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Evaluation of blood circulation of polysaccharide surface-decorated PLA nanoparticles

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Abstract

The aim of our study was to investigate the blood clearance characteristics and biodistribution of polysaccharide surface-decorated PLA nanoparticles. For this purpose, cholesterol-modified dextran was synthesized and used as emulsion stabilizer for preparation of PLA nanoparticles by an o/w emulsion-evaporation technique. The influence of substituted degree (SD) of cholesterol on dextran chains and concentration of dextran–cholesterol on the size of PLA nanoparticles were studied *via* TEM and DLS. It was found that the optimal conditions were SD = 5% and $C_{DEX-CH} = 0.5$ mg/ml to prepare dextran–cholesterol-coated PLA nanoparticles (DEX–CH/PLA) with the size about 105 nm and a narrow size distribution. The coating of polysaccharide on the surface of PLA nanoparticles was demonstrated by ζ-potential measurement, and the existence of polysaccharide remarkably reduced non-specific protein absorption. These polysaccharide-decorated PLA nanoparticles were injected intravenously into S–D rats and their blood clearance and biodistribution *in vivo* was studied using scintillation counter. The results showed that although the polysaccharide coating inhibits BSA absorption, but does not help in prolonging the blood circulation time compared with PVA stabilized PLA nanoparticles. The DEX–CH/PLA nanoparticles were captured mainly by liver, spleen, and lungs within 5 h after injection, while they were barely found in brain.

Keywords: Dextran; PLA; Nanoparticles; Surface-decoration; Blood clearance; Conformation

1. Introduction

Biodegradable polyester nanoparticles represent a new large class of drug carriers, such as poly(lactic acid) (PLA), poly(lactic-co-glycolic acid) (PLGA), and poly(ε-caprolactone) (PCL). But none of actual available polymers/copolymers are satisfactory. For example, particles made from PLA and stabilized with surfactants, such as sodium cholate or human serum albumin (HSA), their half-life in blood when injected intravenously into rats is only 2 min (Verrecchia et al., 1995) due to their surface′s activation of the complement system, which leads to unwanted liver accumulation. Poly(ethylene glycol) (PEG) surface-modification can significantly prolong the circulation of nanoparticles *in vivo* (Gref et al., 1994), but they do not possess reactive groups at their structure allowing ligand coupling (Gref, Rodrigues, & Coubvreur, 2002).

One of the most promising alternative to PEG is polysaccharide which has actually been widely investigated. Rouzes et al. synthesized amphiphilic dextran, modified by covalent grafting of phenoxy groups (Rouzes & Gref, 2000). Then they prepared dextran-coated PLA nanoparticles using alkyl dextran derivatives (Dex C_n) as stabilizers (Rouzes & Leonard, 2003). Passirani, Barratt, Devissaguet, and Labarre (1998) developed long circulating nanoparticles bearing heparin or dextran covalently bound to poly(methyl methacrylate). Both types of nanoparticles proved to be long-circulating: both circulated for more than

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48 h. The half-life of heparin nanoparticles was 5 h, while bare PMMA nanoparticles were proved to have a half-life of only 3 min. The advantage of polysaccharide coating is steric protection of nanoparticles against non-specific interactions with proteins and insured particle stability in the blood circulation system. Additionally, as polysaccharides offer many available reactive groups, active targeting could be obtained by grafting ligands onto the nanoparticles surface (Lemarchand, Gref, & Couvreur, 2004). However, several papers (Lemarchand & Gref, 2005; Lemarchand & Gref, 2006) investigated polysaccharide-coated nanoparticles *in vitro* and suggested that the polysaccharide surface may act as a weak or strong activator of complement system. This seems to conflict with the *in vivo* studies presented above in this paper.

In order to get a better understanding about the roles played by polysaccharide coatings, the aim of our study was to investigate the blood clearance characteristics and biodistribution of polysaccharide-coated PLA nanoparticles. For these purposes, a series of cholesterol hydrophobically modified dextran (DEX–CH) was synthesized, and DEX–CH-coated poly(lactic acid) (PLA) nanoparticles were prepared using an emulsion-evaporation method, taking advantage of the anchoring of cholesterol onto the surface of PLA core. The clearance characteristics of these nanoparticles after i.v. administration into rats were investigated using the technique of scintillation counter.

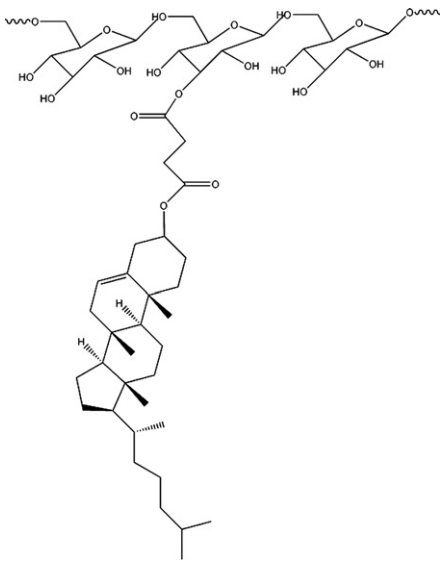
2. Experiment

2.1. Materials

Dextran (average molecular weight: 100 kDa), Bovine serum albumin (BSA) and fluorescein isothiocyanate (FITC) were purchased from Sigma. Cholesterol was purchased from Tianjin Chemical Reagent Co., China, and recrystallized in ethanol before use. PLA was obtained from Department of Medical Polymers Shandong Institute (Jinan, China). Technetium-labeled diethylene-triamine-pentacetate (99mTc-DTPA) was purchased from Beijing Kesheng Co., China. DMSO-d_6 was obtained from Beijing Huateng Chemical Co., China. Other chemicals used were purchased from Tianjin Kewei Reagent Co., China. Additionally, triethylamine, anhydrous dimethyl sulfoxide and sulfoxide chloride were refined before use.

2.2. Synthesis of cholesterol hydrophobically modified dextran

The synthesis of DEX–CH (structure shown in Scheme 1) was performed according to the procedure reported previously (Gu & Yuan, 2007). Briefly, cholesterol was esterified with succinic anhydride in anhydrous pyridine. The resulting cholesterol 3-hemisuccinate was then reacted with $SOCl_2$ in anhydrous chloroform to obtain cholesterol 3-hemisuccinyl chloride. DEX–CH was obtained by esterification of dextran with cholesterol 3-hemisuccinyl chloride using anhydrous dimethyl sulfoxide (DMSO) as a solution and triethylamine as a catalyst. The structure of DEX–CH was confirmed by ^{1}H NMR (DMSO-d_6, Varian UMTY plus400 NMR spectrometer). The substituted degree (SD), determined from ^{1}H NMR spectrum and defined as percent content of cholesterol per 100 glucopyranosidic units, was controlled by varying the amount of cholesterol 3-hemisuccinyl chloride, and the mole ratio of glucopyranosidic units of dextran to cholesterol are listed in Table 1. We synthesized a series of DEX–CH with three different substituted degrees.



Scheme 1. Structure of cholesterol hydrophobically modified dextran (DEX–CH).

2.3. Preparation of PLA nanoparticles bearing dextran derivatives on surface

PLA nanoparticles bearing DEX–CH (DEX–CH/PLA), was prepared by an o/w emulsion-evaporation method.

Table 1
Size of the DEX–CH/PLA nanoparticles[a]

Substituted degrees (%)	Concentration of DEX–CH (g/l)	Mean diameter (nm)	pI
2	0.125	125	0.464
	0.250	180	0.276
	0.500	167	0.287
	1.000	230	0.422
	2.000	254	0.371
5	0.125	137	0.309
	0.250	158	0.312
	0.500	104	0.207
	1.000	181	0.276
	2.000	210	0.321
10	0.125	123	0.147
	0.250	134	0.240
	0.500	125	0.210
	1.000	177	0.242
	2.000	234	0.289

[a] Size of the DEX–CH/PLA nanoparticles were obtained by DLS measurement.

The typical procedure was as follows: a defined amount of DEX–CH was uniformly dispersed in predetermined amount of distilled water; while PLA was dissolved in acetone. Then the PLA solution was added into the former under sonication (pulse mode, 200 W, 10 min in an ice bath) using an ultrasonic cell pulverizer (model 600 W, Xingzi Inc, Zhejiang, China). Finally, acetone was evaporated under mild vacuum (Rotavapor®, 20–30 min at a constant temperature (25 °C)).

For comparison purpose, a similar procedure was used to prepare "uncoated" PLA nanoparticles and PVA/PLA nanoparticles, using sodium cholate (0.3% w/w in water) and PVA (2% w/w in water) as surfactant, respectively.

2.4. Characterization of polysaccharide-coated nanoparticles

2.4.1. Size and size distribution

To evaluate the effect of nanoparticles size on biofunctionalization, size analyses were carried out on a Dynamic Light Scattering (DLS, 90 Plus/BI-MAS nanoparticle size analyzer, USA). The nanoparticles were dispersed in phosphate-buffered saline (PBS) under sonication, then the effective diameter and size distribution were recorded at 90° scattering angle under 25 °C.

2.4.2. Morphology

The morphology of the DEX–CH/PLA nanoparticles was observed using a transmission electron microscope (TEM) (JEM-2000 FX II, Jeol, Japan). A drop of the nanoparticles' suspension was placed on a copper grid coated with carbon film and dried at 25 °C, then the samples were negatively stained with phosphotungstic acid solution (2% w/v). Observation was performed at 80 kV.

2.4.3. ζ-Potential

ζ-potential of various particles were measured in phosphate-buffered saline (PBS) using zeta analyzer (Powereach®, Zhongchen Digital Technology Instrument Ltd., Shanghai, China).

2.5. Adsorption of fluorescein isothiocyante (FITC)-labeled bovine serum albumin (BSA) on nanoparticles surface

BSA (fraction V, Sigma, St. Louis, MO) was labeled with FITC by mixing 1.5 g of BSA and 30 mg of FITC in 5 mL phosphate buffer solution (0.1 M, pH 8.5) for 6 h at 4 °C (Cho & Jeong, 1997). FITC-labeled BSA was then purified by dialysis against distilled water using a cellulose membrane (MWCO 14,000) for 2 days (Kamiyama, Onishi, & Machida, 1999).

BSA-FITC adsorption was carried out in 0.01 M phosphate buffer, pH 7.4. A well-defined amount of particles (10 mg) was put into contact with 40 mL of BSA-FITC solution (25 mg/mL) (Lemarchand & Gref, 2005). After 6 h of incubation at 37 °C, the samples were centrifuged (30 min, 4 °C, 15,000 rpm, Beckmann J2-21 centrifuge, USA) to remove the non-adsorbed BSA-FITC. Then the collected nanospheres were resuspended in water. The procedure was repeated three times (Rouzes & Leonard, 2003). The adsorbed amounts of protein onto the nanoparticles were determined from the re-dispersed nanoparticles suspension using a fluorescence spectrophotometer (F-4500 FL Spectrophotometer, Hitachi, Japan).

2.6. Radiolabeling procedure of microspheres

The radiolabeling method for microspheres was adopted from the procedure described by Illum, Jorgensen, and Bisgaard (1987). The radiolabeling procedure was carried out in the presence of the powerful reducing agent, stannous chloride. The stannous ion reduces 99m-technetium from the +7 oxidation state to the more reactive +5 oxidation state to promote binding. The electron donating functional groups, for example the hydroxyl groups of polymers and phospholipids, are accepting the technetium (Soane, Frier, Perkins, Jones, & Illum, 1999).

Fifty milligrams of nanoparticles was suspended in the labeling medium containing 1.5 ml of normal saline, 1 ml $SnCl_2{\cdot}2H_2O$ (5 mg/ml) and 0.7 ml technetium–99m pertechnetate eluate containing about 6 mCi of activity. The mixture was left under continuous stirring for about 10 min and separated by centrifugation. The labeled nanoparticles were dialysed against 1 L normal saline for 12 h, and then administered in suspension form.

2.7. Blood clearance and biodistribution

Sprague–Dawley rats weighing 200–250 g were selected for the blood clearance and biodistribution studies. In order to obtain the blood clearance curves for DEX–CH/PLA and PVA/PLA nanoparticles, two groups of rats ($n = 6$) were used in these experiments: group 1 was treated with ^{99m}Tc-labeled DEX–CH/PLA nanoparticles and group 2 with ^{99m}Tc-labeled PVA/PLA nanoparticles. First, the two kinds of suspension were injected into the tail vein at a dose of 0.1 ml per rat. Blood samples were drawn from the retro-orbital sinus at various time intervals with a heparinized Pasteur pipette. Then the blood samples were weighed.

The animals were sacrificed by cervical dislocation after 5 h of injection and different organs (liver, kidney, spleen, lung, brain) were removed, washed with normal saline and dried in paper folds. The radioactivity in each organ and blood samples was counted using scintillation counter and expressed as percent injected dose per gram, taking into account of the physical decay in a standard sample.

3. Results

3.1. Physicochemical properties of nanoparticles

3.1.1. Morphology of DEX–CH/PLA nanoparticles

The typical TEM image of DEX–CH/PLA nanoparticles (SD = 5%, $C_{\text{DEX–CH}} = 0.5$ mg/ml) was shown in

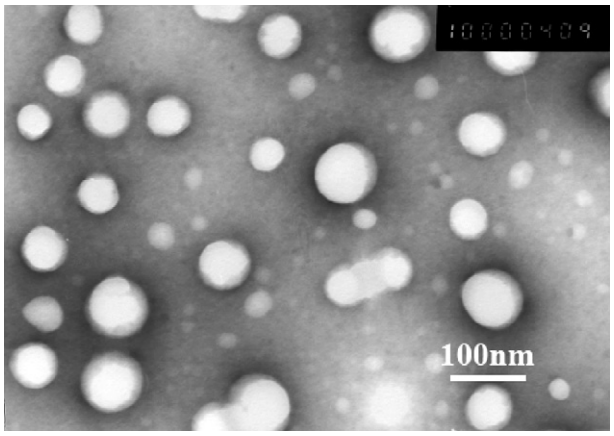


Fig. 1. Morphology of DEX–CH/PLA nanoparticles observed by TEM (SD = 5%, $C_{DEX-CH} = 0.5$ mg/ml).

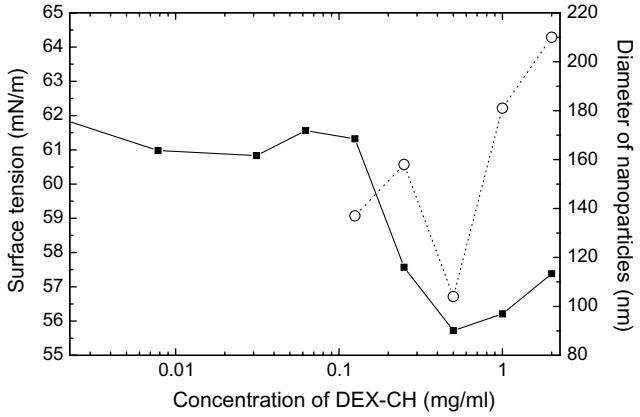


Fig. 3. The relationship between the surface tension (■) of DEX–CH and the diameters of the DEX–CH/PLA nanoparticles (○) (SD = 5%).

Fig. 1. The nanoparticles were found to be almost spherical with size of about 80–90 nm, and a core–shell structure was obviously observed. Since the DEX backbone is hydrophilic whereas PLA is hydrophobic, the shell should be assigned to DEX–CH, which was anchored onto the surface of hydrophobic PLA core through the anchoring of cholesterol pendant groups.

3.1.2. ζ-Potential

ζ-Potential measurement was employed to determine the coating of polysaccharide on surface of PLA nanoparticles. ζ-Potential of PLA, DEX–CH/PLA and DEX nanoparticles were measured, respectively. The pure PLA particles exhibited a ζ-potential value of −35.2 mV, while the DEX–CH/PLA nanoparticles possess a zeta potential of −11.9 mV. This value was very close to that of DEX–CH self-aggregated (DEX) nanoparticles (−11.2 mV), demonstrating the presence of polysaccharide derivative on the PLA nanoparticles.

3.1.3. Size and size distribution of DEX–CH/PLA nanoparticles

PLA nanoparticles with a narrow distribution (Fig. 2) could be obtained through o/w emulsion-evaporation method using DEX–CH as an emulsion stabilizer. The variation of mean diameters of nanoparticles with DEX–CH concentration and SD values are presented in Table 1. The size of DEX–CH/PLA nanoparticles were mainly influenced by the concentration but secretly affected by SD value of DEX–CH. The mean diameter of the nanoparticles decreased with the increase of DEX–CH concentration. These phenomena are in consistent with the results reported by Rouzes and Gref (2000). They found that the nanoparticles with minimum size appeared at the DexP concentration of 1–2 g/L, whatever the phenoxy content in the DexP. Increasing the DexP concentration, as well as using smaller DexP concentrations, resulted in larger particle sizes. In our study, we found that the smallest PLA nanoparticles could be obtained at the concentration when the lowest surface tension appeared (Fig. 3), thus our results demonstrated the hypothesis made by Rouzes, that the size of the nanoparticles prepared through o/w emulsion evaporation method is controlled by surfactant efficiency of the amphiphilic polysaccharide. When the concentration of amphiphilic polysaccharide becomes larger than a critical concentration, self-aggregation will occur between the polysaccharide molecules, resulting in a decrease in adsorption concentration at the o/w interface, which lead to the instability of the emulsion drops, and consequently, the size of the particles increased.

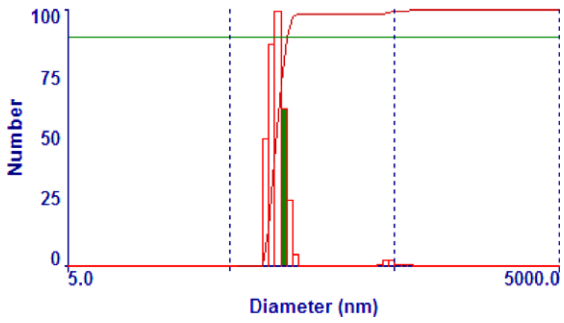


Fig. 2. Histogram of the size distribution of the DEX–CH/PLA nanoparticles obtained by DLS measurement (SD = 5, $C_{DEX-CH} = 0.5$ mg/ml).

3.2. BSA adsorption studies

The adsorption of FITC-labeled BSA (BSA-FITC) onto DEX, DEX–CH/PLA = 3/1, DEX–CH/PLA = 10/1 and uncoated PLA nanoparticles was measured for comparison purpose. As can be seen in Fig. 4, all dextran-coatings significantly decreased the interactions of the nanoparticles surface with proteins (compared with the uncoated PLA particles). The presence of a hydrophilic layer of dextran onto the surface of nanoparticles resulted indeed in protein rejecting abilities. Additionally, it can be seen from the figure that DEX–CH/PLA = 10/1 nanoparticles adsorb the least amount of BSA suggesting that such composition is the optimized one to avoid protein adsorption.

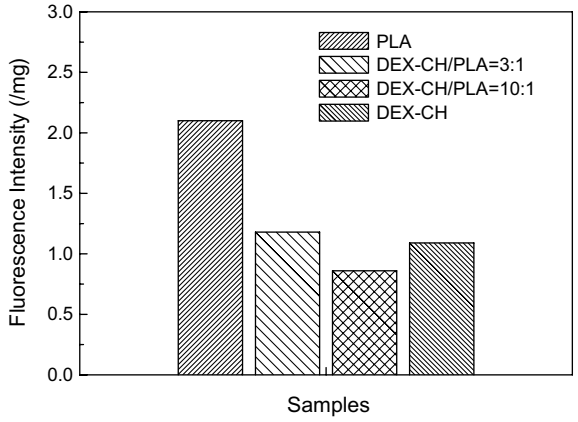


Fig. 4. BSA-FITC adsorption onto nanoparticles.

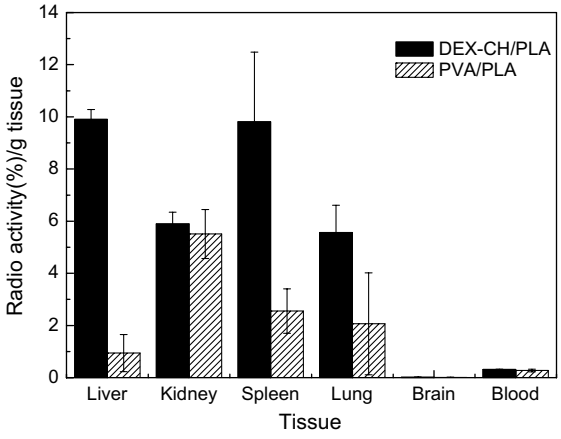


Fig. 6. Biodistribution of nanoparticles after 5 h of *i.v.* injection.

3.3. Blood clearance characteristics and biodistribution

3.3.1. Blood clearance characteristics of the two types of nanoparticles

The DEX–CH/PLA nanoparticles with the minimum size of 104 nm were chosen for *in vivo* studies. Since the uncoated PLA nanoparticles stabilized with sodium cholate were not stable during dialysis against physiologic saline, they aggregated gradually. So this sample was abandoned for *in vivo* study taking into account that they would aggregate quickly in the blood of rats (Verrecchia et al., 1995), just like during the dialysis procedure. Polyvinyl alcohol is commonly used as a polymeric stabilizer and provides particles with a hydrophilic polymer layer, but it is not acceptable for intravenous administration (Rouzes & Leonard, 2003). Despite this limitation, PVA has been used as polymeric stabilizer in several researches. We introduced it here in our research as a stabilizer for PLA just for comparison purpose.

The blood clearance curves for ^{99m}Tc-labeled PVA/PLA and DEX–CH/PLA nanoparticles were shown in Fig. 5. As we can see from this figure, the elimination of both nanoparticles from blood was fast in the first 1 h but then slowed down. The PVA/PLA nanoparticles showed an initial higher blood circulating levels compared with DEX–CH/PLA nanoparticles. Twenty minutes after injection, the radioactivity of DEX–CH/PLA nanoparticles (counted % original dose/g blood) was 0.8, while that of the PVA/PLA nanoparticles was about 2.0. The half-life ($t_{1/2}$) of the PVA/PLA nanoparticles in blood was 13.8 min, while that of the DEX–CH/PLA nanoparticles was 6.1 min.

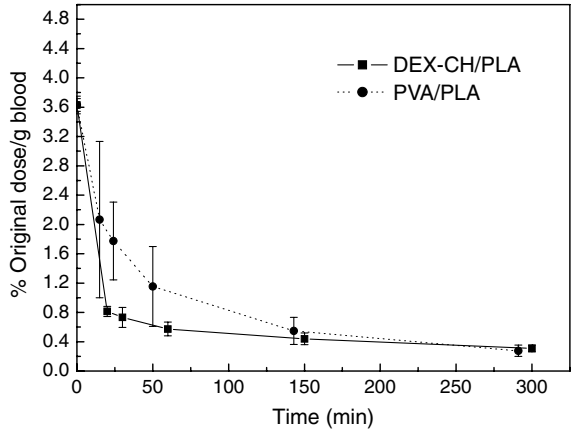


Fig. 5. Blood clearance profiles of DEX–CH/PLA and PVA/PLA nanoparticles.

3.3.2. Biodistribution

The biodistribution profile of ^{99m}Tc-labeled DEX–CH/PLA and PVA/PLA nanoparticles to various tissues after 5 h of *i.v.* injection was shown in Fig. 6. In the case of DEX–CH/PLA nanoparticles, the radioactivity data (counted per gram of organ) in different organs were found to be as follows: liver (10%), spleen (10%), lungs (5.6%), kidney (5.9%), blood (0.3%), while only 0.01% activity was found in brain. In the case of PVA/PLA nanoparticles, the data were as follows: liver (1%), spleen (2.6%), lungs (2%), kidney (5.6%), blood (0.27%) and brain (0.009%).

4. Discussion

Longevity in the bloodstream is a highly demanded feature for a drug delivery system, giving the carriers enough time to reach their targets and to release their cargos in a continuous and controlled fashion (Gref et al., 1994). To avoid rapid blood clearance, administered particles must retard opsonization and macrophage recognition which results in sequestration by RES organs, such as liver and spleen. Furthermore, injected particles (and their aggregates) should be small and deformable enough to avoid filtration through the lung capillary bed and the splenic interendothelial cell slits (Moghimi, Hunter, & Murray, 2001). In conclusion, the blood circulation time and biodistribution of nanoparticles depend on their size and surface characteristics including hydrophilicity, flexibility, and surface charges.

From the size and morphology studies, we finally found the optimum condition to obtain appropriate dextran-coated PLA nanoparticles. The obtained nanoparticles were spherical in shape and uniform in size about 100 nm. The BSA adsorption study demonstrated that the dextran coating significantly decreased the interaction of PLA nanoparticles with BSA protein. This result affirmed the hypothesis that the amphiphilic dextran coating provides a steric protection for nanoparticles against non-specific interactions with proteins.

However, the *in vivo* study showed almost the opposite results. The blood circulation time of DEX–CH/PLA nanoparticles was not significantly prolonged compared with uncoated PLA nanoparticles and their organ accumulation was rather much. That's because when it comes to polysaccharide, the situation is even more complicated as their conformation is involved in as a key factor to determine its destiny in blood (Labarre et al., 2005; Lemarchand & Gref, 2005; Lemarchand & Gref, 2006; Passirani et al., 1998).

Radioactivity of ^{99m}Tc-labeled DEX–CH/PLA nanoparticles recorded in the highly perfused organs, such as liver, spleen and lungs could be accounted for as the combined activity of the circulating blood passing through organs as well as that due to particle uptake by cells of the reticoendothelial system (RES) of these organs (Banerjee, Mitra, Somh, Sharma, & Maitra, 2002). The majority of surface-coating polymers used to date are rich in hydroxyl groups, which may eventually trigger the activation of both the alternative and classical pathways of the complement system. Not only the presence of hydroxyl groups at the nanoparticles′ surface, but also the DEX conformation (loops or brush) in the shell are key factors involved in the complement activation process (Lemarchand & Gref, 2006). This was also demonstrated by Labarre et al., recently. They suggested that when polysaccharide chains arranged as "loops" and "trains" it will activate the complement system while as a "brush" will not (Labarre et al., 2005). In the present study, dextran was modified by grafting cholesterol onto their backbones. Then DEX–CH anchored by several points resulting in the formation of loops and trains very close to the core of the nanoparticles, corresponding to more dextran chains becoming collapsed onto the nanoparticles surface. Therefore, the dextran surface may act as a strong activator of the complement system. Thus results in particle fixation by opsonizing complement fragments (e.g., C3 degradative products) and stimulation of particle recognition by activated complement receptors of phagocytic cells.

The PVA/PLA nanoparticles accumulated much less than DEX–CH/PLA nanoparticles in RES organs, such as liver and spleen, suggesting that the PVA coating on the particles surface provides a hydrophilic layer, which significantly decreases the interaction of particles with blood proteins. However, it is worth noticing that the blood retention of PVA/PLA is lower than DEX–CH/PLA nanoparticles at the end of the observation. That can be explained by a high excretion of the PVA/PLA nanoparticles (Park & Kwon, 2006). The kidney accumulations of the two particles are almost the same.

Additionally, the brain accumulations of both nanoparticles were found to be close to zero, suggesting that neither nanoparticle has the brain-targeting property. This result offers an evidence to deny the hypothesis that DEX coated nanoparticles may be helpful for the blood–brain barrier (BBB) targeting and recognition (Lemarchand & Gref, 2006).

5. Conclusions

Polysaccharide surface-decorated PLA nanoparticles were obtained using cholesterol hydrophobically modified dextran as emulsion stabilizer, and particles with size about 105 nm was obtained at SD value of 5% and emulsion concentration of 0.5 mg/ml. The existence of polysaccharide on the nanoparticles obviously decreased the protein adsorption, but did not significantly increase the blood circulation time of the nanoparticles mainly due to the "loops" conformation of the polysaccharide chains which was demonstrated to be a strong complement activator. So it can be concluded that graft-modified polysaccharide should not be used as surface coatings in long-circulating drug delivery system.

Acknowledgement

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