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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, poly(vinyl alcohol) (PVA) residual on nanoparticles surfaces as well as drug loading. The macrophage uptake and biodistribution including plasma clearance kinetics following intravenous administration in mice of the NPs labeled by 6-coumarin were evaluated. The results showed that, except for the particles size, the 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 ($t_{1/2}$) 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.

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 chains 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

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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 (MPM), 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 (50 W for 15 s) to form a primary oil-in-water emulsion. This initial emulsion was further mixed in the stabilizer of PVA-containing aqueous solution to make a w/o/w double emulsion with a high-pressure homogenizer. After that, the double emulsion was poured into 110 ml water solution and the system was stirred for the removal of the solvent under atmospheric pressure at room temperature.

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 a 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 were 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 solved 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 (NICOLET5700 (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 I_2/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 (Formulaction, 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