In vitro macrophage uptake and in vivo biodistribution of long-circulation nanoparticles with poly(ethylene-glycol)-modified PLA (BAB type) triblock copolymer

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Abstract

The effect of the PEG-grafted degree in the range of 0–30% on the in vitro macrophage uptake and in vivo biodistribution of poly(ethylene glycol)–poly(lactic acid)–poly(ethylene glycol) (PELE) nanoparticles (NPs) were investigated in this paper. The prepared NPs were characterized in terms of size, zeta potential, hydrophilicity, superficial charges and in vitro phagocytosis amount of NPs are dependent on the PEG content in the copolymers greatly. The higher of the PEG content, the more hydrophilicity and the nearer to neutral surface charge was observed. And the prolonged circulation half-life of the PELE NPs in plasma was also strongly depended on the PEG content with the similar trend. In particular for PELE30 (containing 30% of PEG content) NPs, with the lowest phagocytosis uptake accompanied the highest hydrophilicity and approximately neutral charge, it had the longest half-life in vivo with almost 12-fold longer and accumulation in the reticuloendothelial system organs close to 1/2-fold lower than those of reference PLA. These results demonstrated that the PELE30 NPs with neutral charge and suitable size has a promising potential as a long-circulating oxygen carrier system with desirable biocompatibility and biofunctionality.

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1. Introduction

A major barrier for achieving effective drug targeting to specific sites in other organs than liver and spleen is the sequestration of intravenously administered polymeric drug delivery [1]. It is well established that this sequestration effect is owing to the rapid elimination of foreign nanoparticles by the cells of the mononuclear phagocyte system (MPS), which is defined as a cellular phenomena ‘phagocytosis’. Therefore, it is clear that one pre-requisite for engineering long-circulating NPs is to ensure that NPs with a surface that can avoid elimination by the MPS [2].

Previous researches have revealed that the nanoparticles with highly hydrophobic surface [3], positive charge [4] and large diameter [5] are very affinity to the opsonic proteins, and thus lead to high macrophage uptake and rapid clearance from blood circulation after intravenous (i.v.) administration. It has been proved that the proper particle size threshold of intravascular long-circulating nanoparticles is within 70–220 nm. Also, the opsonization of hydrophobic, negative nanoparticles, as compared to that of hydrophilic, neutral nanoparticles, may occur more quickly due to the enhanced adsorption of plasma proteins on their surfaces. Owing to the flexibility and electrical neutrality of the chains, PEG has been widely used as a successful strategy to incorporate hydrophilic polymers or to change the surface charges to/of NPs, resulting in a decreased non-specific interaction of complexes with serum components and an increased blood circulation time [6]. In particular, the PEG segments bound to the NPs surface can form a large water-cloud layer by linking two to three water molecules with each PEG molecule, resulting in a ‘brush’ or ‘mushroom’ configuration and sterically repel the deposition of large proteins [7].

Due to the unique flexibility and hydrophilicity, double-sided PEGylated triblock (BAB type) copolymers have attracted great attentions in recent years. Totally different from the configuration of the diblock copolymer, such as PLA–PEG, BAB type copolymer containing hydrophobic PLA or PCL (A-block) domains and hydrophilic polyester PEG (B-block) is more prone to form a U-shape with PLA...
units concentrated in the core and the PEG tails projecting out into the water [8]. In the same way, the NPs formed by BAB type copolymer are with higher flexible PEG coverage, and thus repel the protein adsorption more effectively. Therefore, the BAB type copolymer has been widely used as carriers for drug target in recent years [9,10]. In the past few years, a large amount of studies on BAB type copolymer NPs have been reported on their preparation [11], drug controlled release [9], degradation [12] and biocompatibility [13]. However, seldom studies emphasized on the longevity and biodistribution of BAB type NPs in vivo, including the PEGylation degree effect.

Herein, the main objective of this work was to investigate the effect of the PEGylation degree on the in vitro macrophage uptake and in vivo blood circulation time of BAB type copolymer NPs. The NPs, with the bovine hemoglobin as a model drug, were fabricated by a five-step double emulsion method from pure PLA and BAB type mPEG–PLA–mPEG copolymers (PELE for short) with different PEG-grafted degree. The physicochemical properties with respect to the particle size, apparent electrical charge, suspension stability and drug loading were characterized. The in vitro macrophage uptake, in vivo pharmacokinetics and biodistribution following i.v. administration of nanoparticles labeled by 6-coumarin were analyzed. To mimic the phagocytosis in vivo, the primary culture of mouse peritoneal macrophages (MFP), a classical phagocytic cell line model [14], was selected to carry out the in vitro macrophage uptake experiment. The pharmacokinetic analysis about nanoparticles circulation longevity in blood and organs accumulation especially in MPS studies is conducted with ICR mice. Besides, in vitro cytotoxicity of nanoparticles was also studied.

2. Materials and methods

2.1. Materials

dl-Poly((L)-lactide) (dl-PLA) (Mw 40,000) and PELE copolymers with different PEG content were purchased from Jinan Daigang Biomaterial Co., Ltd. In detail, PELE copolymers are synthesized with a monomer ratio of [LA] to [EG] 95 to 5 and an average molecular weight (Mw) of 70 kDa (PELE5), 85 to 15, Mw 35 kDa (PELE15) and 70 to 30, Mw 16 kDa (PELE30). Lyophilized Bovine Hemoglobin (Hb) was purchased from Yuanju Biotechnology Company, Shanghai. 6-Coumarin with laser grade was obtained from Acros. Other chemical reagents (methylene chloride, acetone, acetic ether, poly(vinyl alcohol) (PVA)) were all analytical grade.

Male ICR mice of 25 ± 2 g body weight were obtained from Shanghai Animal Center (Chinese Academy of Science, Shanghai, China). Fetal bovine serum (FBS, non-heat inactivated) was purchased from Gibco Laboratories (Lenexa, KS).

2.2. Preparation of Hb-loaded nanoparticles

 Nanoparticles (NPs) containing model drug of hemoglobin and coumarin-6 were formulated using a modified multiple emulsion–solvent evaporation technique as described previously [15]. In brief, 10 mg polymer, 6-coumarin (10 μg) and 0.15 g Span80 were dissolved in mixture of methylene chloride, acetone and acetic ether (5 ml) as organic phase. Then an aqueous solution of hemoglobin (0.15 g/ml, 0.5 ml) was emulsified in the organic phase using a probe sonicator (50W for 15 s) to form a primary oil-in-water emulsion. This initial emulsion was further mixed in the suspension stability tests of NPs suspensions in phosphate buffer saline (PBS) at pH 7.4 by ultrasonication. The size distributions and zeta potentials of the NPs suspensions were determined at 25 °C by Dynamic Light Scattering (DLS) using Zetasizer Nano ZS (Malvern Instruments Ltd., UK). Morphology of particles was observed by transmission electron microscope (TEM, Hitachi H-7500, Japan).

2.3. Characterization of the nanoparticles

2.3.1. Size distribution and zeta potential

Approximately 100 mg NPs were re-dispersed in 10 ml PBS (pH 7.4) for several minutes using an ultrasonic bath. The size distributions and zeta potentials of the NPs suspensions were determined at 25 °C by Dynamic Light Scattering (DLS) using Zetasizer Nano ZS (Malvern Instruments Ltd., UK). Morphology of particles was observed by transmission electron microscope (TEM, Hitachi H-7500, Japan).

2.3.2. Hydrophilicity investigation of the NPs

2.3.2.1. Contact angle measurement of NPs

Hydrophilicity of the NPs was evaluated by measuring the static contact angle of particle films as previously described by Cao et al. [16] with some modifications. Briefly, NP suspension (1 mg/ml Millipore water) was spin-coated onto a cleaned glass slide (100 mm × 100 mm × 1 mm) at 1500 rpm for 45 s. And then, advancing sessile drop water contact angles were measured on PEG modified and unmodified surfaces using JJC-1 static contact angle equipment (Changchun No. 5 Optical Instrument Co. Ltd.). Milli-Q water was used with a drop volume of approximately 0.02 ml. Results are presented as an average of 5 measurements on at least three different sites.

2.3.2.2. Water swelling behavior of the corresponding polymers

The polymers were dissolved in methylene chloride and the suspension was spin-coated on the surface of glass slides, and then the glass slides were allowed to evaporate the solvent for the polymers films formation at 50 °C for 24 h. Then, the dynamic swelling properties of the polymer matrix were measured by a gravimetric method. Polymer films with different PEGylation degree were swollen in PBS (pH 7.4) solution at 37 ± 0.5 °C in an incubator. At pre-determined time, samples were removed from solution and blotted dry with tissue paper, then weighed in predetermined time until no weight change was observed. The swelling ratio was defined by the weight ratio of the net liquid uptake to the dried polymer sample.

2.3.3. Measurement of the drug loading

A Fourier transform infrared spectrophotometer (NICOLET7500 (Thermal Nicolet, USA)) was employed to investigate the hemoglobin loading efficiency in the NPs using the KBr pellet by calibration curves method with internal standard polyacrylonitrile (PAN).

2.4. Determination of the PVA residual on nanoparticles surfaces

Residual amount of PVA on the surface of NPs was determined using a colorimetric method [17–18]. Briefly, 2 mg of lyophilized nanoparticles was treated with 2 ml of 0.5 M NaOH at 60 °C. After 15 min of incubation, each sample was neutralized with 900 μl of 1N HCl and the volume was diluted into 5 ml with distilled water. Then 3 ml of a 0.65 M solution of boric acid, 0.5 ml of I2/KI (0.05/0.15 M) solution, and 1.5 ml of distilled water were added. Finally, the absorbance of the samples was measured at 690 nm after 15 min incubation to make sure that the color reaction completely finished. A standard plot of PVA was prepared under identical conditions.

2.5. Suspension stability of NPs

Suspension stability tests of NPs suspensions in phosphate buffer saline (PBS) at pH 7.4 were performed by an analyzer of physical destabilization of concentrated liquid dispersions (Formulation, L’Union, France). In detail, suspensions of NPs (0.1 g/ml) were measured in a glass measurement cell at 37 °C for 5 days, and
the backscattered and transmitted flux rates analyses were carried out at various time points.

2.6. MTT assay

Cytotoxicity of the NPs was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay in human umbilical vein endothelial cell-derived cell line, ECV304. ECV304 cell suspensions (100 μl, 2 × 10^4 cells) were dispensed (four wells for each particle type) into 96-well plates (Costar, New York, USA) and incubated overnight (12 h) to allow for cell adherence. Culture medium was replaced with 100 μl of nanoparticles/culture medium suspensions with different concentrations of 0.03125, 0.0625, 0.125, 0.25, 0.5, 1, and 2 mg/ml and incubated at 37 °C. ECV304 cells without nanoparticles were used as the control. After 72 h incubation, 30 μl of MTT (5 mg/ml) solution was added into each well and was allowed to react at 37 °C for 4 h. Then, the solution was removed and 100 μl of dimethylsulfoxide (DMSO) was added into each well. The plate was incubated for 15 min at 37 °C and optical densities (OD) at 490 nm was measured with Spectra max PLUS384 ( Molecular Devices Corp., USA). The cell viability was calculated by the following formula:

\[
\text{Cell viability} (\%) = \frac{OD_{\text{sample}} - OD_{\text{control}}}{OD_{\text{control}}} \times 100\%
\]

2.7. Quantitative and qualitative analysis of phagocytic uptake

Phagocytic uptake of NPs was determined by using murine peritoneal macrophage cell (MPM) harvested from ICR mice (weight in 25 ± 1 g). 1 ml mercaptoacetaate was first injected into the abdominal cavity of mice. After 30 min later, the mice were sacrificed and the peritoneal cells were collected by flushing the peritoneal cavity with 10 ml of 0.9% NaCl. Then, the MPM were isolated by centrifugation at 1000 rpm for 5 min and re-suspended in culture medium (cell density). After that, the MPM (1 ml/well) were transferred into 24-well plate and incubated at 37 °C in atmosphere containing 5% CO₂. After 24 h, the cells were then washed twice with cell culture medium to remove the non-adherent cells and adherent cells were further incubated in culture medium containing 0.1% (v/v) FBS for possible opsonization events.

And then, 300 μl of coumarin-6 labeled NPs isotonic Na chloride suspension (500 μg/ml) were added and incubated with the MPM at 37 °C for 30 min. The phagocytized particles were observed using a fluorescence microscope (Nikon TE2000, Nikon Instruments, Melville, NY).

With respect to the quantification analysis, the macrophages were washed and rinsed twice with ice PBS to remove the non-phagocytized particles. Then to each well, 300 μl of mixture of triton X 100 at 10% in acetoneitrile was added to extract the coumarin-6 entrapped in the NPs. Finally, the fluorescent intensity of the 6-coumarin-6-containing extraction was analyzed by a fluoroskan ascent reader (Thermo Labsystems, Finland). And the percentage of uptake was calculated by division of the fluorescent intensity of total NPs introduced into the cells by the fluorescent intensity of the nanoparticle-phagocytosed cells.

2.8. In vivo pharmacokinetics and biodistribution

Following intravenous administration to ICR mice (25 ± 1 g), the blood circulation time and tissues distribution of the NPs labeled with coumarin-6 were determined as our previously procedure in detail [15]. Briefly, the mice were injected in tail vein at the dose of 10 ml/kg (body weight of mice) of NPs suspension in saline of 150 mg/ml. At the indicated time intervals post-injection, blood samples (approximately 0.5 ml) were collected from fossa orbitalis and added into heparinized microcentrifuge tubes. Subsequently, the mice were sacrificed. The organ samples, consisting of the common organs such as heart, brain, spleen, and the typical MPS organs such as liver, lung, and kidney were removed, quickly rinsed with distilled water and weighted. After that, the fluorescent intensity of the coumarin-6 extracted by acetonitrile from the blood and the organs samples were assayed by spectrophluorimeter (Thermo Lab-systems, Finland) at λex 485 nm and λem 538 nm to calculate the blood clearance and biodistribution of the NPs.

In order to avoid the errors derived from the different coumarin-6 loading in the NPs fabricated from different polymer, in the experiment, for every specific nanoparticles, a serials standard curves of the coumarin-6 extracted from blood and different organs leaching liquor containing determined NPs content were established to assay the coumarin-6 extracted from the NPs left in the corresponding blood and organs, respectively. Then, the nanoparticles concentration left in blood and organs after specific period was expressed by the amount of coumarin-6 in nanoparticles extracted by acetonitrile and calculated from the corresponding standard curves. And the percentages of every nanoparticles residual amount were calculated from the ratios of coumarin-6 amount in the blood and organs to the total amount of coumarin-6 on nanoparticles injected into the tail vein. Triplicates of mice were intravenously administration for each sample point. PK parameters were estimated using Kinetic software (InnaPhase Corporation, Copyright © 1997–2006).

2.9. Statistic analysis

All data were generated in three independent experiments with two or three repeats. Data were analyzed with student’s t-test. The difference between treatments was considered significant when \( p < 0.05 \) in a two-tail analysis.

3. Results

3.1. Physicochemical properties of nanoparticles

The physicochemical properties, including particle size, polydispersity index, ζ potential and drug loading of the NPs with different PEGylation degree are presented in Table 1. It can be found that the composition of the copolymers had no obvious effect on the particle size and polydispersity index. Fig. 1 shows the typical TEM image of the NPs encapsulated in this study. The NPs appeared spherical with a size range of 70–220 nm, which was in accordance with the size distribution determination (Table 1). But the surface electrical potential of the NPs varied with the PEGylation degree.

Table 1 Physicochemical properties of hemoglobin-loaded NPs encapsulated by copolymers with different PEGylation degree.

<table>
<thead>
<tr>
<th>Samples</th>
<th>Type of polymer (PEGylation in copolymer %)</th>
<th>Mean diameter (nm)</th>
<th>Polydispersity index</th>
<th>Drug loading (%)</th>
<th>ζ Potential (mV)</th>
<th>PVA residence (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PELEG NPs</td>
<td>di-PLA(0)</td>
<td>1877 ± 15.6</td>
<td>0.215</td>
<td>24.79 ± 1.67</td>
<td>−37.67 ± 0.5</td>
<td>12.30 ± 0.9</td>
</tr>
<tr>
<td>PELE5 NPs</td>
<td>mPEG-PLA-mPEG(5)</td>
<td>193.0 ± 8.3</td>
<td>0.203</td>
<td>23.05 ± 2.17</td>
<td>−22.8 ± 1.2</td>
<td>10.07 ± 2.5</td>
</tr>
<tr>
<td>PELE15 NPs</td>
<td>mPEG-PLA-mPEG(15)</td>
<td>189.9 ± 12.5</td>
<td>0.209</td>
<td>33.17 ± 3.33</td>
<td>−13.6 ± 0.7</td>
<td>10.03 ± 2.3</td>
</tr>
<tr>
<td>PELE30 NPs</td>
<td>mPEG-PLA-mPEG(30)</td>
<td>171.5 ± 4.9</td>
<td>0.198</td>
<td>16.77 ± 1.45</td>
<td>−2.23 ± 0.3</td>
<td>7.06 ± 1.5</td>
</tr>
</tbody>
</table>

All data were measured triplicates and represented by mean ± standard deviation (SD).
degree pronouncedly. NPs obtained were all negatively charged, and the absolute \( \zeta \) potential monotonically decreased from \(-37.67\) to \(-2.23\) mV with the PEG-grafted degree increasing from 0% to 30%. As for the Hb loading efficiency in NPs, which were calculated according to the calibration curves with good linear correlations \( R^2 \) and excellent reliability (not shown), respectively, the results suggested that when the PEG percentage were lower than 5%, the Hb loading efficiency almost remained constant. Whereas, when the PEG percentage in copolymer increased from 5% to 15%, the drug loading capacity was improved obviously. But further increase the PEG-grafted degree led to the sharp decrease of the Hb loading, indicating a typical PEG-grafted degree-dependent manner. In addition, the results in Table 1 also demonstrated that the surface associated PVA decreased from 12.3% (w/w) to 7.06% (w/w) depending on the increase of the PEGylation degree in PELE copolymers. The dynamic swelling behaviors and contact measurements of pure PLA and copolymers in PBS solution at 37\(^\circ\)C (in Fig. 2) indicated that the hydrophilicity of nanoparticles increased with the increasing of the PEGylation degree from 0% to 30%.

3.2. Suspension stability of nanoparticles

Fig. 3 demonstrates the BS intensity and the transmission variation as a function of the height of the samples and the detection time. It can be seen that a very instable suspension in the middle (5–30 mm) of the PLA NPs was easily detected. According to Fig. 3(c and d), for PELE15 and PELE30 NPs, the BS intensity was all constant over the sample height at the determined time point with no macroscopic creaming at the top of sample or sedimentation at the bottom of samples during the period of scanning [19]. However, with respect to PELE5 displayed in Fig. 3(b), a typical sedimentation phenomenon was observed at the bottom of the sample characterizing the concentration enhancement in dispersed phase, and decreased at the top of the signals characterizing a reduced concentration.

In addition, the transmission variations over the height of the samples were also detected and the results are illustrated in Fig. 3(e). After 5 days scanning, the decrease of the transmission for PLA was 11.02%, much higher than the other samples (7.69% for PELE5, 4.90% for PELE15 and 1.29% for PELE30), indicating that more flocculation and coalescence occurred in PLA NPs than the PELE copolymer NPs.

3.3. MTT assay

The cytotoxicities of the NPs fabricated by different polymer were investigated with ECV304 as the model cell line. The morphology observed by microscopy demonstrated that after incubation with 250 \( \mu \)g/ml of PELE30 NPs suspensions for 72 h, ECV304 all exhibited typical cobblestone-like appearance and reached confluence (not shown). From Fig. 4, it can be seen that after 72 h incubation, the cell viabilities exposed to the PEGylation NPs were all higher than that exposed to the PLA NPs significantly, suggesting that PEGylation, to some extent, can decrease the cytotoxicity of the NPs.

3.4. In vitro macrophage uptake of NPs

In this study, coumarin-6, which had been used as a fluorescent marker of polymeric nanoparticles extensively [18,20,21], was introduced as a marker of NPs to study NPs internalized in phagocytosis as well as biodistribution. In order to verify the reliability of the data obtained from the \textit{in vitro} and \textit{in vivo} experiments, the 6-coumarin release experiment first carried out in simulated body fluid (SBF, pH 7.4) and the bovine fresh blood in this study (data not shown). The results demonstrated that the total released amount of 6-coumarin from the NPs with different PEGylation degree were not more than 1% during the 48-h period tested, suggesting that the 6-coumarin incorporated in the NPs as a marker could not interfere with their inherent biological behaviors \textit{in vitro} and \textit{in vivo}.

Fig. 5 shows the cellular uptake of 6-coumarin-labeled NPs made from different copolymers by MPMs after 30 min incubation, respectively. As shown in Fig. 5, significant decreases of the NPs with emitting much weaker fluorescent light by phagocyte were observed with the increase of PEGylation degree. Fig. 6 shows the percentage of the cellular uptake of NPs with different PEG-grafted degree by MPMs. It can be seen that compared to the PLA NPs, the uptake percentage of PEGylated NPs decreased significantly. In particular for the PELE30 NPs, a near 5-fold decrease of the macrophage cellular uptake was observed.

3.5. Plasma pharmacokinetics of nanoparticles

The plasma residences of the NPs with different PEG content were investigated and the plasma pharmacokinetic parameters...
were also calculated by Kinetica 4.4 software. The plasma residence and the corresponding pharmacokinetic parameters are presented in Fig. 7 and Table 2, respectively.

As shown in Fig. 7, after intravenous administration, the PLA NPs were quickly cleared from the plasma (see “clearance” row in Table 2) with falling to half its original dose within 30 min. In contrast, the residual amount of PEGylated nanoparticles in plasma was improved significantly (Fig. 7), and the extent of the enhancement increased with the increasing of the PEGylation degree. Moreover, the total area-under-the-curve (AUC$_{0–36}$) and MRT in plasma, which determine the therapeutic efficiency and the residence in plasma respectively, were much higher than that of the pure PLA nanoparticles (Table 2). This is particular true for the PELE30 NPs, which indicated the highest dose remaining in plasma and the half-life. Even after 36 h, up to 45% of the injected dose was recovered from the plasma. This indicated that the NPs prepared from PELE copoly-

### Table 2

<table>
<thead>
<tr>
<th>NPs</th>
<th>PELE0</th>
<th>PELE5</th>
<th>PELE15</th>
<th>PELE30</th>
</tr>
</thead>
<tbody>
<tr>
<td>AUC$_{0–36}$ plasma (%Dose h) $^a$</td>
<td>306.266</td>
<td>1221.96</td>
<td>1331.49</td>
<td>1733.29</td>
</tr>
<tr>
<td>Cl (h$^{-1}$) $^b$</td>
<td>0.1280</td>
<td>0.0161</td>
<td>0.0073</td>
<td>0.0051</td>
</tr>
<tr>
<td>MRT$_{0–36}$ plasma (h) $^c$</td>
<td>17.1955</td>
<td>110.879</td>
<td>234.939</td>
<td>256.564</td>
</tr>
<tr>
<td>AUC$_{0–36}$ heart (%Dose h)</td>
<td>15.617</td>
<td>–</td>
<td>–</td>
<td>5.4691</td>
</tr>
<tr>
<td>AUC$_{0–36}$ lung (%Dose h)</td>
<td>168.835</td>
<td>–</td>
<td>–</td>
<td>25.2454</td>
</tr>
<tr>
<td>AUC$_{0–36}$ liver (%Dose h)</td>
<td>1582.82</td>
<td>–</td>
<td>–</td>
<td>33.1046</td>
</tr>
<tr>
<td>AUC$_{0–36}$ spleen (%Dose h)</td>
<td>402.739</td>
<td>–</td>
<td>–</td>
<td>33.1046</td>
</tr>
<tr>
<td>AUC$_{0–36}$ kidney (%Dose h)</td>
<td>250.876</td>
<td>–</td>
<td>–</td>
<td>37.4176</td>
</tr>
</tbody>
</table>

$^a$ AUC$_{0–36}$ plasma = the area under the plasma concentration versus time curve, from time 0–36 h concentration; Dose = the original injection dose.

$^b$ Cl = clearance.

$^c$ MRT = mean residence time.
Fig. 4. In vitro cytotoxicity of NPs on ECV304 cells. The NPs were fabricated by PLA and PELE copolymers with different PEG content. ECV304 cells were incubated with the NPs for 72 h. Cell viability (%) was determined based on the ratio of optical density of treated cells to that of the untreated cells. Data presented are mean ± SD from 3 separate experiments of triplicate wells. Compared to the control sample of non-PEGylated nanoparticles, there was a significant difference in cellular toxicity between PEGylated and non-PEGylated nanoparticles (p < 0.05 by the Student’s t-test, n = 3).

3.6. Biodistribution of NPs in vivo

The NPs prepared from PELE30 exhibiting the most prolonged blood circulating property was selected to investigate the biological fate of NPs in vivo after i.v. administration. The PLA NPs was also studied for control. The major organs (such as heart, lung, liver, spleen and kidneys) pharmacokinetic obtained from these NPs were analyzed by Kinetta 4.4 software and the relevant parameters are summarized in Table 2. As shown in Fig. 8 and Table 2, liver was the major cumulative organ for both nanoparticles. In detail, PLA NPs were concomitantly the majority accumulated in liver (from 56.65% of the injected dose at the first 5 min after intravenously injection to 23.22% at 36 h), followed by lung (from 12.62% to 1.28%), kidneys (from 2.25% to 8.52%) and spleen (from 6.6% to 10.97%) (Fig. 8(a)). In contrast, in the case of PELE30 NPs, 23.38% and 36 h later, respectively, which was dramatically lower than that of PLA NPs during the whole period of determination. Similarly, the concentrations of the PELE30 NPs in other organs investigated seemed also lower than the PLA NPs: in lung (from 3.80% to 0.61%), in kidneys (from 1.94% to 0.83%), in spleen (from 4.24% to 0.58%) (Fig. 8(b)). Additionally, after 36hrs administration, the accumulation percentage of the PLA NPs in heart and brain were 0.20% and 0.95%, a little higher than that of the PELE30 NPs (0.12% and 0.79%, respectively).

The differences in major MPS tissues (i.e. liver, spleen and lungs) as a function of time after intravenously administration were also calculated and compared in Fig. 8(c). With respect to PLA NPs, the sequestration in the MPS tissues was very rapid, in particular in the first 5 min, over 75% of nanoparticles were captured by phagocytosis. Furthermore, the uptake nanoparticles were eliminated by metabolism rapidly, with only 35.5% of original injected dose residual after 36 h later, decreased about 40%. In contrast, PELE30 NPs had a more steady experience with 31.4% found in the MPS in 5 min after injection. During 36 h of blood circulation, the accumulation in the MPS decreased for 22.7%. Thus, an advantage of PEG with double side-grafted to hydrophobic chains for avoiding MPS uptake was clear. With the same PEG chain length (5 kDa), the higher density on the surface resulted in a lower uptake by MPS.

4. Discussion

In the past few years, double-sided PEGylated triblock (BAB type) copolymers have been developed and used as drug carriers. There are many reports on the preparation, degradability, and drug-controlled properties of the BAB type copolymer, in particular the mPEG–PLA–mPEG and mPEG–PCL–mPEG. In present work, amphiphilic BAB type copolymers were applied to fabricate intravenously administered long-circulation NPs, with a hydrophobic core and a hydrophilic shell. The effects of the PEGylation degree on the physicochemical characteristics, in vitro macrophage uptake as well as in vivo biodistribution and plasma clearance kinetics of the NPs were investigated. For comparison purpose, conventional PLA nanoparticles were also included in this study.

Based on the requirements of the long-circulating NPs, we first tailored all the NPs around 100–200 nm. Within this size range, the surface charge, hydrophilicity, suspension stability, PVA residual as well as the drug loading all showed a typical PEGylation degree-dependant manner. It has been broadly accepted that PEG is a classical polymer with uncharged, hydrophilic and nonimmunogenic properties that can be physically adsorbed onto or, preferably, covalently attached to the surface of hydrophobic colloids [23]. As we mentioned above, indeed, when PEG chains are bounded at the surface, the high affinity for water of the flexible chains would improve the hydrophilicity of particles membrane and sterically repel plasma protein adsorption. Furthermore, PEG coating builds a shift in the shear plane on the surface, leading to neutralization of the apparent charge of the NPs. In present work, with the hydrophobic PLA serving as core reservoirs for drug and the hydrophilic PEG as the shell projecting into the aqueous environment, the copolymer chains appeared a U-shape of brush-like conformation. The presence of the hydration ‘brush’, with a particularly effective steric repulsion, is thought to be essential for improving the physicochemical properties such as zeta potential and hydrophilicity on the surface of nanoparticles. When PEG chains introduced into the negative PLA segments, the neutral charged PEG segments occupy the negative site on the surface of nanoparticles, with more PEG chains resulting in less negative charges. In the same patterns, the hydrophilicity of copolymers was gradually increased in association with the elevation of PEG content in copolymer. These results were consistent with that obtained in the common diblock copolymers [24].

The data obtained here showed that NPs made of PELE copolymer effectively repelled the macrophage uptake in vitro and prolonged the circulatory half-life in vivo. And the extent of the suppression of the macrophage uptake and the prolongation of the circulatory half-life monotonically increased with the PEGylation degree in the copolymers ranging from 0 to 30 wt%. In addition, the pharmacokinetics in plasma and the MPS organs of the NPs with different PEGylation degree highlighted that the sequestration of the NPs by MPS organs, such as liver, lungs and spleen, predominantly determined the plasma clearance efficiency of NPs. In detail, for PLA NPs, a low experimental area under the curve (AUC) and the mean residence time (MRT) in plasma were observed, which suggested a short residence time in blood, and a large amount of NPs was observed in MPS organs. In contrast, with respect to NPs containing PEG content of 30% (PELE30 NPs), a much longer circulation time was obtained involved in a lower NPs accumulation in MPS organs, as listed in Table 2. These results were in accordance with the results obtained in the preliminary macrophages uptake test in vitro. In phagocytosis study, a remarkable decline of cellular uptake from pure PLA with 0% PEGylation to PELE30 with 30%
PEGylation was found (Fig. 6), with the corresponding phagocytosis fluorescent image presenting much weaker luminescence (Fig. 5).

To our great knowledge, a favorable/unfavorable response to foreign particles after intravenous administration is controlled by the adsorption of opsonins proteins, and subsequent phagocytic recognition in vitro and in vivo. This is especially true for NPs. Owing to the considerable specific surface area and surface energy, NPs are preferred to adsorb proteins to minimize the surface energy [25]. From this viewpoint, the reason for the reduction trend of macrophage uptake and subsequent blood clearance of the NPs derived from the PEGylated PLA with the PEGylation degree from 0 to 30 wt% in the current experiment was considered.

Firstly, it has been proposed that the surface coverage and the configuration of PEG molecules have a large effect in reducing phagocytosis instead of the PEG chain length [25]. A ‘brush’ configuration compared to a ‘mushroom’ can form a mobility of PEG
molecules with a higher cloud density and uniformity that may enhance the shielding effect [26]. It has been elucidated that the PEG chains with ‘brush-like’ conformation can effectively minimize the interfacial energy and weaken the attraction of plasma proteins by means of altering the surface properties such as zeta potential and hydrophilicity of NPs which are the key factors controlling their biological properties [27–28]. It was pointed out that at the PEG chain length of 5 kDa, the diblock copolymer PLA–PEG preferred to shape a ‘mushroom’ configuration [7]. Totally different from the PLA–PEG copolymer, BAB type copolymers, He et al. [8] found large amount of PEG segments migrated to the surface of NPs fabricated by BAB type copolymers, spontaneously. Therefore, even with the same molar ratio of LA/EG and PEG chain length, there is a higher PEG coverage onto the surface of mPEG–PLA–mPEG NPs than that onto the PLA–PEG NPs. One could hypothesize that the PEG chains on the mPEG–PLA–mPEG NPs are prone to assuming ‘brush-like’ structure. And with the PEGylation degree increased, the polymer is more preferred to the ‘brush-like’ configuration, leading to more pronouncedly on suppression the opsonization by plasma proteins.
Secondly, the ζ potential monotonically enhanced from −37.67 to −22.37 mV with the PEG-containing increased in polymer (Table 1) indicating that the PEG modification strongly influence the superficial charges of NPs. The mechanism of PEG ζ-effect on NPs has been studied by other researchers [29]. In the same way, the higher negative ζ potential of PELE copolymers may also be attributed by the PEG segment sheltering the ionized carboxyl end groups of PLA on the nanoparticles surface which could be indicative of few negative charges on the surface of the PLA core. Therefore, owing to this ‘shelter effect’, the ζ potential could be assigned to an increase in the more and more thickness of the PEG barrier as the density of PEG increases [30]. Gbadamosi et al. [31] had successfully established a linear correlation between the surface charge and the extent of phagocytosis by macrophage cells of the nanoparticles. They found that the negative charged NPs with lower ζ potential were more prone to phagocytosis which supported our results in vitro and in vivo. In conclusion, the extent of phagocytosis and ζ potential data were in good agreement and shown that the PEGylation could reduce both ζ potential and recognition by phagocytic cells, with the higher PEG content in copolymer, the ζ potential becomes more close to neutrality and the more phagocytic cells are repelled.

Thirdly, another surface property responsible for suppressing plasma proteins approach to NPs is hydrophilicity. The osmoporation existing between the protein and the NPs were more prone to occur on the hydrophilic surfaces than on hydrophobic ones [7,32]. Therefore, for hydrophobic surface, a strategy for making it more hydrophilic was to introduce the hydrophilic chains onto the surface. It is true in this work that hydrated PEG chains remarkably altered the hydrophilic properties of PLA and decrease hydrophobic interactions with phagocytic cells. The hydrophilicities of copolymers were compared in terms of swelling behavior values (Fig. 2). It was showed that as the PEGylation degree increased in hydrophobic PLA chains from 0% to 30%, the equilibrium-swelling capacity of the water-uptake significantly increased. Owing to linking two to three water molecules with each PEG molecule brush, the higher grafted degree makes more PEG molecules exist which adsorbed more water molecules into the macromolecules structure. Therefore, the NPs surface possessing more of these hydrated layers might be more beneficial for remain camouflaged or invisible to phagocytic (Figs. 5 and 6).

In addition, in our study, a special phenomenon was found that the stability of NPs was improved in associated with the increase of PEG content. Because, in general, the electrostatic repulsion between particles with the same electrical charge prevents the aggregation of the particles [33–34], with the larger of the absolute zeta potential the more stable the dispersion should be [35]. This interesting phenomenon could be owing to the presence of PEG ‘brush’ conformation ensuring a more efficient steric protection on the particles surface to avoid flocculation and coalescence phenomena (Fig. 3) superior to the electrostatic affect. It is suggested that the PEG density played an important role in NPs suspension stability, with more PEG density on the surface tending to the more significant steric stability.

Taken together, it has been demonstrated that the plasma clear- and MPS accumulation amount of PELE30 NPs significantly lower than that of other polymers, suggesting that NPs with PEG-grafited degree of 30% have the possibility of the best efficiency in long-circulation in bloodstream.

5. Conclusions
The nanoparticles fabricated by PELE copolymers were found to have prolonged the longevity in bloodstream, especially for PELE30 NPs with a PEG chains length of 5 kDa. They could also avoid uptake by phagocytic cells existing in MPS organs more significantly than other NPs with or without PEG did. The study illustrated that the zeta potential and hydrophilicity of nanoparticles dramatically were changed by PEG chains content in polymer resulting in repelling the phagocytic cells and extending the residence time in blood. PELE NPs improved their fate in vivo with increasing of PEG content significantly, suggesting that the ‘stealth’ NPs prepared by double side PEGylated copolymer is a useful strategy to service for long-circulating drugs, as in the case in some blood therapies.

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