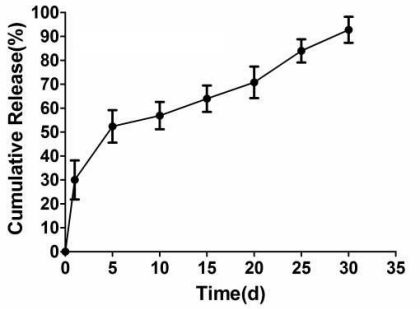


Fig.5 *In vitro* release profile of CA4P from CA4P-MS of Formulation 5.

***In vitro* degradation of microspheres**

To identify the effect of the microsphere degradation on CA4P release, meanwhile, we removed CA4P-MS from the release medium at the certain time intervals and observed their physical structure changes by SEM during the drug release for 1 month. As depicted in **Fig. 6**, the microspheres maintained intact structure on the first day and began to disintegrate slowly after dispersion in PBS buffer after 5 days. The external section of microspheres began to degrade and pores were observed on the microsphere's surface. Moreover, until 15 days, the microparticles converted from original smooth spheres to non-regular form with a rough surface. On the 28th day, SEM pictures showed the breakdown products due to the polymers degradation and drug release, and the spherical form was completely collapsed. Conventional microspheres prepared by solvent evaporation method with polymer PLGA (LA:GA=50:50) led to a slow drug release of about 40 days,[31] much slower than our novel CA4P-MP. So we could draw a conclusion that the addition of PELA accelerated the degradation of microsphere due to the formation of numbers of hydrophilic pores as discussed above.

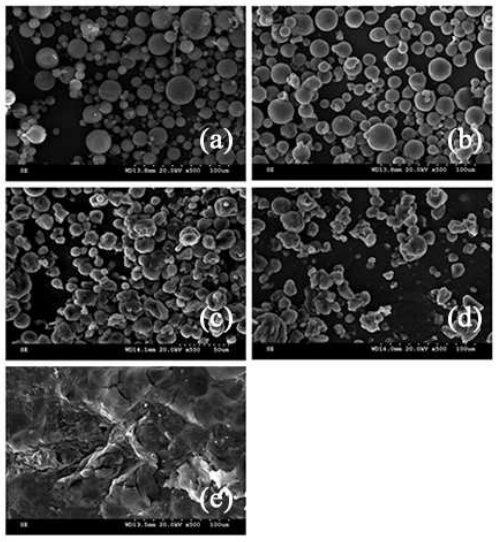


Fig. 6 SEM images of CA4P-MS after degradation: (a) 0 d, (b) 1 d, (c) 5 d, (d) 15 d and (e) 28 d.

***In vivo* pharmacokinetics and antitumor studies**

The pharmacokinetic parameters were measured by fitting the data to a two-compartment model. As clearly shown in **Fig. 7A**, the plasma drug concentration of CA4P solution group increased sharply and dropped rapidly during each administration period to form six peak-valley fluctuations due to its short half-life. However, the plasma concentration of CA4P-MS group maintained at a relatively stable level after drug burst release stage during the whole therapy. The half-life of distribution phase ($t_{1/2\alpha}$) for CA4P solution was calculated as 21.6 min while that of CA4P-MS was extended to 36.04 d. In addition, for CA4P-MS, the AUC was 4.69-fold larger, the CL was 6395.9-fold shorter, and the MRT was much longer than that of the CA4P solution. These parameters demonstrate sustained release of CA4P encapsulated in this novel microsphere, which would contribute to the sustained inhibition effect of CA4P on tumor growth.

In vivo tumor inhibition was shown in **Fig. 7B**. It clearly demonstrates that the tumor size of CA4P-MS-treated group was significantly smaller than that of CA4P solution-treated mice,

though the total dose of CA4P was maintained the same for two groups. During the first week, the continuous infusion of CA4P solution inhibited the tumor growth greatly. In the microsphere group, the initial burst release offered effective drug concentration to suppress the tumor growth. However, in the long term of one month, the tumor sizes rapidly increased in CA4P solution group while the sustained drug release of CA4P-MS provided continuous tumor suppression during 30-day period. In addition, no mice died and a steady increase of mice body weight (**Fig. 7D**) was observed after administration of the CA4P-MS suspension, suggesting the safety for its *in vivo* application. **Fig. 7C(a)** revealed that tumor tissues in control (normal saline) display numerous vessels with the lumen structure. However, those treated with CA4P-MS displayed few tumor vessels (**Fig. 7C(b)**), indicating that the sustained-release of CA4P from CA4P-MS significantly destroyed tumor neovasculature. CA4P could not only collapse abnormal vasculature in tumors but also inhibit tumor cell proliferation by binding to tubulin and arresting mitosis. Therefore, we used Ki67, a marker of overexpression in proliferating cancer cells, to assess the proliferation. As shown in **Fig. 7C(c)** and **Fig. 7C(d)**, CA4P-MS induced apparent proliferation-inhibition, whereas the control group showed no effect on tumor growth.

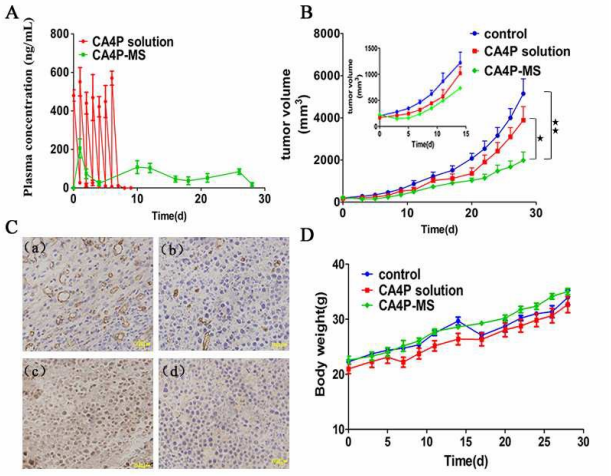


Fig. 7 (A) Plasma concentration-time curves in mice treated with CA4P solution and CA4P-MS. All data are means ± SD for five mice per group. (B) S180 tumor growth curves in mice treated with CA4P solution and CA4P-MS. * $P < 0.05$; ** $P < 0.01$ (C) Immunostaining with anti-CD31 was used to detect tumor vasculature: (a) Control and (b) CA4P-MS； Tumor sections were immunostained with anti-Ki67 nuclear antigen for cell proliferation: (c) Control and (d) CA4P-MS. (D) Body weight of mice after the various treatments.

Conclusion

In summary, we successfully developed a novel reverse micelle-in-microsphere to encapsulate water soluble small molecular drug CA4P with EE as high as 92.88%. More interestingly, significant sustained-drug release was achieved, which exhibited a sustained-inhibition effect on S180 tumor during 30-day period with one-time administration. In summary, our study established a biodegradable polymer-based platform to construct microspheres for water-soluble small molecular compounds with sustained release characteristics.

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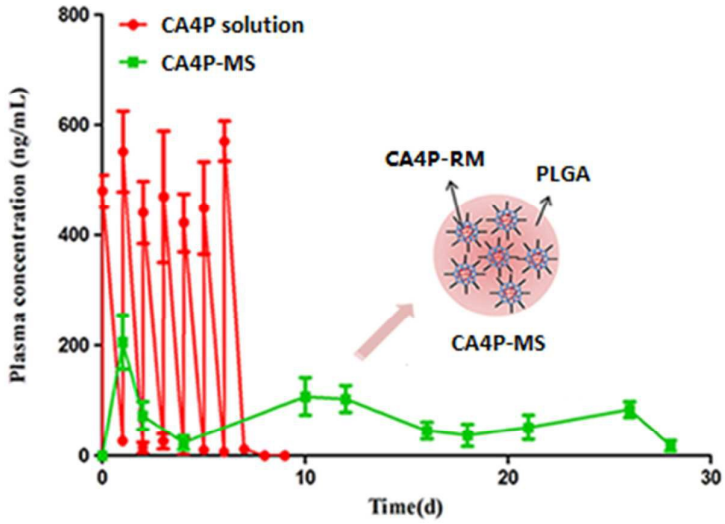
Graphic abstract



A CA4P-loaded microsphere (CA4P-MS) composed of PELA reverse micelle (CA4P-RM) and PLGA with sea-island structure was prepared. This unique construction can greatly improve the encapsulation efficiency of water-soluble CA4P and provide sustained drug release and action for cancer therapy.