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Long-circulating polymeric nanoparticles bearing a combinatorial coating of PEG and water-soluble chitosan

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ABSTRACT

A major obstacle in the development of polymeric nanoparticles (NPs) as effective drug delivery vesicles is the rapid clearance from blood. In order to realize a significant prolongation in blood circulation, a combinatorial design, covalent attachment of polyethylene glycol (PEG) to polylactic acid (PLA) and physical adsorption of water-soluble chitosan (WSC) to particle surface, has been developed for surface modification of PLA NPs. Two types of WSC, cationic partially deacetylated chitin (PDC) and anionic *N*-carboxy propionyl chitosan sodium (CPCTS) were investigated. All the NPs formulated in the size range of 100–200 nm were prepared by a modified w/o/w technique and physicochemically characterized. *In vitro* phagocytosis by mouse peritoneal macrophage (MPM), *in vivo* blood clearance and biodistribution following intravenous administration in mice, of these NPs labeled with 6-coumarin, were evaluated. The presence of WSC, whether alone or with PEG, highly improved the surface hydrophilicity as well as suspension stability of NPs. Their surface charge was greatly affected by the WSC coating, being close to neutrality for PEG/PDC NPs and highly negative in the case of PEG/CPCTS NPs. In comparison to NPs treated with PEG or WSC alone, the synergistic action of PEG and WSC strongly inhibited the macrophage uptake and extended the circulation half-life ($t_{1/2}$) with concomitant reduced liver sequestration. Particularly, PEG/PDC NPs showed the most striking result with regard to their performance *in vitro* and *in vivo*. Calculated $t_{1/2}$ of PEG/PDC NPs and PEG/CPCTS NPs was 63.5 h and 7.1 h, respectively, much longer than that of control PEG/PVA NPs (1.1 h). More WSC materials need to be evaluated, but the present data suggest that, a combinatorial coating of PEG and PDC greatly prolongs the systemic circulation of NPs and represents a significant step in the development of long-circulating drug delivery carriers.

1. Introduction

Over the past few decades, there has been considerable interest in developing polymeric nanoparticles (NPs) as effective drug delivery devices. NPs, mainly composed of poly(lactic acid) (PLA) or poly(lactide-*co*-glycolide) (PLGA), are biocompatible and biodegradable, and exhibit excellent incorporation capability for hydrophilic drugs, proteins and plasmid DNA [1–3]. However, a major obstacle for achieving effective drug targeting to its therapeutic sites within the body is the rapid opsonization and following sequestration of intravenously administered NPs by cells of mononuclear phagocytic system (MPS) [4]. Adsorption of plasma proteins (opsonins) onto the surface of a NP, also know as opsonization, is critical to the process of phagocytic recognition [5,6]. The design of long-circulating NP is therefore reliant upon an 'invisible' surface that can avoid opsonic adsorption and the subsequent removal from the blood by phagocytic cells.

Chemical and physical properties of the NPs, including size, surface charge and surface chemistry are key factors that determine their *in vivo* fate after intravenous (i.v.) administration [7,8]. The desired diameter of intravascular long-circulating NPs has been reported within a relatively narrow range (70–200 nm) [9–11]. A widely used method of camouflaging or masking NPs is the use of surface adsorbed or grafted shielding groups which can hinder the

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hydrophobic and electrostatic interactions that help plasma proteins bind to particle surface [12–14]. Some examples of polymer systems have been tried in the literature as shielding groups, including polyethylene glycol (PEG), polyvinyl alcohol (PVA), polysaccharides, etc. Among them, PEG appeared as an ideal candidate, and was extensively used to coat the surface of NPs, since it has been shown to successfully weaken the MPS uptake and lead to prolonged blood circulation time [4,6,15,16]. Polysaccharides are nowadays recognized for having a high potential in the development of long-circulating systems. Hydrophilic polysaccharide coatings, such as heparin and dextran, have been shown to decrease the uptake of particulate carriers by the MPS [17–19]. Particular attention has been given to chitosan due to its attractive biocompatibility, biodegradability, non-toxicity and low cost [20]. However, the quite low solubility of chitosan is considered to limit its application in drug delivery system. In order to overcome the problem of poor solubility, various studies were conducted to prepare water-soluble chitosans (WSCs), which were endued with excellent hydrophilicity without impairing their native biological properties [21,22]. However, to our great knowledge, to date, there has been no report examining the effect of surface modification of NPs with WSC on their longevity and biodistribution *in vivo*.

On these premises, our idea in here was to develop a unique combinatorial coating of PEG and WSC for the sake of effectively improving the longevity of PLA NPs in blood as well as the stability of the oily cores in the physiological fluids. For this purpose, the surface treatment of PLA NPs was carried out through covalent attachment of PEG to PLA polymer and physical adsorption of WSC to particle surface. Two types of WSC, cationic partially deacetylated chitin (PDC) with approximate 50% deacetylation degree and anionic *N*-carboxy propionyl chitosan sodium (CPCTS) were selected. For comparison, PVA, the most commonly used stabilizer for emulsion system [23] was also included as control. Then we report the investigation on how altering the surface properties of these NPs affected their interaction with macrophages *in vitro*, blood clearance and biodistribution characteristics *in vivo*. *In vitro* cytotoxicity assay was also involved to determine the biocompatibility of these WSC-based NPs. In this study, bovine hemoglobin (Hb) was selected as a model protein.

2. Materials and methods

2.1. Materials

PDC (M_v 2.8×10^5 g/mol, the degree of deacetylation 42.74%) was synthesized as previously described in our group [24]. CPCTS (M_v 3.0×10^5 g/mol, the degree of carboxylation >63.8%) was supplied by Golden-Shell Biochemical Co., Ltd, Zhejiang, China. PVA (1750 ± 50 DP, 87–89% hydrolyzed) was purchased from Sinopharm Chemical Reagent Co., Ltd, Shanghai, China. PLA (M_w 40 k) and PLA–PEG diblock copolymer (M_w 110 k, $M_{w_{PLA}} : M_{w_{PEG}} = 80 : 20$) were supplied by DaiGang Biotechnology Co. Ltd., Jinan, China. Purified bovine Hb in lyophilized form was purchased from YuanJu Biotechnology Company, Shanghai, China. ICR mice were obtained from Shanghai Animal Center, Chinese Academy of Science, Shanghai, China. Ultrapure water (Millipore, Bedford, MA, USA) was used throughout the experiment. All other reagents and solvents were of analytic grade.

2.2. Methods

2.2.1. NPs preparation

Hb-loaded NPs were prepared by an improved $w_1/o/w_2$ solvent diffusion/evaporation method as previously described by Zhao et al. [11] with some modifications. In brief, 0.5 mL Hb solution (w_1) with the concentration of 150 mg/mL was emulsified in 5 mL of organic solvent (o) containing PLA-based polymer (10 mg) and 6-coumarin (10 μg) by ultrasonic (JYD-900, Zhixin Instrument Co., Ltd., Shanghai) for 12 s. Thereafter, the primary emulsion was poured into 50 mL aqueous solution (w_2) followed by two steps of re-emulsification by a high pressure homogenizer (AH110D, ATS Engineering Inc., Canada). The double emulsion was subsequently added to 150 mL aqueous solution (w_2) and then was vacuumed to completely remove the solvents. The NPs were collected by centrifugation (GL-21M, Shanghai Centrifuge Institute Co., Ltd., Shanghai) at 21,000*g* for 60 min, followed by washing three times with Millipore water to remove excessive emulsifier and fluorescent marker before lyophilization. The entire process was maintained at 4 °C by thermostatted water bath.

2.2.2. Surface modification of NPs

For the preparation of NPs coated with PEG alone (PEG NPs), PEG was co-incorporated into the NP matrix as copolymer. In this way, 10 mg PLA–PEG was dissolved in the o phase during the NP formulation process. For the preparation of NPs coated with PVA, PDC or CPCTS alone (PVA NPs, PDC NPs and CPCTS NPs), 10 mg PLA was dissolved in the o phase as matrix polymer and 0.5% (w/v) PVA, PDC and CPCTS was added in the w_2 phase as modifier, respectively. For the preparation of NPs coated with the combined PEG and PVA, PEG and PDC, or PEG and CPCTS (PEG/PVA NPs, PEG/PDC NPs, PEG/CPCTS NPs), 10 mg PLA–PEG was dissolved in the o phase as matrix polymer and 0.5% (w/v) PVA, PDC and CPCTS was added in w_2 phase as modifier, respectively.

2.2.3. Characterization of NPs

The morphology of the freeze-dried NP powders was observed under scanning electron microscopy (SEM) (JSM-6360LV/Falcon, JEOL/EDAX, Japan). Samples were transferred onto a small metal cylinder and coated with gold prior to examination.

Particle size and polydispersity index (PI) were analyzed by photon correlation spectroscopy (PCS) at 25 °C under an angle of 90°. Zeta potential was determined by laser Doppler anemometry (LDA) at 25 °C. For measurements, samples were diluted to the appropriate concentration with Millipore water. The PCS and LDA analyses were performed using a Zetasizer® Nano ZS (Malvern Instrument, Malvern, UK), respectively. Each batch was analyzed in triplicate.

Hydrophilicity of NP surface was evaluated by measuring the static contact angle of particle film as previously described by Cao et al. [25] with some modifications. Briefly, NP suspension (1 mg/mL Millipore water) was spin-coated onto a cleaned glass slide ($100 \times 100 \times 1$ mm^3) at 1500 rpm for 45 s. Thereafter, the glass slides were allowed to dry at 60 °C for 24 h to remove the dispersion medium for the formation of the particle film. In each experiment, a drop of deionized water (5 μL) was placed on the glass slide sample and the contact angle measurements were taken by direct reading from the contact angle goniometer (OCA20, Germany) at 25 °C. Each value is the average of five measurements conducted on different parts of the sample surface.

2.2.4. In vitro suspension stability of NPs

Taking into account that these NPs were developed as carriers for i.v. administration of drugs, we considered it important to evaluate their suspension stability in a physiological medium. The suspension stability assay was performed using an optical analyzer Turbiscan (Formulaction, France) on the basis of turbidity measurements [26]. Briefly, the NP suspension (5 mL) was incubated in 5 mL of fetal bovine serum (FBS) (SiJiQing Biomedical Inc., Hangzhou, China) at 37 °C for 120 h. During incubation, they were scanned by a light beam emitted in near infrared (880 nm) wavelength at each predetermined time point. Two synchronous optical sensors received respectively light transmitted through the sample and light backscattered by the sample. The variation of backscattering data (ΔBS) was directly dependent on the mean particle size and their volume fraction, which was used to evaluate the stability of NP suspension.

2.2.5. Cytotoxicity studies

To investigate the biocompatibility of NPs with different polymer coatings, an *in vitro* experiment was performed using human vascular endothelial cell (EVC-304) culture. EVC-304 was seeded at 30,000 cells per well in DMEM complete medium containing 10% FBS followed by incubation in 5% CO_2 incubator at 37 °C for 24 h. The cytotoxicity of the polymer was evaluated by determining the viability of the cells after incubation with different concentrations of PEG NPs, PDC NPs, CPCTS NPs, PEG/PDC NPs or PEG/CPCTS NPs (from 0.0625 mg/mL to 1 mg/mL) for 72 h. The number of viable cells was determined by the estimation of their mitochondrial reductase activity using the tetrazolium-based colorimetric method. This assay depends on the cellular reductive capacity to metabolise the yellow tetrazolium salt, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide dye (MTT), to a highly colored formazan product. At the end of the incubation period with NPs, cells were incubated with 100 μL of a MTT solution (5 mg/mL) for 4 h at 37 °C. One hundred microliters of DMSO were then added in order to dissolve the formazan crystals. The UV absorbance of the solubilized formazan crystals was measured spectrophotometrically (MULTISKAN MK3, Thermo Electron Corporation, USA) at 490 nm. Cell viability was expressed as the ratio between the amount of formazan determined for cells treated with the different NP suspensions and for control non-treated cells. Each point was performed in quadruplicate.

2.2.6. In vitro phagocytosis test

2.2.6.1. Harvesting and culture of macrophages. The mouse peritoneal macrophage (MPM) was selected to evaluate the phagocytic uptake of NPs *in vitro*. Elicited macrophages were harvested from the peritoneal cavity of male ICR mice (25 ± 5 g) 4 days after intraperitoneal injection of 1 mL 2.9% thioglycolate medium (Sigma Chemical Co.). Cells were isolated by centrifugation at 2000 rpm for 10 min and then re-suspended in RPMI 1640 supplemented with 10% heat-inactivated FBS, penicillin

G (100 U/mL) and streptomycin (100 μg/mL). Thereafter, the cells were seeded into 24-well Costar® culture plates (Corning, NY, USA) at a density of 1.3×10^5 cells/cm^2. After incubated in a humidified atmosphere containing 5% CO_2 at 37 °C for 6 h, non-adherent cells were washed off with phosphate-buffered saline (PBS, pH 7.4) and then culture under the same conditions until they formed a confluent monolayer. Media used in all incubations with NPs contained 10% FBS to account for possible opsonization events.

2.2.6.2. Phagocytic uptake experiments. Quantitative studies were determined by analyzing the cell lysate of MPM. Upon reaching confluence, cell uptake of NPs was initiated by adding 100 μL of specified NP suspension (200 μg/mL in HBSS) and incubating the cells at 37 °C for 1 h. The experiment was terminated by washing the cell monolayer five times with PBS at 4 °C to ensure the removal of particles adhered to the cell surface. Then, cell membrane was permeabilized with 10% Triton X-100 in acetonitrile solution to expose the internalized NPs for quantitative measurement. Cell-associated NPs were quantified by analyzing the cell lysate in a fluroskan ascent reader (Thermo Labsystems, Finland) using $\lambda_{ex} = 485$ nm, $\lambda_{em} = 530$ nm. Uptake was expressed as the percentage of fluorescence intensity associated with cells versus the amount of fluorescence intensity present in the feed solution.

2.2.7. Blood clearance and biodistribution in vivo

Female ICR mice, body weight between 25 ± 5 g, were used for blood clearance and biodistribution investigations. The mice were fasted overnight but had free access to water. The mice, three per group, were injected intravenously into the tail vein corresponding to 10 mL/kg (body weight of mice) with the various NP suspensions of 10 mg/mL. After i.v. injection at designated time points (5 min, 15 min, 30 min, 1 h, 2 h, 3 h, 6 h, 12 h, 24 h, 36 h, 48 h, 72 h), the mice were sacrificed. Blood samples (0.5 mL) were withdrawn by cardiac puncture in pre-weighed heparinized capillary tubes. Major organs such as heart, liver, spleen, lung, kidney and brain were excised and rinsed by Millipore water.

Blood clearance and biodistribution of NPs were determined by measuring the fluorescence of 6-coumarin after their extraction of NPs from blood or organs into acetonitrile. The extraction procedure in detail was described in Ref. [11]. The survival amount in blood and cumulative amount in organs then can be calculated. The area under the concentration–time curve (AUC) was calculated using the linear trapezoidal rule during the experimental period ($AUC_{[0-72]}$). A logarithmic regression analysis allowed us to calculate the half-life ($t_{1/2}$).

2.2.8. Statistical analysis

Results were expressed as mean ± SD. All data were generated in three or four independent experiments. Statistical analysis was performed with one-way analysis of variance (ANOVA). Differences were considered to be significant at a level of $P < 0.05$.

3. Results

3.1. Characterization of the NPs

The typical morphological appearance of PEG/PDC NPs in freeze-dried form was depicted in Fig. 1. SEM image reveals their regular spherical shape with smooth surface.

The results of the physicochemical characteristics of the prepared NPs were shown in Table 1. For comparison, conventional PVA NPs and PEG/PVA NPs were also included as controls. Particle size distribution by dynamic light scattering showed the unimodal distribution (PI < 0.2) with a size range of 100–200 nm for all the NPs coated with PEG, PDC, CPCTS, PEG/PDC or PEG/CPCTS. As can be seen, the sizes of PDC-based formulations or CPCTS-based formulations were larger than that of the controls. This increase in particle size was a first indication of the attachment of WSC to the surface of the oil core. Moreover, the sizes of PEG/CPCTS NPs and CPCTS NPs were slightly larger than that of the corresponding PEG/PDC NPs and PDC NPs, respectively. An additional observation is there was no significant difference in particle size between PDC-based formulations or CPCTS-based formulations regardless of PEGylation, suggesting the contribution of PEG to particle size could be negligible.

Differences on the NP characteristics due to the effects of charged WSC chains arranged at the surface of polymer particle appeared more clearly considering the zeta potential of NPs. In the case of PEG-based NPs, the control PEG/PVA NPs had a negative charge of −18.9 mV which, substituting PDC for PVA, was inverted

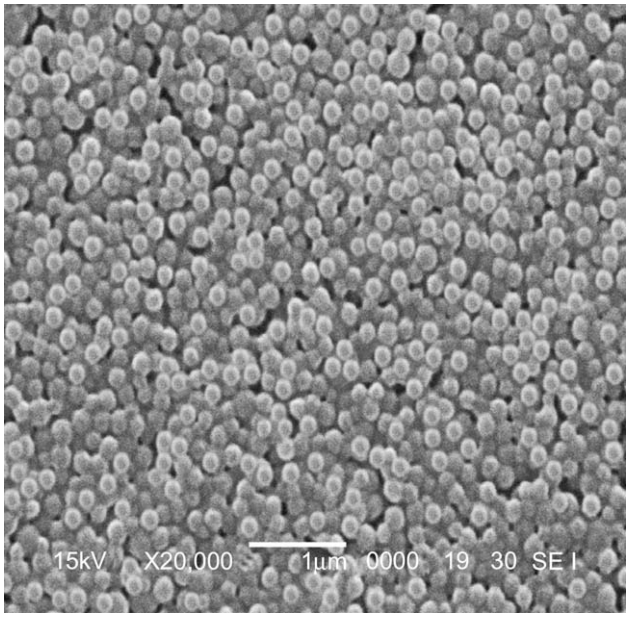

Fig. 1. SEM image of freeze-dried PEG/PDC NPs.

to positive value (+4.9 mV). On the contrary, it can be noted that a strongly negative charge of −38.3 mV occurred in the case of PEG/CPCTS NPs. Comparable results could also be seen for the NPs coated with control PVA, PDC or CPCTS alone. This diversity in superficial charge of the system is the second evidence of the presence of WSC coating. In addition, the slight influence of PEGylation could be an indication of the presence of both PEG and WSC around the nanostructure for PEG-based formulations.

The third evidence of WSC coating comes from the surface hydrophilicity measurement. A high improvement in surface hydrophilicity of NPs was observed for both PDC- and CPCTS-based formulations in comparison with the controls as evidenced by their significantly decreased static contact angles. The slight lack of influence of the PEGylation could be another indication that the hydrophilicity of WSC was much higher than that of PEG.

3.2. Suspension stability of NPs

It is known that NPs are susceptible to aggregation in media and the surface composition of NPs plays an important role in its stability. In this study, we have investigated the effect of WSC coating at improving the stability of nanoemulsion in the presence of blood serum. The ΔBS as a function of time for NPs with different surface properties (PEG, PDC, CPCTS, PEG/PDC or PEG/CPCTS) were shown in Fig. 2. PVA NPs and PEG/PVA NPs were also included for comparison. After 120-h incubation, the variation of ΔBS for PEG NPs was 9.13%, much higher than other formulations, indicating the

Table 1
Physicochemical characteristics of various surface-modified NPs.

Type of NPs	Size (nm)	Polydispersity index	Zeta potential (mV)	Contact angle (°)
PEG NPs	143.5	0.196	−16.7 ± 0.5	55.88 ± 0.61
PVA NPs	131.4	0.152	−32.5 ± 1.3	72.56 ± 0.58
PDC NPs	159.1	0.176	13.1 ± 0.3	19.92 ± 1.04
CPCTS NPs	172.2	0.189	−46.7 ± 0.8	11.13 ± 1.12
PEG/PVA NPs	127.6	0.120	−18.9 ± 0.7	62.59 ± 1.37
PEG/PDC NPs	151.9	0.129	4.9 ± 0.4	23.31 ± 0.97
PEG/CPCTS NPs	168.6	0.151	−38.3 ± 1.2	14.06 ± 1.18

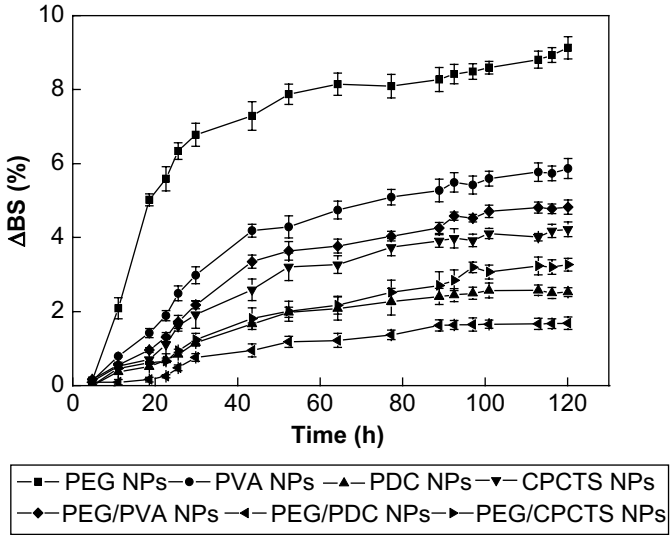

Fig. 2. Variation of backscattering data (ΔBS) of NPs with various coatings (PEG, PVA, PDC, CPCTS, PEG/PVA, PEG/PDC or PEG/CPCTS) in the presence of blood serum as a function of time (0–120 h). Data represent mean ± SD, $n = 3$.

lowest stability. It can also be found that, both PDC- and CPCTS-coated NPs were more stable than the corresponding PVA-stabilized NPs irrespectively of PEG. In particular, PEG/PDC NPs exhibited the highest stability of all.

3.3. Cytotoxicity of the NPs

Human vascular endothelial cells (EVC-304) were chosen as the *in vitro* model to assess the cytotoxicity of various formulations, which mimics the endothelial lining of the blood vessel wall. The cytotoxicity of NPs modified by the two types of WSC should be emphasized since it is the first report on i.v. administration of PDC- and CPCTS-coated NPs. The cells were incubated with different NPs varied in surface properties (PEG, PDC, CPCTS, PEG/PDC and PEG/CPCTS) for a period of 72 h at graded doses of the NPs (from 0.0625 mg/mL to 1 mg/mL), and the results of MTT assay were shown in Fig. 3. All the NPs developed were found to be non-toxic at majority of concentrations studied. We did not observe a significant difference ($P > 0.05$) in the toxicity of different formulations at any of the concentrations used, although there was a slight reduction in cell viability at higher concentrations. Notably, the combination of PEG together with PDC or CPCTS could help in improving the biocompatibility of NPs coated with PDC or CPCTS alone. Average cell viabilities were between 75 and 120% of control at the concentrations studied, indicating that there was no significant cytotoxicity observed for NPs coated with PEG, PDC, CPCTS, PEG/PDC or PEG/CPCTS. Also, during the 72-h period *in vivo*, the animals did not present any visible symptoms of toxicity related to the injection of these NPs. These results imply that PDC and CPCTS have a high potential in clinic use to the body.

3.4. In vitro phagocytosis test

6-Coumarin has extensively been used as a fluorescent marker of polymeric particles [27,28]. Therefore, in this work, 6-coumarin was employed as the fluorescent marker of NPs to evaluate their biological behaviors. In order to verify the reliability of the data obtained from the *in vitro* and *in vivo* experiments, *in vitro* release of 6-coumarin from the labeled NPs was examined preliminary in this study (data not shown). The results revealed that less than 1% of the dye was released for all the NPs coated with PEG, PDC, CPCTS, PEG/PDC or PEG/CPCTS in the presence of serum during the 72-h period tested. Therefore, 6-coumarin incorporated in the NPs as a marker could not interfere with their inherent biological behaviors *in vitro* and *in vivo*.

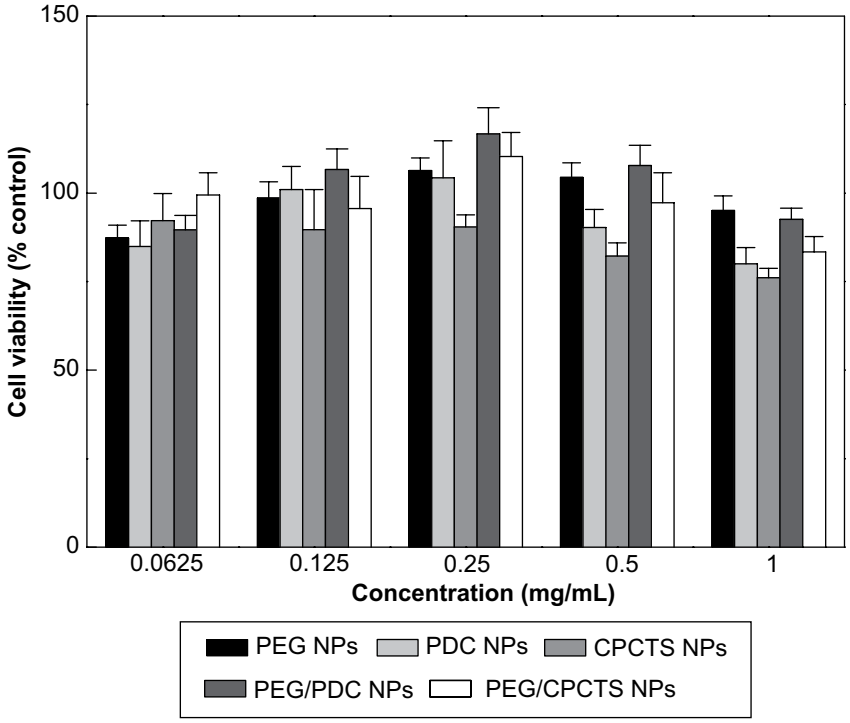

Fig. 3. Cytotoxicity of NPs with various surface coatings (PEG, PDC, CPCTS, PEG/PDC or PEG/CPCTS) analyzed by MTT assay. ECV-304 cells were cultured with 0, 0.0625, 0.125, 0.25, 0.5, 1 mg/mL of NPs for 72 h at 37 °C. Data represent mean ± SD, $n = 4$.

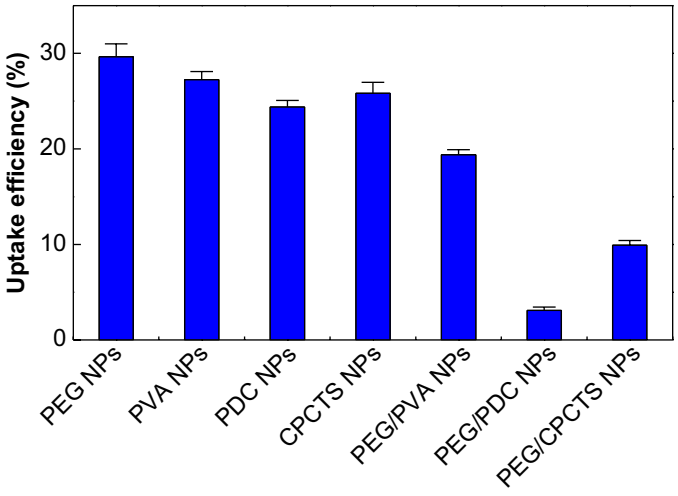

Fig. 4. Phagocytic uptake efficiency of NPs coated with PEG, PVA, PDC, CPCTS, PEG/PVA, PEG/PDC or PEG/CPCTS, which was measured after 1 h incubation with MPM in the serum containing medium at 37 °C. Data represent mean ± SD, $n = 4$.

The effect of surface properties on the degree of 6-coumarin-labeled NPs association with macrophages was characterized quantitatively by fluorometric method. The percentage of cells associated with fluorescence will be the definition for the percent of uptake. Fig. 4 shows the uptake efficiencies of NPs with different surface coatings (PEG, PDC, CPCTS, PEG/PDC or PEG/CPCTS) by MPM after 1 h incubation at 37 °C. For comparison, PVA NPs and PEG/PVA NPs were also included. Our results illustrated that, the presence of PEG, PDC or CPCTS coating alone failed to reduce the phagocytic uptake of NPs. The uptake percentage of PEG NPs, PDC NPs and CPCTS NPs was 29.64%, 24.39% and 25.83%, respectively. Interestingly, combining PEG with PDC or CPCTS clearly attenuated the NP-cell association, which represented only 3.09% and 9.92% of cell uptake, respectively. The uptake percentage of PEG/PDC NPs was 7.9-fold/9.6-fold lower than that of the corresponding PDC NPs and PEG NPs, respectively. The capture of CPCTS NPs and PEG NPs by MPM exceeded 2.6-fold/3-fold over that of PEG/CPCTS NPs, respectively. By contrast, only a slight effect was observed when surface modification of NPs with the combined PEG and PVA.

3.5. Blood clearance and biodistribution in vivo

To fully describe how the body handles the foreign NPs, the *in vivo* blood clearance and biodistribution of the formulated NPs were monitored and compared. The blood concentration–time profiles after the i.v. administration of different formulations (PEG NPs, PDC NPs, CPCTS NPs, PEG/PDC NPs and PEG/CPCTS NPs) at a dose of 10 mL suspension/kg (weight of mouse) in mice are shown in Fig. 5. The half-lives of various formulations are presented in Fig. 6. Conventional PVA NPs and PEG/PVA NPs were also included for comparison. It could be seen that, NPs coated with PEG, PDC or CPCTS alone exhibited a rapid elimination from the bloodstream and the remaining dose at 3 h post-injection was 15.46% and 29.81% and 25.56%, respectively. After combining PEG with PDC or CPCTS, a significant prolongation of NPs in blood occurred. For PEG/PDC NPs and PEG/CPCTS NPs, 49.03% and 37.67% of the fluorescence-associated NPs were still found in the systemic circulation at 72 h after injection, respectively. The half-life ($t_{1/2}$) calculated for PEG/PDC NPs was 63.5 h, 302-fold as long as that of PEG NPs and 96-fold as long as that of PDC NPs. By comparison, the present study clearly illustrated that PEG/CPCTS NPs did not exhibit the same long-circulating property as PEG/PDC NPs, disappearing from the blood with a $t_{1/2}$ of 7.1 h. Control PEG/PVA NPs possessed

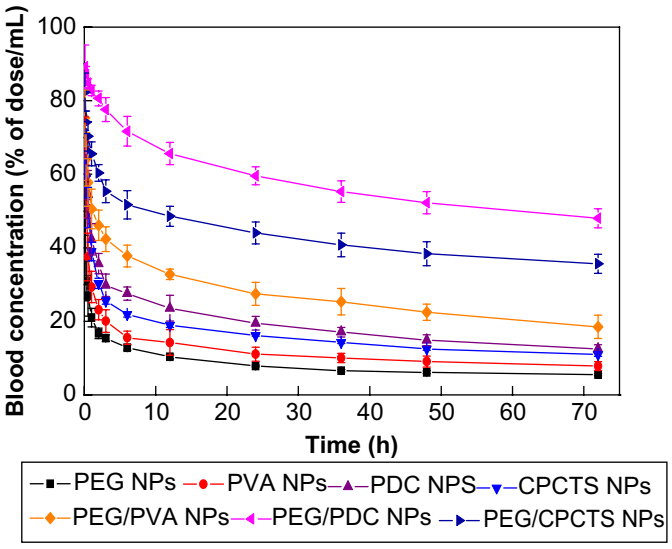

Fig. 5. Blood clearance profile of surface-modified NP formulations (PEG NPs, PVA NPs, PDC NPs, CPCTS NPs, PEG/PVA NPs, PEG/PDC NPs and PEG/CPCTS NPs) after i.v. injection of 10 mg/mL of NP suspensions (10 mL/kg) in mice. Data represent mean ± SD, $n = 3$.

an elimination $t_{1/2}$ of 1.1 h. Furthermore, it could be seen that all the NPs showed the biphasic clearance profile. Particularly for PEG/PDC NPs, an obvious retardation in clearance from the blood could be observed during the initial 3 h after administration.

In this study, PEG/PDC NPs exhibiting the most prolonged blood circulating property was chosen to investigate the fate of NPs *in vivo* after i.v. administration. Conventional PEG/PVA NPs was included for comparison. The distributions of PEG/PDC NPs and PEG/PVA NPs in various organs for a 72-h period post-administration in mice are illustrated in Fig. 7 and AUCs are summarized in Table 2. The accumulation and clearance of the NPs in different organs varied. Besides the blood, the NPs were predominantly found in the liver, followed by the kidney, brain and spleen and, to a lesser extent, in the lung and heart. In the case of PEG/PDC NPs, the cumulative amounts compared with those of PEG/PVA NPs dramatically reduced in liver and kidney, changed little in lung and heart, and exhibited slightly higher in spleen and brain in terms of AUCs. Within the liver, PEG/PDC NPs and PEG/PVA NPs exhibited different distribution profiles. For PEG/PDC NPs, they displayed a fast accumulation in the first 5 min with the dose of 8.52% followed by progressive clearance out to 72 h. However, PEG/PVA NPs followed an abrupt hepatic accumulation until 15 min with the dose up to 24.46% after administration followed by hepatobiliary excretion out to 72 h. The rate of accumulation in liver for PEG/PDC NPs was 3.3-fold lower than that of PEG/PVA NPs at the initial 15 min. This reduction in uptake of PEG/PDC NPs by liver macrophages (Kupffer

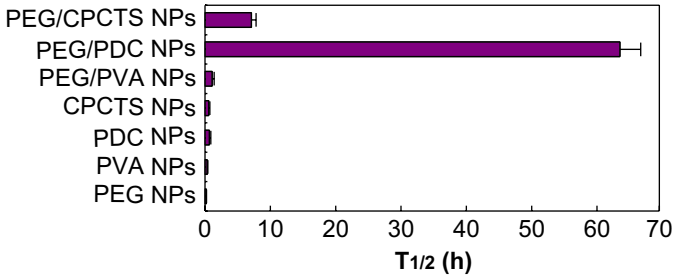

Fig. 6. Half-life in blood circulation of various NP formulations (PEG NPs, PVA NPs, PDC NPs, CPCTS NPs, PEG/PVA NPs, PEG/PDC NPs and PEG/CPCTS NPs). Data represent mean ± SD, $n = 3$.

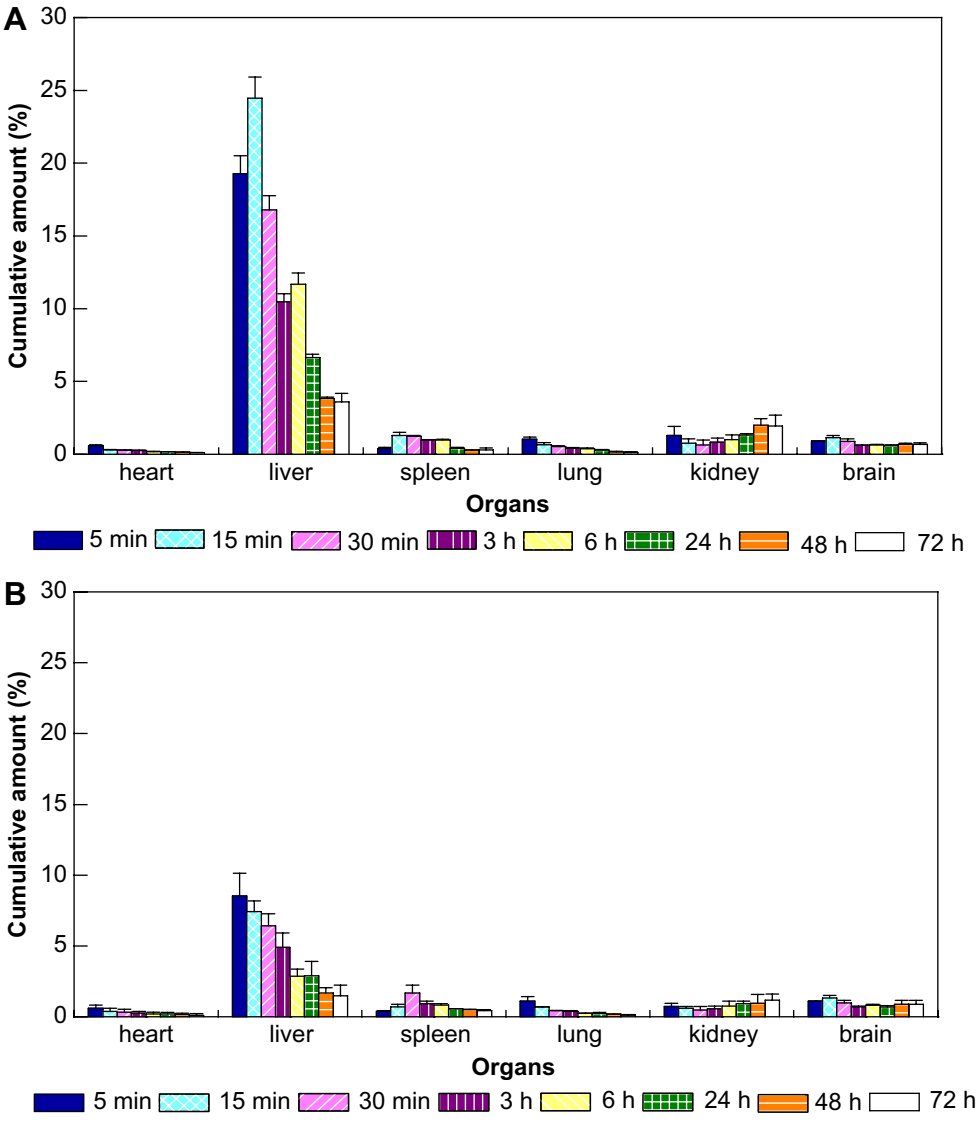

Fig. 7. Biodistribution of NPs in main organs over time. (A) PEG/PVA NPs, (B) PEG/PDC NPs. Data represent mean ± SD, $n = 3$.

cells) correlated well with a noted retention of circulatory level in the blood at the initial period. Furthermore, a dramatic decrease of the fluorescence in the kidney was observed for PEG/PDC NPs. The AUC for PEG/PDC NPs in kidney was 61% as much as that of PEG/PVA NPs. The uptake of PEG/PDC NPs by spleen was 1.39-fold higher than that of PEG/PVA NPs in terms of AUC. In addition, a slight higher accumulation in brain could be observed for PEG/PDC NPs than that for PEG/PVA NPs. However, accumulated amounts of NPs in lung and heart were found to be negligible for the tested formulations.

4. Discussion

It is well accepted that, successful *in vivo* drug delivery for i.v. administration may require not just a well-defined nanostructure but also long-circulating efficacy. Previously, our group has demonstrated that surface treatment of NPs by co-incorporation of PEG as matrix polymer was beneficial for prolonged blood residence when compared with naked NPs (7.2 fold increase) [11]. In this work, we continued these studies in order to realize a further prolongation in systemic circulation. In comparison to the primary work, a unique combinatorial design of PEG and WSC (PDC or CPCTS) was applied to engineer a 'stealth' core-corona NP, with the hydrophobic PLA serving as the core for drug reservoir and the hydrophilic PEG and WSC cooperative as the corona projecting towards the outer aqueous environment. Since literature suggested PVA as the most popular stabilizer for the production of polyester NPs [29], PVA-based NPs, PVA NPs and PEG/PVA NPs, were also included for comparison.

Table 2
Tissue distributions of PEG/PVA NPs and PEG/PDC NPs at 72 h post-injection of 10 mg/mL of NP suspensions in mice at a dose of 10 mL/kg.

Tissue	PEG/PVA NPs		PEG/PDC NPs		Ratio[c]
	AUC_{0-72}[a]	T/P ratio[b]	AUC_{0-72}[a]	T/P ratio[b]	
Plasma	1857.06	1	3962.86	1	2.13
Heart	10.52	0.006	11.04	0.003	1.05
Liver	367.48	0.198	151.62	0.038	0.41
Spleen	26.91	0.014	37.32	0.009	1.39
Lung	13.84	0.007	13.79	0.003	1.00
Kidney	123.23	0.066	74.95	0.019	0.61
Brain	46.71	0.025	59.11	0.015	1.27

[a] AUC_{0-72} (%·h), area under the drug concentration–time curve from 0 to 72 h.
[b] Tissue-to-plasma ratio based on AUCs.
[c] Ratio of AUCs (PEG/PDC NPs to PEG/PVA NPs).

The comparison of the physicochemical features of the different formulations evaluated and listed in Table 1, can be of help to rationalize the physicochemical parameters that determine the following biological behaviors. Physical adsorption of surface-active molecules from the external aqueous phase to the particle surface in an emulsion-based preparation procedure seems to be simple, since no chemical synthesis is necessary. In this study, the increase in particle size, reversal of surface charge and enhancement of surface hydrophilicity can be easily understood by the fact that effective 'insertion' of the PDC or CPCTS in the outer PEG layer of the NPs was achieved, appending the anchor, via PEG spacer, to the particle surface. The PDC coating of the NPs was formed probably due to electrostatic interactions between the positively charged PDC and the negatively charged PLA. Furthermore, owing to its amphiphilic nature [24], PDC molecules were prone to form monolayer arrangement at the surface of NPs. The adsorption of negative CPCTS on the particle surface might be a consequence of the hydrophobic and other weak interactions of its propionyl chains with the dissolved PLA chains.

PVA is known to form a stable network on the NPs, which cannot be removed during the washing steps and protects the particles from aggregation [23,30]. According to our knowledge, there has been seldom description on the stability of anchoring of WSC to the surface of NPs. A potential explanation for the positive role of the WSC coating at improving particle stability was based on an efficient steric repulsion effect produced by the hydrophilic chains of the macromolecular WSC projecting towards the external phase, which inhibited coalescence or flocculation. By contrast, NPs coated with PEG alone were in an instable state probably due to the lack protection by the stabilizer such as PVA, PDC or CPCTS. Similar results were also observed by Coombes et al. [31]. Notably, despite having a surface charge close to neutrality, a slight higher stability for PEG/PDC NPs was observed compared with PEG/CPCTS NPs and control PEG/PVA NPs. Since it has been found that PDC has good emulsifying capacity better than widely used surfactant Tween-60 near the concentration of 0.5% (w/v) [24], it is therefore plausible to ascribe this to not only the steric repulsion but also amphiphilic nature of PDC, reducing the interfacial tension between the aqueous and organic phases to form a more stable emulsion.

Circulatory half-life is the key parameter determining the efficacy of drug delivery system for i.v. administration. Meanwhile, phagocytic uptake of NPs by cells of MPS is thought to be responsible for the major loss of NPs in the systemic circulation. In this work, prior to the *in vivo* experiment, *in vitro* macrophage uptake assay was carried out to predict the phagocytosis of NPs by the cells of MPS. More specifically, since the absorption of plasma proteins on particle surface is regarded as a key factor to the process of phagocytic recognition, *in vitro* experiment was performed in the presence of blood serum to account for possible opsonization events for the purpose of describing the substantial process of phagocytosis *in vivo*. A comparison of the *in vitro* macrophage uptake data and the *in vivo* blood clearance data obtained with the different compositions clearly revealed that, the lower the phagocytosis *in vitro*, the longer the systemic circulation *in vivo*. That is to say, the behavior of NPs *in vivo* correlated well with their *in vitro* interaction with macrophages. Therefore, it can be inferred that the *in vitro* preliminary phagocytic uptake experiment can be used to predict the circulation longevity of NPs *in vivo*.

The noticeable differences with regard to macrophage uptake, blood clearance as well as biodistribution between PEG/WSC NPs and control PEG/PVA NPs arouse our attentions. In this study, the PEG/WSC NPs and PEG/PVA NPs were prepared by the same process and possessed comparable average diameters in terms of long-circulating requirement [9–11]. Therefore, it can be inferred that the different performances *in vitro* and *in vivo* between PEG/WSC NPs and PEG/PVA NPs were supposed to mainly result from the post-adsorbed WSC and PVA. Introduction of highly hydrophilic WSC coating, may possibly adsorb more water molecules around the nanostructure to form a 'cloud' of hydrated layer as evidenced by their significantly reduced contact angle, which might effectively avoid the hydrophobic interactions with plasma protein in an aqueous environment. By contrast, given the fact that PVA is much less hydrophilic than WSC, we speculate that the lack protection of PVA coating against opsonization could be related to its relatively high hydrophobicity. A higher protein adsorbability of hydrophobic relative to hydrophilic surfaces has been shown to enhance the uptake of more hydrophobic particles by phagocytes *in vitro* and rapid removal of hydrophobic particles *in vivo* [32,33]. In this sense, one could further argue that their lack of efficacy for repelling opsonins with the PVA coating could be associated with the partial mask of PEG 'stealth' properties by PVA. Indeed, it is plausible that PVA partially hides the flexibility and hydrophilicity of PEG when forming an interconnected network around the nanostructure [30]. On the other hand, we can hypothesize that, the way by which such a high-molecule-weight WSC molecule adsorbs onto the surface of NPs may differ from that of the PVA molecule, which was supposed to have a different impact on the surface-bound PEG. The results suggest that PEG could retain its inhibiting properties towards opsonization after combination with WSC. These properties might even be increased by an unknown mechanism, perhaps as a result of cooperative effects between PEG and WSC chains bound side by side on the same particle.

Interestingly, surface treatment of NPs with PEG, PDC or CPCTS alone failed to reduce the macrophage uptake and to prolong the circulatory half-life. However, after combination, whether with PDC or CPCTS, the PEGylated NPs all exhibited substantial positive effect on phagocytic uptake inhibition and blood circulation prolongation, a markedly synergistic action of PEG and WSC. It is also worthwhile to note that, there was an apparent difference between PEG/PDC NPs and PEG/CPCTS NPs in terms of their behaviors *in vitro* and *in vivo*. This may arise from the differences between PDC and CPCTS. Since both types of WSC coatings are highly hydrophilic in nature, which was supposed to provide a sufficient hydrated layer around the nanostructure, the hydrophilicity of particle surface should not play a decisive role in the NP/opsonin interactions. Taking into account these considerations, two explanations for the above phenomena are possible.

The first, we believe, is related to the different surface charges of the NPs in the presence of WSC coatings. A correlation between surface charge and opsonization has been demonstrated *in vitro* that neutrally charged particles have a much lower opsonization rate than charged particles of the same size [6,34]. In our experiment, PDC possessing the protonated amine groups tailored the particles surface charge close to neutrality, whereas CPCTS, due to the presence of terminal carboxylic groups, rendered the particles bearing more negative charges. Therefore, the surface modification of NPs using the combined PEG and PDC inhibited the electrostatic interactions that help opsonins bind to particle surface. Their lack of binding with opsonins and then evading phagocytosis would be likely responsible for their prolonged persistence in the blood circulation. On the contrary, PEG/CPCTS modified NPs with a higher absolute zeta potential would possess a stronger affinity for plasma proteins, accounting for its relatively higher phagocytic uptake.

The second may be in regard to the structural differences of the outmost surface between PEG/PDC NPs and PEG/CPCTS NPs, since three-dimensional organization has already proved to play an important role in opsonization events [35]. The potential mechanism behind this was supposed concerning the surface chain density and molecular conformation of polymer coating on the particle surface, and these two aspects are much more interrelated

[36]. It has been postulated that the surface-bound PEG would take on either a 'mushroom' or a 'brush' conformation depending on the surface density of the PEG, and the latter conformation would create more effective blocking or repulsion of opsonins than the former one [5,37]. Considering this background, we thus hypothesize that, owing to its desirable amphiphilic nature, an extremely strong surface affinity of anchor PDC block may overcome the osmotic repulsions between PEG chains, resulting in greater surface coverage. Consequently, the steric hindrance created by the relatively rigid PDC chains might facilitate more stretching of PEG chains from the surface. Thus, the surface-grafted PEG in combination with PDC was supposed to assume a dense brush-like conformation and act to enhance the opsonins resistant properties. On the other hand, one can hypothesize that, the adsorption of CPCTS, via PEG chains, on oil core is dominated by osmotic repulsion between the PEG chains. This leads to a relatively larger separation between the anchoring sites and hence a lower surface coverage, which in turn causes less stretching of the PEG chains from the surface, where on average they will be located closer to the surface of the particle. Therefore, PEG molecules might assume a mushroom-like conformation when combined with CPCTS. To have better understanding of the mechanisms, the conformational hydrodynamics of PEG molecules combined with PDC or CPCTS molecules on the particle surface have to be elucidated and will be the emphasis of our further study. Summarily, although it has been found a great improvement in surface hydrophilicity both by PEG/PDC and PEG/CPCTS coatings, nevertheless, the shielding effect will be more pronounced with neutral-charged PEG/PDC NPs bearing a dense brush-like conformation.

The major pathway for the removal of NPs from blood appeared to be the NP capture in MPS tissues, and especially in the liver. As liver is the main MPS organ, there seems logical to exist a correlation between the level of increased blood retention and their reduced sequestration by liver. The significant reduction in liver for PEG/PDC NPs was probably due to the 'invisible' surface created by PEG/PDC coating, with neutral-charge and brush-like conformation, which could effectively avoid the recognition of liver macrophages and thus reduce the hepatic deposition. A slight high spleen uptake was considered to be a consequence of the prolonged systemic circulation of PEG/PDC NPs, which could reach other MPS organs, such as spleen [38]. The reason for a reduction in renal sequestration of PEG/PDC NPs remains to be clarified, but the cooperative interaction of PEG and PDC might result in a compact and dense particle shell wall, which may be responsible for preventing desorption of fluorescence from intact and/or degraded NPs into kidney and eliminated by urinary excretion during the long circulation time, in good agreement with the results of *in vitro* fluorescence release. A relatively high accumulation of particulates in brain was observed compared with PEG/PVA NPs. This indicates the successful delivery of Hb to brain and also an enhanced ability to pass the blood-brain barrier (BBB) depending on the PEG/PDC coating. The low lung fluorescence observed for both PEG/PDC NPs and PEG/PVA NPs may be attributed to the lack of mechanical filtration by the capillary bed due to the small size less than 200 nm [39], indicating that probably no notable particulate flocculation occurs in the bloodstream during 72 h.

5. Conclusions

A new class of PLA NPs bearing a combinatorial coating of PEG and WSC was prepared in the present study. The resultant particles appeared spherical and were homogeneously sized (100–200 nm). Surface charge, surface hydrophilicity and particles stability were greatly affected by the nature of the coatings. PEG, when combined with WSC, provided an effective shielding character to the NPs. Notably, PEG/PDC NPs exhibited the unexpected combination of high suspension stability and surface hydrophilicity as well as charge-neutral surface. *In vitro*, PEG/PDC NPs notably avoided the macrophage uptake. *In vivo*, PEG/PDC combinatory coating led to a dramatic prolongation in blood circulation ($t_{1/2} = 63.5$ h), with only a small amount of the NPs sequestered by the liver. Based on the above results, these stealth polymeric NPs conferred by a combinatorial coating of PEG and PDC might suffice for good long-circulating efficacy and will be promising prospects as efficiently targeted drug delivery systems.

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References

[1] Dong YC, Feng SS. Methoxy poly(ethylene glycol)–poly(lactide) (MPEG–PLA) nanoparticles for controlled delivery of anticancer drugs. Biomaterials 2004;25:2843–9.
[2] Munier S, Messai I, Delair T, Verrier B, Ataman-Onal Y. Cationic PLA nanoparticles for DNA delivery: comparison of three surface polycations for DNA binding, protection and transfection properties. Colloid Surf B Biointerfaces 2005;43:163–73.
[3] Li YP, Pei YY, Zhang XY, Gu ZH, Zhou ZH, Yuan WF, et al. PEGylated PLGA nanoparticles as protein carriers: synthesis, preparation and biodistribution in rats. J Controlled Release 2001;71:203–11.
[4] Gref R, Minamitake Y, Peracchia MT, Trubetskoy V, Torchilin V, Langer R. Biodegradable long-circulating polymeric nanospheres. Science 1994; 263:1600–3.
[5] Owens III DE, Peppas NA. Opsonization, biodistribution, and pharmacokinetics of polymeric nanoparticles. Int J Pharm 2006;307:93–102.
[6] Zahr AS, Davis CA, Pishko MV. Macrophage uptake of core-shell nanoparticles surface modified with poly(ethylene glycol). Langmuir 2006;22:8178–85.
[7] Stolnik S, Illum L, Davis SS. Long circulating microparticulate drug carriers. Adv Drug Delivery Rev 1995;16:195–214.
[8] Alexis F, Pridgen E, Molnar LK, Farokhzad OC. Factors affecting the clearance and biodistribution of polymeric nanoparticles. Mol Pharmacol 2008;5:505–15.
[9] Chang TMS, Powanda D, Yu WP. Analysis of polyethylene-glycol-polylactide nano-dimension artificial red blood cells in maintaining systemic hemoglobin levels and prevention of methemoglobin formation. Artif Cells Blood Substit Immobil Biotechnol 2003;31:231–47.
[10] Fang C, Shi B, Pei YY, Hong MH, Wu J, Chen HZ. In vivo tumor targeting of tumor necrosis factor-α-loaded stealth nanoparticles: effect of MePEG molecular weight and particle size. Eur J Pharm Sci 2006;27:27–36.
[11] Zhao J, Liu CS, Yuan Y, Tao XY, Shan XQ, Sheng Y, et al. Preparation of hemoglobin-loaded nano-sized particles with porous structure as oxygen carriers. Biomaterials 2007;28:1414–22.
[12] Gref R, Lück M, Quellec P, Marchand M, Dellacherie E, Harnisch S, et al. 'Stealth' corona-core nanoparticles surface modified by polyethylene glycol (PEG): influences of the corona (PEG chain length and surface density) and of the core composition on phagocytic uptake and plasma protein adsorption. Colloid Surf B Biointerfaces 2000;18:301–13.
[13] Dunn SE, Coombes AGA, Garnett MC, Davis SS, Davies MC, Illum L. In vitro cell interaction and in vivo biodistribution of poly(lactide-*co*-glycolide) nanospheres surface modified by poloxamer and poloxamine copolymers. J Controlled Release 1997;44:65–76.
[14] Gessner A, Lieske A, Paulke BR, Müller RH. Influence of surface charge density on protein adsorption on polymeric nanoparticles: analysis by two-dimensional electrophoresis. Eur J Pharm Biopharm 2002;54:165–70.
[15] Avgoustakis K, Beletsi A, Panagi Z, Klepetsanis P, Karydas AG, Ithakissios DS. PLGA–mPEG nanoparticles of cisplatin: in vitro nanoparticle degradation, in vitro drug release and in vivo drug residence in blood properties. J Controlled Release 2002;79:123–35.
[16] Fontana G, Licciardi M, Mansueto S, Schillaci D, Giammona G. Amoxicillin-loaded polyethylcyanoacrylate nanoparticles: influence of PEG coating on the particle size, drug release rate and phagocytic uptake. Biomaterials 2001; 22:2857–65.
[17] Lemarchand C, Gref R, Couvreur P. Polysaccharide-decorated nanoparticles. Eur J Pharm Biopharm 2004;58:327–41.
[18] Jaulin N, Appel M, Passirani C, Barratt G, Labarre D. Reduction of the uptake by a macrophagic cell line of nanoparticles bearing heparin or dextran covalently bound to poly(methyl methacrylate). J Drug Target 2000;8:165–72.
[19] Passirani C, Barratt G, Devissaguet J-P, Labarre D. Long-circulating nanoparticles bearing heparin or dextran covalently bound to poly(methyl methacrylate). Pharm Res 1998;15:1046–50.

[20] Illum L. Chitosan and its use as a pharmaceutical excipient. Pharm Res 1998;15:1326–31.
[21] Sashiwa H, Kawasaki N, Nakayama A, Muraki E, Yamamoto N, Aiba S. Chemical modification of chitosan. 14: synthesis of water-soluble chitosan derivatives by simple acetylation. Biomacromolecules 2002;3:1126–8.
[22] Chen CL, Wang YM, Liu CF, Wang JY. The effect of water-soluble chitosan on macrophage activation and the attenuation of mite allergen-induced airway inflammation. Biomaterials 2008;29:2173–82.
[23] Sahoo SK, Panyam J, Prabha S, Labhasetwar V. Residual polyvinyl alcohol associated with poly (D,L-lactide-*co*-glycolide) nanoparticles affects their physical properties and cellular uptake. J Controlled Release 2002;82: 105–14.
[24] Wang J, Liu CS, Chi P. Aggregate formation and surface activity of partially deacetylated water-soluble chitin. Res Chem Intermed 2008;34:169–79.
[25] Cao SS, Liu BL, Deng XB, Luo R, Chen HL. A novel approach for the preparation of acrylate–siloxane particles with core-shell structure. Polym Int 2007; 56:357–63.
[26] Mengual O, Meunier G, Cayre I, Puech K, Snabre P. Characterisation of instability of concentrated dispersions by a new optical analyser: the TURBISCAN MA 1000. Colloids Surf A Physicochem Eng Asp 1999;152:111–23.
[27] Panyam J, Sahoo SK, Prabha S, Bargar T, Labhasetwar V. Fluorescence and electron microscopy probes for cellular and tissue uptake of poly(D,L-lactide-*co*-glycolide) nanoparticles. Int J Pharm 2003;262:1–11.
[28] Win KY, Feng SS. Effects of particle size and surface coating on cellular uptake of polymeric nanoparticles for oral delivery of anticancer drugs. Biomaterials 2005;26:2713–22.
[29] Zambaux MF, Bonneaux F, Gref R, Maincent P, Dellacherie E, Alonso MJ, et al. Influence of experimental parameters on the characteristics of poly(lactic acid) nanoparticles prepared by a double emulsion method. J Controlled Release 1998;50:31–40.
[30] Boury F, Ivanova T, Panaiotov I, Proust JE, Bois A, Richou J. Dynamic properties of poly(DL-lactide) and polyvinyl alcohol monolayers at the air/water and dichloromethane/water interfaces. J Colloid Interface Sci 1995;169:380–92.
[31] Coombes AGA, Tasker S, Lindblad M, Holmgren J, Hoste K, Toncheva V, et al. Biodegradable polymeric microparticles for drug delivery and vaccine formulation: the surface attachment of hydrophilic species using the concept of poly(ethylene glycol) anchoring segments. Biomaterials 1997;18:1153–61.
[32] Lück M, Paulke B-R, Schröder W, Blunk T, Müller RH. Analysis of plasma protein adsorption on polymeric nanoparticles with different surface characteristics. J Biomed Mater Res Part A 1998;39:478–85.
[33] Illium L, Hunneyball IM, Davis SS. The effect of hydrophilic coatings on the uptake of colloidal particles by the liver and by peritoneal macrophages. Int J Pharm 1986;29:53–65.
[34] Roser M, Fischer D, Kissel T. Surface-modified biodegradable albumin nano- and microspheres. II: effect of surface charges on in vitro phagocytosis and biodistribution in rats. Eur J Pharm Biopharm 1998;46:255–63.
[35] Peracchia MT, Vauthier C, Passirani C, Couvreur P, Labarre D. Complement consumption by poly(ethylene glycol) in different conformations chemically coupled to poly(isobutyl 2-cyanoacrylate) nanoparticles. Life Sci 1997;61:749–61.
[36] Kenworthy AK, Simon SA, Mcintosh TJ. Structure and phase behavior of lipid suspensions containing phospholipids with covalently attached poly(ethylene glycol). Biophys J 1995;68:1903–20.
[37] Vonarbourg A, Passirani C, Saulnier P, Benoit J-P. Parameters influencing the stealthiness of colloidal drug delivery systems. Biomaterials 2006;27:4356–73.
[38] Peracchia MT, Fattal E, Desmaële D, Besnard M, Noël JP, Gomis JM, et al. Stealth PEGylated polycyanoacrylate nanoparticles for intravenous administration and splenic targeting. J Controlled Release 1999;60:121–8.
[39] Gèze A, Chau LT, Choisnard L, Mathieu J-P, Marti-Batlle D, Riou L, et al. Biodistribution of intravenously administered amphiphilic β-cyclodextrin nanospheres. Int J Pharm 2007;344:135–42.