Preparation of Sterically Stabilized Human Serum Albumin Nanospheres Using a Novel Dextranox-MPEG Crosslinking Agent

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Human serum albumin (HSA) nanospheres with a size less than 200 nm in diameter were prepared using a modified coacervation method and crosslinking with methyl polyethylene glycol modified oxidized Dextram (Dextranox-MPEG) which created a sterically stabilizing polyethylene oxide surface layer surrounding the nanospheres. The crosslinking efficiency and the surface characteristics of glutaraldehyde and Dextranox-MPEG crosslinked HSA nanospheres were determined and compared. The zeta potential of the Dextranox-MPEG crosslinked particles was significantly lower than that of glutaraldehyde stabilized particles. The existence of a hydrated steric barrier surrounding the nanospheres was confirmed by an electrolyte and pH induced flocculation test. The Dextranox-MPEG crosslinked nanospheres showed a significantly reduced plasma protein adsorption on the particle surface compared with glutaraldehyde crosslinked nanospheres.

KEY WORDS: human serum albumin; nanospheres; crosslinking agent; surface characteristics.

INTRODUCTION

Colloidal drug delivery systems such as microspheres, liposomes and emulsions are now used as intravenously injected carriers for delivery of drugs to specific organs or target sites within the body (1). The biodistribution of these colloidal systems and delivery of incorporated drugs to target sites after intravenous administration are mainly determined by their physicochemical characteristics such as size and surface hydrophobicity through their recognition or non-recognition by the body's immunological defence system (2,3,4). Particles that are small enough to escape the capillary beds of the lungs are normally sequestered rapidly by the cells of the reticuloendothelial system (RES), particularly the Kupffer cells of the liver (5). The rapid removal of colloidal particles by the Kupffer cells has been correctly identified as a major obstacle to targeting other cells or tissues elsewhere in the body such as the bone marrow and solid tumours (6). Several methods have been investigated in an attempt to overcome this problem such as the changing of surface characteristics by coating the particles with block copolymers (2,4). The adsorption of poloxamer and poloxamine surfactants on to small polystyrene particles can form a steric polyethylene oxide (PEO) barrier that dramatically reduces the uptake of the particles by the RES and provides particles with a significantly longer circulation half life (2) or particles that deposit in a specific organ site such as the bone marrow (4) and the spleen (3,7).

Human serum albumin (HSA) is widely used as a microsphere material since it is considered to be nonantigenic and biodegradable, and is readily available (8). HSA microspheres or nanospheres can easily be prepared using emulsion or coacervation methods and stabilized by using either a chemical crosslinking agent or heat denaturation (9,10). However, since the surface of HSA nanospheres is more hydrophilic than that of polystyrene latex, poloxamer or poloxamine surfactants are poorly adsorbed on the surface of the HSA particles, neither has it proven successful to incorporate the surfactant during the preparation process. Studies have been published by Chen et al (11) where PEO chains have been linked to the surface of albumin microspheres by post-treating the microspheres with cyanuric chloride-actived PEG. However, so far the preparation of nanosphere sized albumin spheres sterically stabilized with PEO chains has not been reported.

This paper describes a novel method for the preparation of HSA nanospheres (less than 200 nm in diameter) sterically stabilized with grafted PEO chains. The nanospheres were prepared using a modified coacervation method and crosslinking with methyl polyethylene glycol substituted oxidized dextran (Dextranox-MPEG). The crosslinking efficiency and the surface characteristics of glutaraldehyde and Dextranox-MPEG crosslinked HSA nanospheres were determined and compared. The difference between these two types of the HSA nanospheres with respect to blood protein adsorption is also reported.

MATERIALS AND METHODS

Materials

Dextranox-MPEG (Figure 1) was synthesized and purified by the Biomaterial Group, Department of Organic Chemistry, University of Gent, Belgium. (Dextran MW: 70,000 Da, MPEG MW: 5000 Da, substituted: 1.2 mol%, oxidized: 47 mol %, total MW: approx. 89,000 Da). HSA (Albutein, 20% albumin solution, BP) was supplied by Alpha (UK). Glutaraldehyde (50% solution) was purchased from BDH (UK), guanidine hydrochloride from Aldrich (UK) and phosphotungstic acid from Sigma (UK).

Preparation of HSA Nanospheres

Crosslinked with glutaraldehyde: 4.0 ml 0.01 M phosphate buffer (pH 6.0) was mixed with 2.8 ml of acetone and 0.2 ml of PEG 400. The mixture was stirred using a magnetic stirrer and 0.2 ml of 20 % HSA aqueous solution was added dropwise from a syringe. 100 μl of 4 % glutaraldehyde ethanol solution was added to crosslink the HSA nanospheres at room temperature for 24 hours. The crosslinked nanospheres were subsequently cleaned by centrifuging.

Crosslinked with Dextranox-MPEG: 4.0 ml of 0.5 - 2.0

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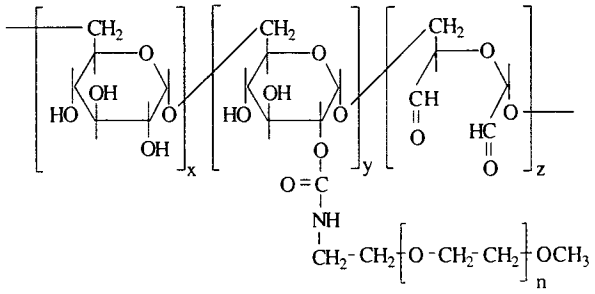

Fig.1. Structure of Dextranox-MPEG.

% Dextranox-MPEG solution (in 0.01 M phosphate buffer (pH 6.0)) was mixed with 2.8 ml of acetone and 0.2 ml of PEG 400. The mixture solution was stirred on a magnetic stirrer and 0.2 ml of 20 % HSA aqueous solution was added dropwise. The HSA particles were crosslinked by a Schiff's base reaction between amino groups in the HSA and aldehyde groups in Dextranox-MPEG at room temperature for 24 hours. The stabilized nanospheres were cleaned by centrifuging.

Crosslinking Efficiency Study

A turbidity ratio test (10,12) was employed to investigate the crosslinking efficiency obtained in the HSA nanospheres by use of glutaraldehyde and Dextranox-MPEG. This method establishes a turbidity ratio, i.e., the turbidity of the HSA nanospheres in a solution of a protein denaturing agent (guanidine hydrochloride, GuHCl) to that in water, as an indicator of the degree of crosslinking in the HSA particles. The test method consisted of the following steps: 0.2 ml of fresh HSA nanosphere suspension was added to 0.8 ml of 7.5 M GuHCl solution or 0.8 ml of distilled water respectively. The samples were incubated at room temperature for 1 hour and the resulting sample turbidity was measured using a spectrophotometer (Uvikon 860, Switzerland) at 540 nm.

Particle Size and Morphological Characteristics

The size and size distribution of the HSA nanospheres were determined by Photon Correlation Spectroscopy (PCS) using a Malvern 4700 submicron particle analyzer system (Malvern instruments, UK) with a Siemens Helium-Neon laser operating at 632.18 nm and 40 mW. The samples were diluted with distilled water and measured at 25° C at a scattering angle of 90 degrees. The size distribution was characterized by a polydispersity index (PI). The morphological characteristics of the HSA nanospheres were examined using transmission electron microscopy (TEM) (Jeol 1200 EX12, Japan). A phosphotungstic acid solution was used as stain.

Zeta Potential Measurements

The zeta potential of HSA nanospheres was determined using the technique of electrophoretic laser doppler spectrometry. The HSA nanospheres were suspended in 0.005 M pH 7.0 phosphate buffer (ionic strength 0.05 M) and subsequently measured using a Zeta Sizer (Model 4, Malvern Instruments UK).

Electrolyte Induced Flocculation of HSA Nanospheres

One mg of glutaraldehyde of Dextranox-MPEG crosslinked HSA nanospheres were incubated in 1.0 ml of Na_2SO_4 solution of different concentrations. The optical turbidity of the resultant suspensions was measured at 600 nm using a spectrophotometer.

pH Induced Flocculation of HSA Nanospheres

One mg of glutaraldehyde or Dextranox-MPEG crosslinked HSA nanospheres were incubated in 1.0 ml of constant ionic strength (0.01M) acetate buffer of pH ranging from 3.5 to 6.0. The optical turbidity of the resultant suspensions was measured. The zeta potentials of the nanospheres at the different pH values were also determined.

Plasma Protein Adsorption on the Surface of the HSA Nanospheres

HSA, rat serum and plasma were used to investigate the adsorption of protein in vitro as models for in vivo plasma protein opsonisation of the HSA nanospheres. Two mg of glutaraldehyde or Dextranox-MPEG crosslinked HSA nanospheres were incubated in 1.5 ml of 0.005M McIlvaine buffer (pH 7.4) containing 0, 2, 5 and 10 % of HSA, rat serum or plasma. The samples were shaken on a vibratory shaker (IKA-VIBRAX-VXR, IKA-labortechnik, Germany) at room temperature for 2 hours. The zeta potential and size of the nanospheres were measured after incubation.

RESULTS AND DISCUSSION

Normally, small HSA nanospheres can be prepared using coacervation methods which involve the addition of coacervation agents to the albumin aqueous solution (10,13,14). The size of the nanospheres is dependent upon the balance between several factors such as the amount of coacervation agent, stirring speed and viscosity of the medium. Since Dextranox-MPEG is a hydrophilic macromolecular crosslinking agent, the addition to the newly formed HSA particle suspension will affect the balance of the system by changing the state of hydration of the HSA particles and increasing the viscosity of the medium and hence influence the size of the HSA nanospheres. In the present study, a modified coacervation method was developed by addition of aqueous HSA solution to Dextranox-MPEG acetone solution to prepare HSA nanospheres. HSA nanospheres with a size less than 200 nm and a relatively narrow size distribution (Table I) can be prepared using this method. The TEM micrograph in Figure 2 reveals that a good spherical shape of HSA nanospheres was obtained.

For the protein crosslinking stage, the amino-aldehyde reaction (Schiff's base reaction) is normally carried out at natural or mildly alkaline pH (15). Increasing the pH can accelerate the reaction and increase the repulsion force between entwined HSA molecules to prepare smaller particles (10). However, since an increase in pH will result in an increase in the solubility of the HSA above that of Dextranox-MPEG in the water-acetone system, in the present study, the crosslinking reaction was carried out at pH 6. Additionally, a larger amount of crosslinking agent and a longer reaction

Table I. HSA Nanospheres Crosslinked with Glutaraldehyde and-Dextranox-MPEG

	Crosslinking Agent	
	Glutaraldehyde[a]	Dextranox-PEG[b]
Particle size (nm)	134.7 ± 13.6	171.6 ± 16.7
Polydispersity index	0.042	0.078
Zeta potential (mV)	−18.8 ± 0.5	−8.2 ± 0.5
Turbidity ratio	0.388 ± 0.017	0.317 ± 0.041

[a] 4 mg glutaraldehyde (aldehyde 0.08 mmol) used to crosslink 40 mg HSA

[b] 40 mg Dextranox-PEG (aldehyde 0.09 mmol) used to crosslink 40 mg HSA

time were used compared with the previously published pH-coacervation method (10).

Using HSA nanospheres as a drug carrier, the particles, although biodegradable, should be relatively stable in circulation and release the drug in the target organ. The degree of crosslinking effects the stability of the particles and drug release characteristics (16). Figure 3 shows the effect of Dextranox-MPEG and glutaraldehyde concentration on the degree of crosslinking and hence the stability of the nanosphere suspension expressed as the turbidity ratio. The results indicate that increasing the concentration of the crosslinking

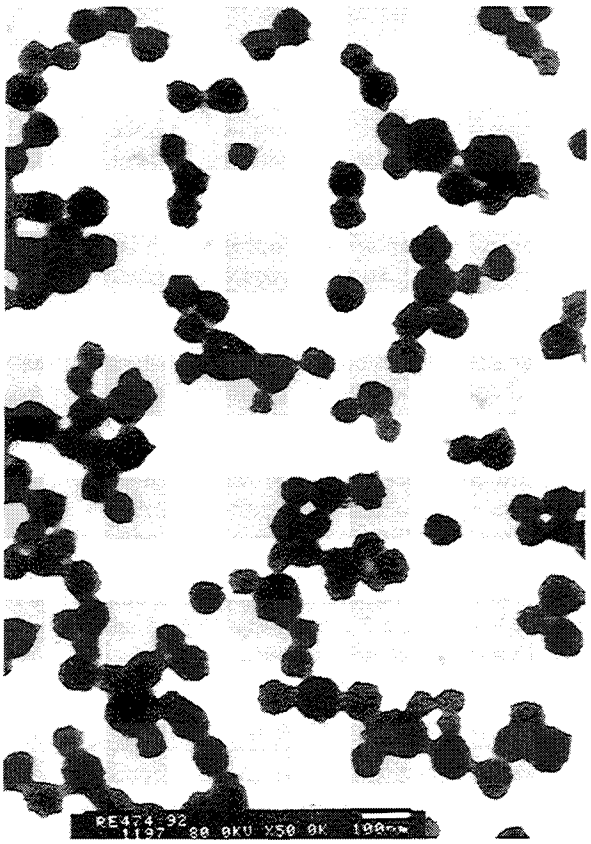

Fig.2. Transmission electron microscope micrography of Dextranox-MPEG crosslinked HSA nanospheres (no difference in particle size and morphology from the nanospheres crosslinked with glutaraldehyde).

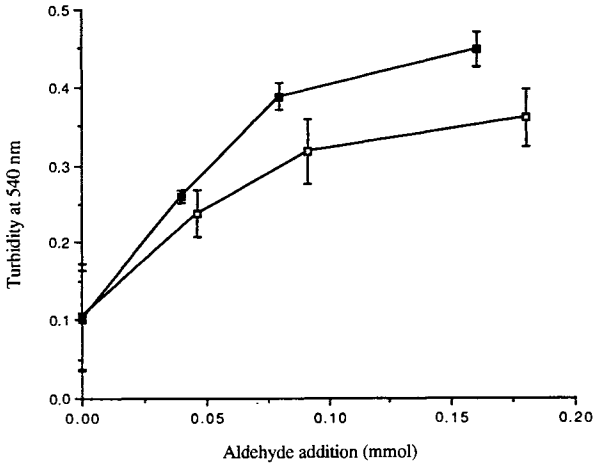

Fig.3. Influence of glutaraldehyde (solid symbol) and Dextranox-MPEG (open symbol) on the crosslinking efficiency of HSA nanospheres. Error bars = ±SD (n = 3).

agent, as expected, increases the degree of crosslinking. The crosslinking degree of Dextranox-MPEG stabilized HSA nanospheres is lower than that of glutaraldehyde crosslinked nanospheres. The probable explanation of this effect is that Dextranox-MPEG is a grafted macromolecular crosslinking agent and its steric structure suppresses the crosslinking reaction.

Surface characteristics have been known for many years to influence the biodistribution of microspheres. Determination of the surface charge of microspheres can give some information as to the nature of the particle surface. Table I shows that the zeta potential of HSA nanospheres at pH 7.0 is around −19 mV, whereas the zeta potential of the HSA nanospheres crosslinked with Dextranox-MPEG is significantly lower (−8 mV). The difference in zeta potential reveals the different surface nature of these two types of colloidal particles.

For the purpose of confirming the presence and effect of a hydrated steric PEO barrier on the surface of Dextranox-MPEG crosslinked nanospheres, the physical stability of the HSA nanosphere suspensions was assessed by electrolyte and pH induced flocculation tests. The physical stability of a colloidal system is mainly dependent upon the competitive processes of attraction (van der Waals forces) and repulsion (either electrostatic repulsive force or steric stabilizing barrier or both) (17). If particles are mainly electrostatically stabilized, adding electrolyte or adjusting pH will be sufficient to destroy the electrostatic double layer surrounding the particles. This results in the aggregation of the particles into clusters known as flocculation with a corresponding increase in optical turbidity. However, if the particles are mainly stabilized by hydrated steric stabilizing barriers, the colloidal system should be stable even if the electrostatic double layers have been destroyed. Figure 4 shows that the turbidity of the suspension of glutaraldehyde crosslinked nanospheres was increased at a low concentration of Na_2SO_4 (less than 0.3 M), whereas that of the suspension of Dextranox-MPEG crosslinked nanospheres remained at base line until the concentration was higher than 1.1 M. These results show clearly that the added electrolyte was

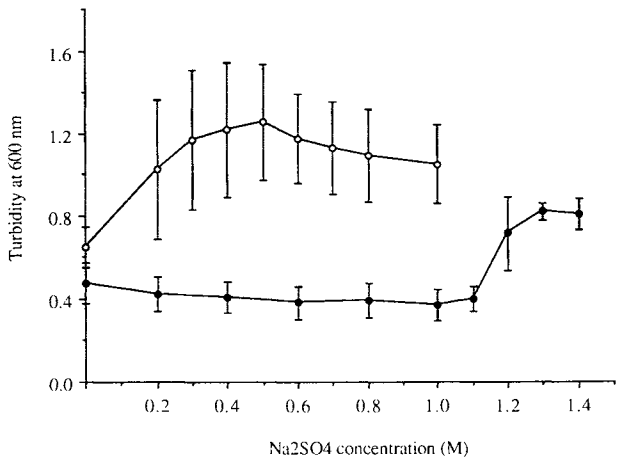

Fig.4. Influence of electrolyte on optical turbidity of dextranox-MPEG crosslinked HSA nanospheres (solid symbol) and glutaraldehyde crosslinked HSA nanospheres (open symbol). Error bars = ±SD (n = 4).

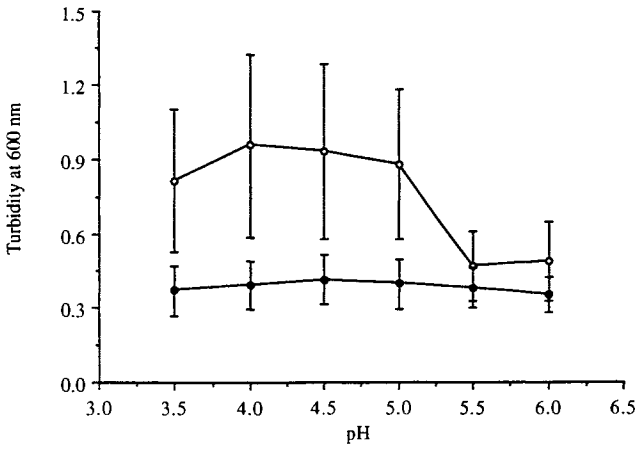

Fig.6. Influence of pH on optical turbidity of dextranox-MPEG crosslinked HSA nanospheres (solid symbol) and glutaraldehyde crosslinked HSA nanospheres (open symbol). Error bars = ±SD (n = 5).

able, at low concentration, to destabilize the glutaraldehyde crosslinked nanospheres by suppressing the electrostatic double layer surrounding and stabilizing these nanospheres. However, due to the sterically stabilizing PEO layer on the surface of Dextranox-MPEG crosslinked HSA nanospheres, no effect on the stability of the nanospheres was found at lower electrolyte concentrations. At high concentrations of Na_2SO_4 (>1.2 M), flocculation could be observed due to the dehydration of the steric layer.

The presence of a sterically stabilizing barrier surrounding Dextranox-MPEG crosslinked nanospheres was also confirmed by the pH induced flocculation test. In this test, the change in turbidity of the HSA nanosphere suspension of different pH values was measured. Figures 5 and 6 show that the zeta potential of glutaraldehyde crosslinked HSA nanospheres was near zero and the nanospheres were flocculated when the pH was around 4.5. However no flocculation was observed for Dextranox-MPEG crosslinked HSA nanospheres although the surface charges of the nanospheres were neutralized. This confirmed the presence of a hydrated steric PEO barrier surrounding the Dextranox-MPEG crosslinked nanospheres that plays an important role in the stability of the colloidal system, whereas the glutaraldehyde crosslinked nanospheres are mainly stabilized by electrostatic repulsive forces.

The rapid and efficient uptake of colloids by the RES is considered to be a consequence of the opsonization of the particles by blood components (18,19). In the present study, HSA, rat serum and plasma were used as models to investigate the opsonization of the HSA nanospheres in vitro. The adsorption of the proteins onto the HSA nanosphere surface was investigated by measuring the hydrodynamic diameter and zeta potential of the particles after incubation with HSA and other plasma proteins. The results show that the addition of HSA to the nanosphere suspensions did not significantly increase the size or change the zeta potential of the HSA nanospheres. It can be assumed that the amount of HSA adsorbed by both types of HSA nanospheres is too

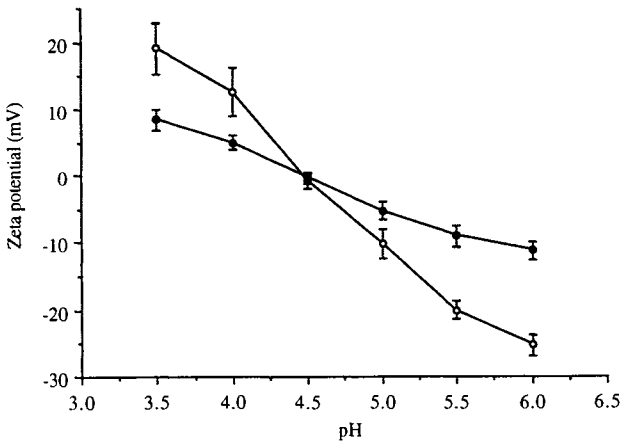

Fig.5. Influence of pH on zeta potential of dextranox-MPEG crosslinked HSA nanospheres (solid symbol) and glutaraldehyde crosslinked HSA nanospheres (open symbol). Error bars = ±SD (n = 5).

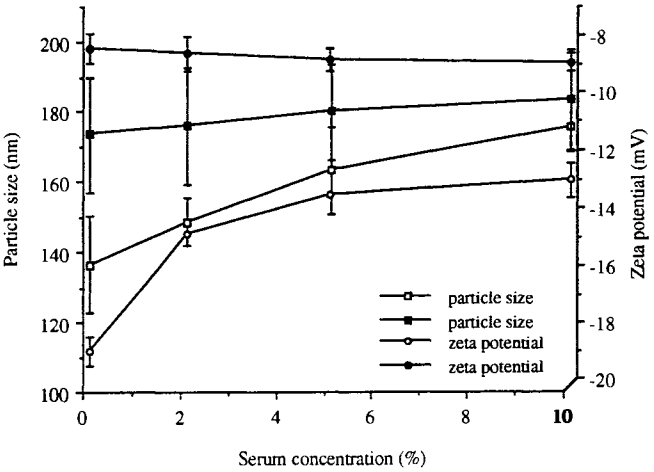

Fig.7. Rat serum adsorption on the surface of glutaraldehyde crosslinked HSA nanospheres (solid symbol) and Dextranox-MPEG crosslinked HSA nanospheres (open symbol). Error bars = ±SD (n = 3).

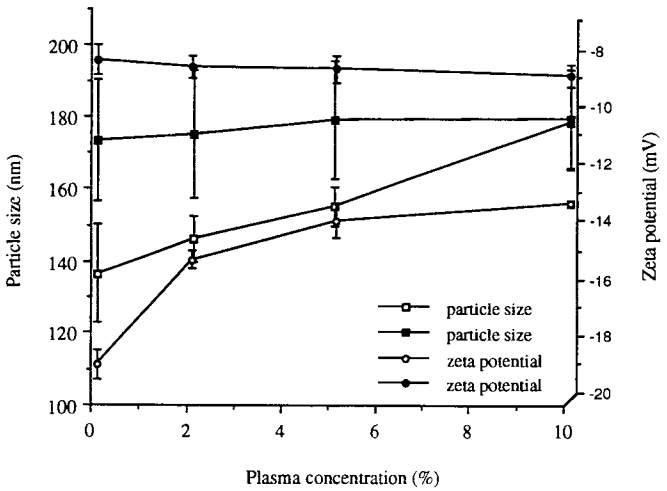

Fig.8. Rat plasma adsorption on the surface of glutaraldehyde crosslinked HSA nanospheres (solid symbol) and Dextranox-MPEG crosslinked HSA nanospheres (open symbol). Error bars = ±SD (n = 3).

small to be detected. Figures 7 and 8 show that when rat serum or plasma were used for studying the opsonisation of the HSA nanospheres, a significant difference between glutaraldehyde and Dextranox-MPEG crosslinked nanospheres was found in the change of both particle size and zeta potential. When glutaraldehyde crosslinked HSA nanospheres were incubated in serum or plasma, the particle size increased markedly as the concentration of serum or plasma increased. A significant change in zeta potential was found as well. On the contrary, only a slight change was found both in particle size and in zeta potential when the Dextranox-MPEG stabilized HSA nanospheres were incubated in serum or plasma. This reveals that the surface of Dextranox-MPEG crosslinked nanospheres is protein resistant compared to that of glutaraldehyde crosslinked nanospheres. The low level of plasma protein adsorption is likely to be due to the steric PEO barriers surrounding the Dextranox-MPEG crosslinked particles. It has been demonstrated that hydrated steric PEO barriers can reduce the adsorption of proteins on the particle surfaces (19,20) and hence reduce the uptake of the particles by RES (2). Since Dextranox-MPEG crosslinked HSA nanospheres have sterically stabilizing PEO chains on the particle surface and a low level of plasma protein adsorption, it is expected that the nanospheres can avoid uptake of by the RES and thus could exhibit a longer circulation life in vivo.

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