Long Circulating Nanoparticles *via* Adhesion on Red Blood Cells: Mechanism and Extended Circulation

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Polymeric nanoparticles have long been sought after as carriers for systemic and targeted drug delivery. However, applications of nanoparticles are limited by their short *in vivo* circulation lifetimes. We report that by attaching polymeric nanoparticles to the surface of red blood cells, it is possible to dramatically improve their *in vivo* circulation lifetime. The particles remain in circulation as long as they remain attached to red blood cells. Particles eventually detach from red blood cells due to shear forces and cell-cell interactions and are subsequently cleared in the liver and spleen. Circulation of red blood cells themselves is not affected by particle attachment procedures. This manuscript reports an in depth analysis of the behavior of nanoparticles bound to red blood cells, especially their circulation characteristics, biodistribution, and mechanisms of clearance. Exp Biol Med 232:958–966, 2007

Key words: nanoparticles; erythrocyte; stealth; evasion; nanotechnology; clearance

Introduction

Polymeric nanoparticles have been proposed for numerous applications in intravascular drug delivery and blood pool imaging (1–6). Encapsulation of therapeutic agents in polymeric nanoparticles provides enzymatic protection and possible sustained release over prolonged time periods (7–9). Circulating nanoparticles also offer a useful modality as contrast agents for blood and tissue imaging (10). However, injected nanoparticles have found limited applications due to their short vascular circulation lifetimes (11, 12). Particles in circulation are opsonized and thus recognized and captured by the reticuloendothelial system (RES). Accordingly, depending on size and surface characteristics, many particles are cleared within short periods after their injection (6, 11, 13–15).

We have demonstrated previously that vascular circulation times of polystyrene particles can be extended by orders of magnitude *via* noncovalent adhesion on red blood cells (RBCs; Ref. 16). This strategy was found to be effective for particles ranging in diameter from 100 nm to 1.1 μm. This strategy was motivated by mammalian pathogens, including hemobartonella and eperythrozoonosis (diameter: ~0.2–2 μm; Ref. 17), that bind to the exterior RBC surface and remain in circulation for several weeks.

Although the circulation time of particles is greatly improved by adhesion on RBCs, the particles are still cleared from the circulation within several hours of injection, limiting their potential for drug delivery applications. To further improve the circulation time of RBC-bound particles, the mechanism of particle clearance must be understood. Our previous studies showed that although particles are removed from vascular circulation, the RBCs to which they were attached remain in circulation (16). However, the mechanisms by which particles detach from the RBC surface and the biologic fate of detached particles are not known.

In this report we show that once in circulation, particles detach passively from RBCs due to shear forces and cell-cell interactions and are subsequently removed from circulation, primarily in the liver and spleen. We further demonstrate that the strength of binding between the particle and the RBC primarily determines the circulation lifetime of particles. Finally, we show that surface modification of the RBC-particle complex using polyethylene glycol (PEG) further increases the circulation half-life of bound particles to over 24 hrs.

Materials and Methods

Particle Preparation. Polystyrene particles (200 nm) and particles modified with carboxyl groups (110 nm,

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220 nm, 450 nm, 830 nm, and 1100 nm) or amine groups (200 nm), all internally labeled with a fluorescent dye (yellow-green 505/515 nm), were purchased from Molecular Probes (Eugene, OR), IDC Latex (Portland, OR), Polysciences (Warrington, PA), or Bangs Labs (Fishers, IN). All particles were washed at least once prior to use by centrifugation if surfactant or sodium azide was added by the manufacturer in the solvent.

Radiolabeled particles were prepared starting with 250-nm divinylbenzene-crosslinked carboxyl–functionalized polystyrene particles. A total of 20 μl of 20% (w/v) particles were swollen in a mixture of 25 μl tetrahydrofurane (Sigma-Aldrich Corp., St. Louis, MO) containing 1 mg/ml coumarin 6 laser dye (Polysciences Inc., Warrington, PA) and 100 μl of 1 mCi/ml ^{3}H-oleic acid (American Radiolabeled Chemicals Inc., St. Louis, MO) and 500 μl water. Particles were incubated for 30 mins at room temperature and then washed with MilliQ water (Millipore, Bellerica, MA) by centrifugation (Eppendorf 5415c microcentrifuge, Hamburg, Germany) at 1600 *g* for 30 mins, repeated 10 times, to remove unincorporated dye and oleic acid. The final radiation in particles was 18 μCi/mg. Stability of the radiolabel in the particles was tested by incubating the particles with serum and checking the amount of radiation that leaked from the particle after 24 hrs. Less than 1% of the radiation leaked into the supernatant in 24 hrs.

Aldehyde-functionalized particles were generated by reacting 200-nm amine-functionalized polystyrene particles with an excess of glutaraldehyde (EM Grade 8%; Polysciences Inc, Warrington, PA) according to manufacturer's suggested protocol (Bangs Labs). Excess glutaraldehyde was removed by repeated centrifugation with MilliQ water.

Polyethyleneimine (PEI)-coated particles were generated by incubating 200-nm plain polystyrene particles with a 10 mg/ml solution of 25-kDa PEI in water (Sigma-Aldrich) overnight. Excess PEI was removed by centrifugation.

Particles were characterized using scanning electron microscopy, dynamic light scattering (DLS), and zeta potential measurements. Scanning electron microscopy images were taken using a FEI XL40 Sirion FEG Digital Scanning Microscope at 3eV (Sirion, Tampa, FL). Particles were dried under vacuum and palladium coated using a Hummer 6.2 sputtering system (Anatech, Hayward, CA), DLS was done using a Brookhaven BI-200SM Research Laser Light Scattering System (Brookhaven Instruments Corp., Holtsville, NY) operated at 90°. Zeta potential measurements were done using a Zeta-Meter 3.0+ (Zeta-Meter Inc., Staunton, VA).

***In Vivo* Circulation on Biodistribution.** Sprague Dawley Rats (500–700 g, Charles River Laboratories, Wilmington, MA) were anesthetized using 1.5%–3% isoflurane gas in oxygen for initial blood draw and particle injection according to the guidelines of the Institutional Animal Care and Use Committee at the University of California, Santa Barbara. Blood (0.5 ml) was taken from the jugular vein into a heparinized syringe. The blood was diluted into 20 ml saline and washed twice by centrifugation for 2 mins at 410 *g* in a TRIAC centrifuge (Becton Dickinson, Franklin Lakes, NJ) to remove plasma. Plasma proteins prevent particle attachment to RBCs. Particles were added to cells at appropriate particle-to-cell ratios (100:1 for 200-nm plain, 220-nm carboxyl, and 200-nm aldehyde) and mixed by inversion and repeated pippetting. Cells were washed by centrifugation and resuspended in saline to a final volume of 0.6 ml. This sample was injected into the lateral tail vein of the same animal. Rats were allowed to recover from anesthesia immediately following particle administration. Subsequent blood samples (10 μl) were taken from the tail vein at several time points. The number of particle-carrying RBCs remaining in circulation was analyzed by flow cytometry using a Partec PASII Flow Cytometer (Munster, Germany) equipped with an argon laser (488 nm).

For biodistribution studies, the same *in vivo* procedures were employed using radiolabeled particles, and the animals were euthanized 2 hrs after particle injection by intraperitoneal injection of 1 ml euthanasia solution (390 mg/ml pentobarbital sodium; Euthasol; Virbac Corp., Forth Worth, TX). Blood was collected by intracardiac stick into a heparinized vacutainer. The liver, spleen, lungs, kidney, and heart were removed, rinsed, and perfused with saline to remove blood, and then weighed and mechanically homogenized. Known volumes (100–200 μl) or masses (80–250 mg) of tissue samples were dissolved using Soluene 350 (PerkinElmer Life and Analytical Sciences, Inc., Boston, MA). Radiation content in the sample was determined using a scintillation counter.

Control experiments were performed by injecting the same number of unbound particles directly into rat tail vein. Blood volume was assumed to be 7% of the animal's body mass for all experiments (18). A minimum of three animals were used for each group.

In some circulation and biodistribution experiments, Poloxamine 908 (a gift from BASF, Mt. Olive, NJ) was injected (150 mg/kg) into the rat 1 hr prior to particle administration. In other experiments, splenectomized rats (Charles River Laboratories) were used. Splenectomized rats were given 2 weeks to recover from surgery.

RBC-Nanoparticle PEGylation. In some experiments, RBCs were PEGylated after particle binding. Cyanuric chloride–functionalized 5-kDa m-PEG (C-mPEG; Sigma-Aldrich) was added to RBCs after 200-nm plain polystyrene particle attachment. The C-mPEG formed covalent bonds with the cells' amine groups (19). The C-mPEG did not react with the particles, because the polystyrene particles do not have any amine groups present on their surface. C-mPEG (20 mg) was added to 20 ml washed and diluted RBC-particle suspension and allowed to react for 30 mins.

Shear Stress–Induced Particle Detachment. Whole blood was obtained from a jugular vein draw of Sprague Dawley rats (500–700 g) into heparinized containers. For particle attachment, 0.5 ml whole blood was

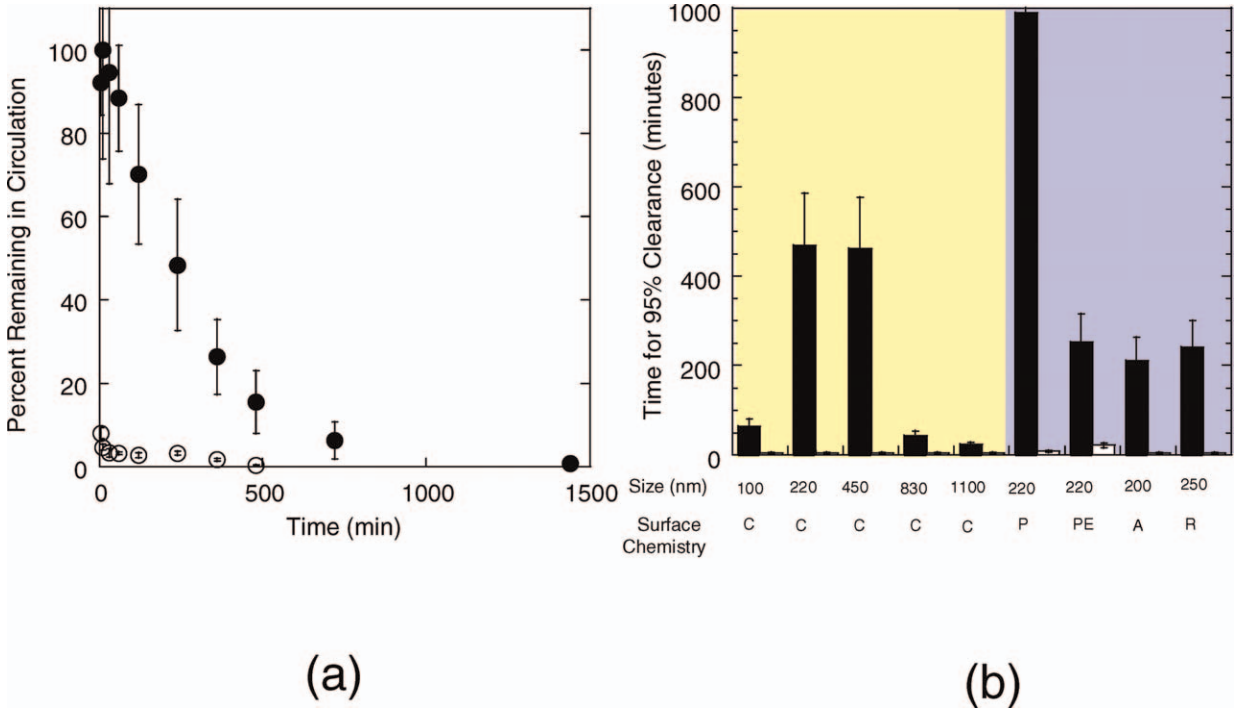

Figure 1. (a) *In vivo* circulation of 220-nm plain polystyrene particles (black circles, RBC bound; white circles, RBC unbound; $n = 3$). The *y* axis is defined as the number of particle-carrying RBCs present in circulation compared with that at time zero. (b) Time of clearance for particles with various sizes and surface chemistries (closed bars, RBC bound; open bars, free; $n = 3$). The yellow region shows particles possessing one specific surface chemistry (carboxyl) and various sizes. Blue region shows particles possessing comparable size (200–250 nm) and various surface chemistries. C, carboxyl; P, plain; PE, polyethyleneimine; A, aldehyde; R, radiolabeled. The surface of R particles was comparable to C. Color figure is available in the online version of the journal.

centrifuged (Eppendorf 5415c microcentrifuge) at 133 *g* for 2 mins. Heparinized plasma was removed and reserved. The remaining cells were diluted into 1 ml saline and washed three more times by centrifugation. Particles were added to washed RBCs at the desired particle-to-RBC ratio and were mixed by inversion. RBC-particle mixtures were centrifuged at 80 *g* to remove unbound particles. The labeled cells were resuspended in reserved plasma to desired hematocrit (10–50). The number of RBCs labeled with particles was determined using flow cytometery.

RBCs labeled with particles were sheared in a 25-mm plate and plate rheometer with a 0.1-mm gap (Paar Physica MCR300; Anton Paar, Ashland, VA). A solvent trap was employed to prevent water evaporation. Cells were sheared at a constant shear stress of 1, 5, or 10 Pa for 30 mins. The number of cells labeled with particles was analyzed before and after shearing using flow cytometry as described above.

Results

Adhesion of Nanoparticles on RBCs. All particles used in this study adhered to RBCs, depending on size and surface chemistry. The concentration of adherent particles increased with increasing particle-to-RBC ratios during incubation. However, adding excessive amounts of particles resulted in cell aggregation. For each particle, the particle-to-RBC ratio was determined so as to maximize the fraction of particle-labeled RBCs without causing aggregation (approximately 40% of incubated RBCs were labeled by particles). The precise site of particle adhesion on RBCs is not clear, although it is likely to be mediated by nonspecific interactions, since adhesion was carried out in a serum-free environment. Accordingly, opsonins and complement are unlikely to contribute significantly. Presence of serum or albumin inhibited particle adhesion in a concentration-dependent manner (data not shown). However, once attached, addition of plasma or albumin did not cause particle detachment for more than 24 hrs *in vitro*. The inability of particles to bind to RBCs in the presence of serum proteins allowed us to quantitatively study their detachment and elimination from circulation *in vivo*.

Circulation of RBC-Adherent Nanoparticles. Intravascularly injected free nanoparticles (200–220 nm in diameter and all chemistries as reported in this study) were rapidly cleared from circulation (typically, >95% cleared in less than 30 mins). Such rapid clearance of free polystyrene nanoparticles is consistent with data in the literature (11, 13). However, adhesion of nanoparticles on RBCs significantly delayed their vascular clearance (Fig. 1a). For example, RBC-bound 220-nm plain polystyrene particles remained in circulation for almost a day, an improvement of more than 100-fold over their free counterpart. Similar results were obtained for particles of all sizes and surface chemistries tested (Fig. 1b). For a given surface chemistry—for example, carboxyl-functionalized polystyrene—the cir-

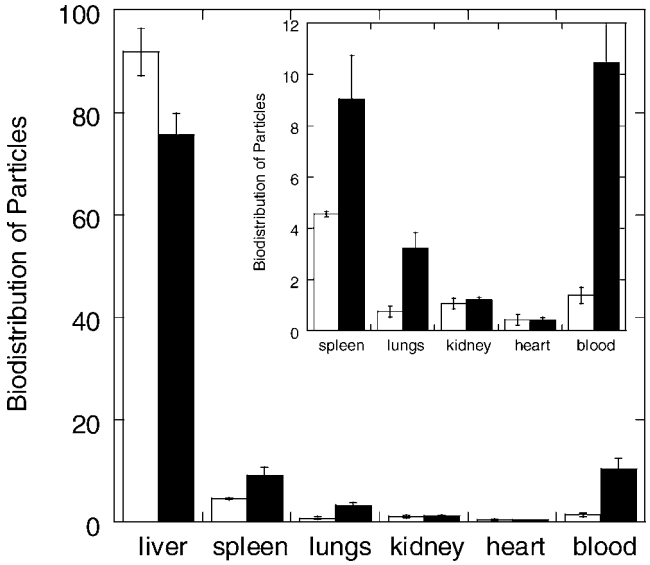

Figure 2. Biodistribution of radiolabeled particles (250 nm) when injected as RBC-bound (closed bars) and free (open bars; $n = 3$). All numbers indicate amounts at the end of 2 hrs. Amounts in blood, liver, spleen, and lungs are statistically different when RBC-bound and free values are compared ($P < 0.05$). Inset shows a zoomed-in view of the same data.

culation time was maximal for intermediate sizes (200–500 nm). At a given size—for example, 200–250 nm—circulation time varied significantly with surface chemistry. Particles with different surface chemistries are likely to adhere to RBC *via* different mechanisms, including hydrophobic, electrostatic, and covalent interactions (20, 21); however, in all cases RBC-bound particles exhibited extended circulation compared with their free counterparts. Although particles were eliminated from circulation, the RBCs to which the particles were attached were not removed. This was established in our previous studies through dual labeling of particles and RBCs (22). Note that we do not distinguish between particles attached to or internalized by RBCs. We collectively refer to them as particles associated with RBCs.

Biodistribution. To gain further insight into mechanisms of particle clearance, biodistribution was assessed using 250-nm carboxyl-functionalized radiolabeled particles. This assessment was performed at the end of 2 hrs after injection. Accumulation of RBC-bound particles in blood was 10-fold higher compared with free particles (Fig. 2). About 10% of injected dose remained in circulation after 2 hrs, most of which was still bound to RBCs (data not shown). RBC-bound particles showed reduced accumulation in the liver and significantly increased accumulation in spleen and lungs compared with free particles ($P < 0.05$; Fig. 2). Since a majority of particles ended up in the liver and spleen, their role in particle clearance was investigated.

Role of Spleen in Particle Clearance. The role of spleen was assessed first, since the assessment can be done in a relatively straightforward way. Specifically, clearance of RBC-bound particles was assessed in splenectomized rats. The spleen has been shown to play an important role in the clearance of larger particles injected intravascularly due to physical entrapment in the tight fenestrations (11, 15, 23). Due to the tight nature of splenic fenestrations, the spleen may cause physical detachment of particles from RBCs. Splenectomy did not reduce the rate of particle clearance (Fig. 3a), which indicates that the spleen does not play a prominent role in particle detachment or clearance.

Role of Liver in Particle Clearance. Understanding the role of liver in particle clearance is somewhat more challenging, since the liver function cannot be completely blocked. Nevertheless, studies have shown that poloxamine can reduce the liver uptake of polystyrene particles by as much as 10-fold *via* two modes: immobilization of poloxamine on particle surface and intravenous injection of poloxamine prior to particle injection (23, 24). Poloxamine's effect on free particles was confirmed prior to testing its influence on clearance of RBC-bound particles. In these experiments, rats were injected with poloxamine 908 prior to injection of free 220–nm particles. This procedure significantly altered particle clearance (Fig. 3b, note the logarithmic scale). Specifically, liver accumulation was reduced by 4-fold, blood accumulation was increased by 7-fold and, most prominently, spleen accumulation was increased by 10-fold. Poloxamine clearly reduced macrophage clearance in liver, thereby making spleen the primary site of clearance.

Having confirmed the ability of poloxamine 908 to influence liver clearance in our experimental system, we adsorbed poloxamine 908 on the exposed surface of RBC-bound particles. Results based on flow cytometry analysis showed that adsorption of poloxamine 908 on exposed parts of particles did not increase the circulation lifetime of RBC-bound particles (data not shown). Next, we preinjected rats with poloxamine 908 under the same conditions as reported for Fig. 3b and, once again, the flow cytometry results showed that preinjection of poloxamine 908 did not influence clearance of RBC-bound nanoparticles (Fig. 3c). The effect of poloxamine preinjection was further tested by studying biodistribution of radiolabeled particles (Fig. 3d, note the logarithmic scale). These studies showed that poloxamine preinjection had a moderate effect on clearance of RBC-bound nanoparticles. Specifically, poloxamine preinjection reduced liver uptake by only 38% (compared with 77% in the case of free particles), increased spleen uptake by 96% (compared with 938% in the case of free particles), and increased blood accumulation by 125% (compared with 646% in the case of free particles). These results indicate that uptake by liver macrophages is not the rate-limiting step in clearance of RBC-bound nanoparticles. This led us to the hypothesis that the rate-limiting step in particle clearance is their detachment from the RBC. This was assessed next.

Particle Detachment from RBCs. RBCs are constantly exposed to shear stresses in the range of 0.1 to 14 Pa

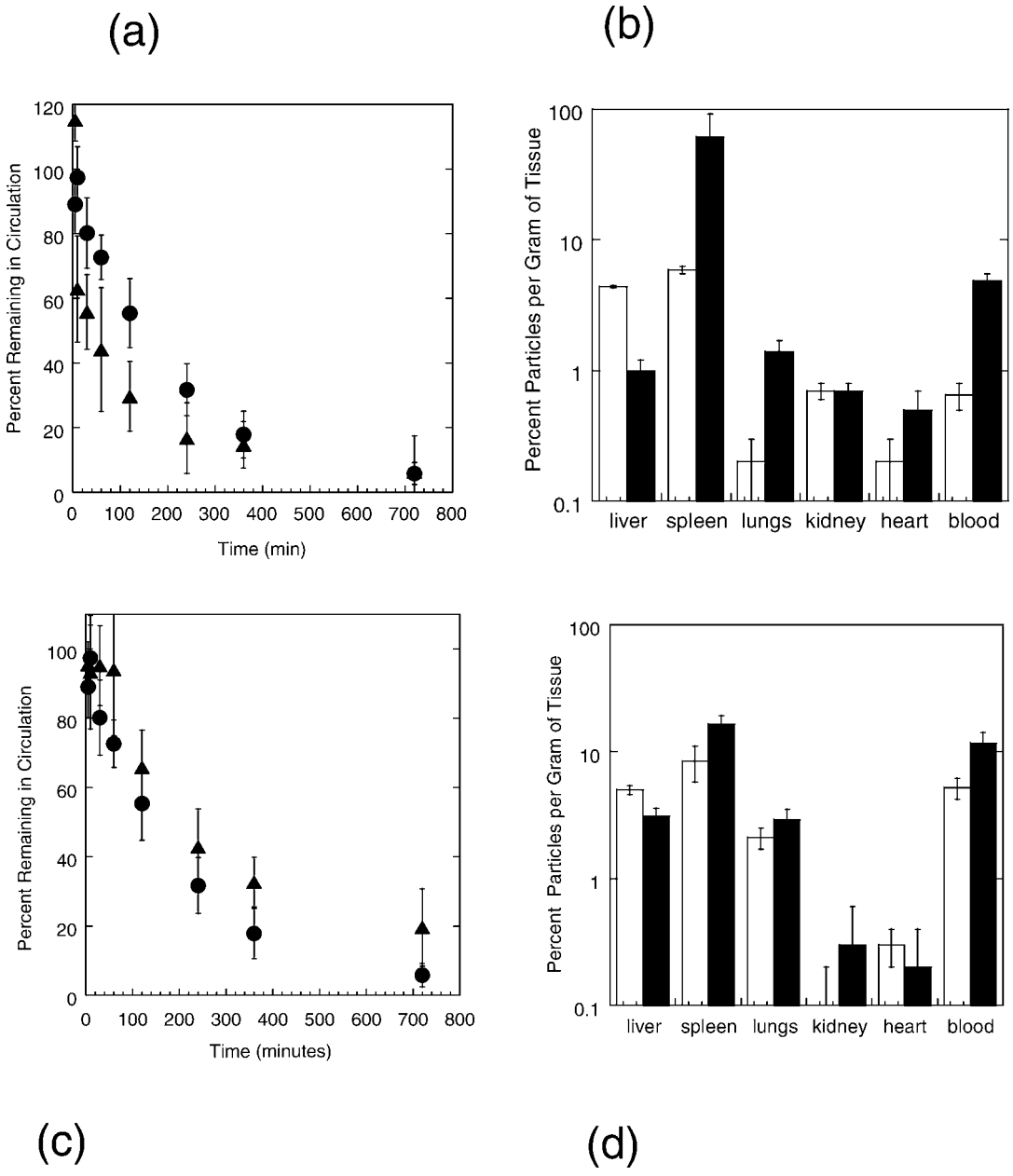

Figure 3. (a) Effect of splenectomy on circulation of RBC-bound 220-nm carboxyl-functionalized nanoparticles (black circles, controls; black triangles, splenectomized; $n = 3$). The y axis is defined as the number of particle-carrying RBCs in circulation compared with that at time zero. (b) Effect of poloxamine preinjection on biodistribution of free particles (open bars, control; closed bars, poloxamine preinjection; $n = 3$). (c) Effect of poloxamine on circulation of RBC-bound nanoparticles (black circles, controls; black triangles, poloxamine preinjected; $n = 3$). The y axis is defined as the number of particle-carrying RBCs in circulation compared with that at time zero. (d) Effect of poloxamine preinjection on biodistribution of RBC-bound nanoparticles (open bars, control; closed bars, poloxamine; $n = 3$).

in vascular circulation (25–29). These shear forces can dislodge the particles from the RBC surface. Additionally, collisions of RBCs with themselves and with the capillary walls may also detach particles from surfaces. To assess this hypothesis, particle-laden RBCs were exposed to a range of shear stresses in a plate and plate rheometer at a constant shear stress for 30 mins at 1, 5, or 10 Pa. The number of particles that remained attached to RBCs before and after the shear was determined using flow cytometry. Particles indeed detached from RBCs in a stress-dependent manner (Fig. 4a). Once detached, the particles cannot reattach to RBC due to presence of plasma proteins. The extent of shear-induced detachment of particles increased with increasing RBC concentration, clearly indicating that cell-cell interactions contribute significantly to particle detachment (Fig. 4b). The extent of particle detachment after 30 mins of shear at 10 Pa *in vitro* varied with particle size and surface chemistry (Fig. 5a). However, regardless of the size and chemistry, *in vitro* detachment was directly proportional

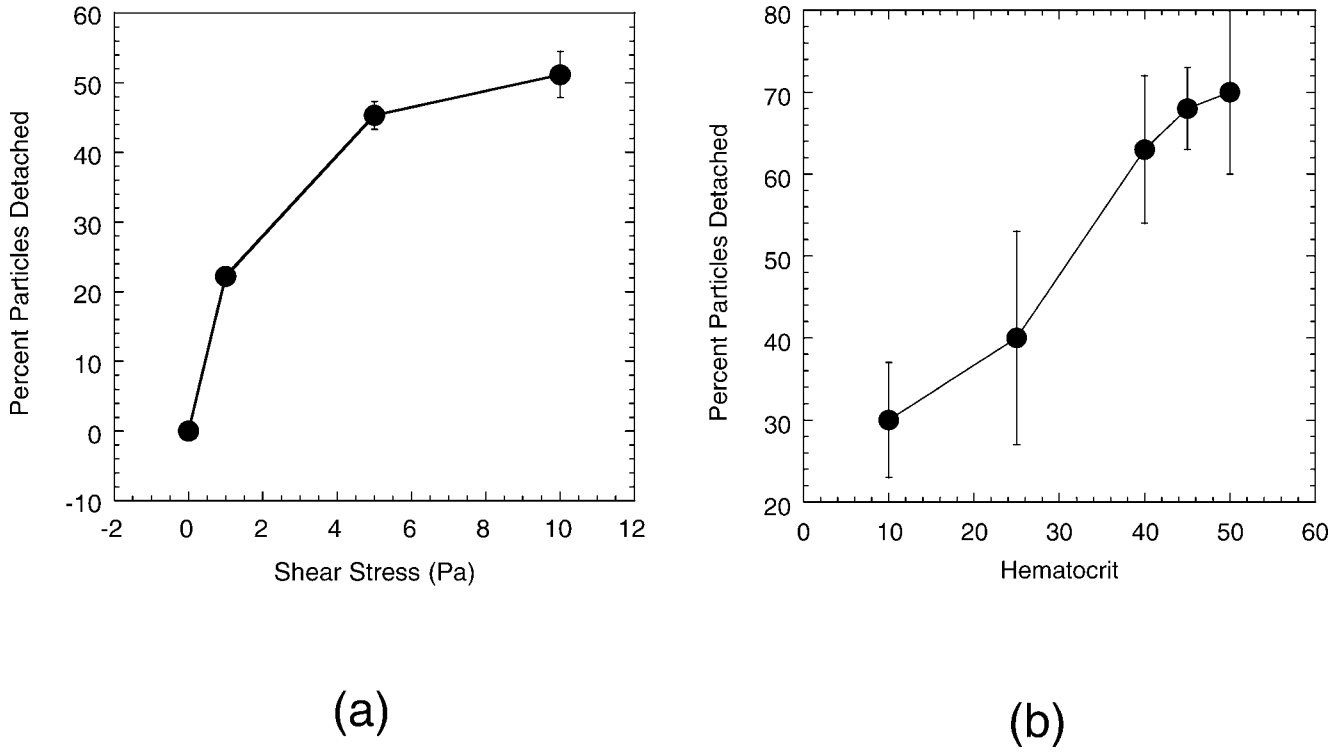

Figure 4. (a) Dependence of particle detachment on shear stress *in vitro* ($n = 3$). (b) Dependence of particle detachment on RBC concentration (hematocrit; $n = 3$). The *y* axis is defined as the number of particle-carrying RBCs at any time compared with that at time zero.

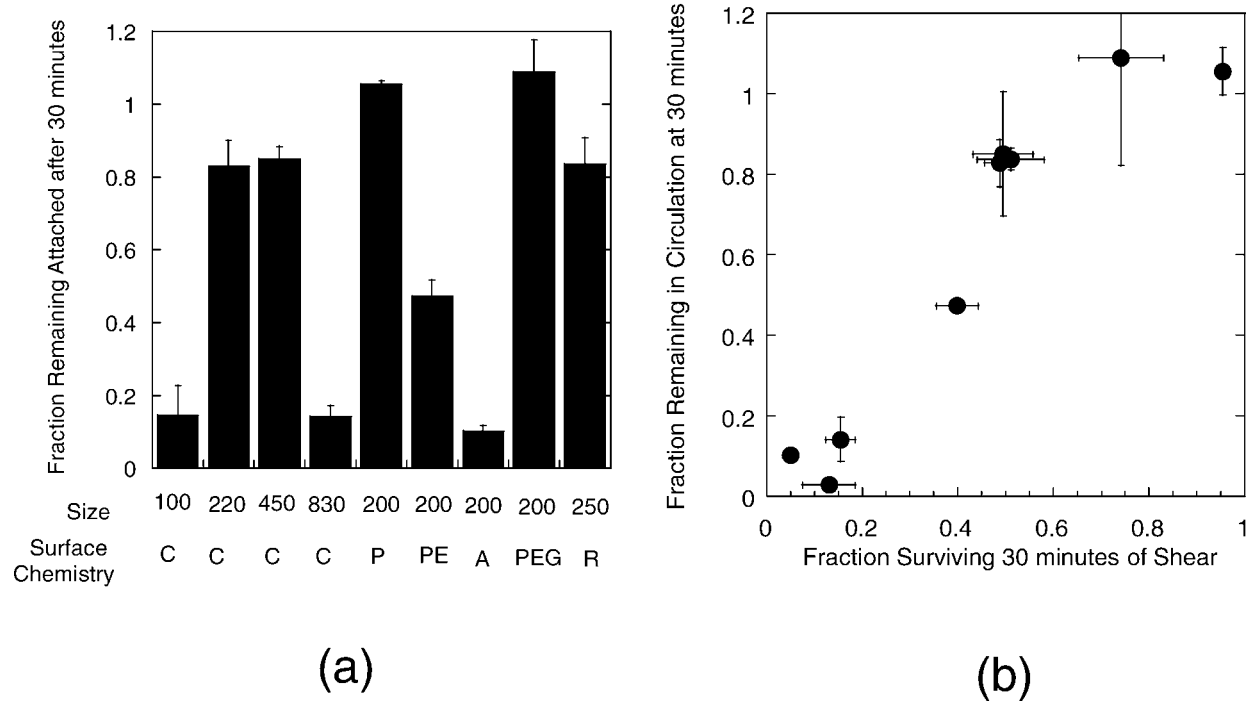

Figure 5. (a) Dependence of shear-induced particle detachment on size and surface chemistry ($n = 3$). Shear stress of 10 Pa was applied for 30 mins, and *in vivo* circulation was measured after 30 mins (PEG-nanoparticles first were attached to RBCs, and RBCs then were modified with PEG). Particle attachment was monitored by flow cytometry. (b) Correlation between particle detachment *in vitro* (taken from Fig. 4a) and fraction of particle-laden cells remaining in circulation *in vivo* after 30 mins ($n = 3$).

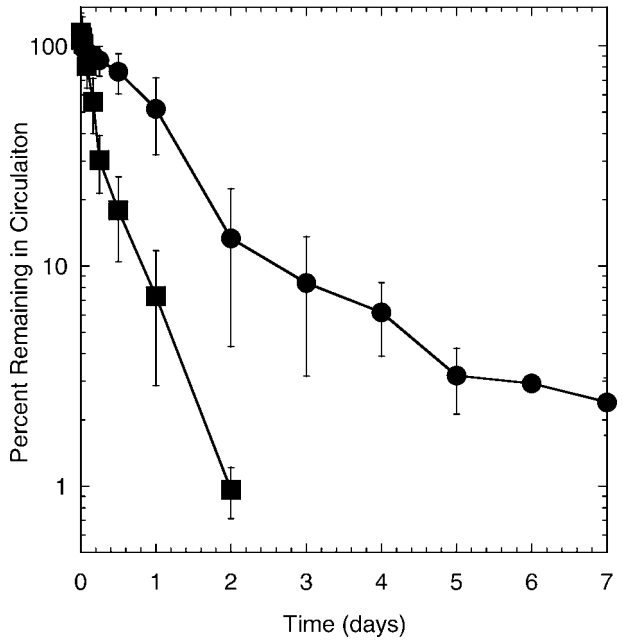

Figure 6. *In vivo* circulation of RBC-bound 220-nm plain polystyrene particles (black squares, $n = 3$) and PEGylated RBC-particle complexes (black circles, $n = 3$). Circulation was monitored by flow cytometry. The *y* axis is defined as the number of particle-carrying RBCs at any time compared to that at time zero.

to the number of particles remaining in circulation *in vivo* after 30 mins ($r^2 = 0.9$ for a linear fit; Fig. 5b).

Since cell-cell interactions appear to contribute significantly to shear-induced particle detachment, reduction of cell-cell contact may further prolong circulation of RBC-bound particles *in vivo*. The cell-cell interactions between RBCs can be reduced by introducing a steric barrier onto the cell surface. This has been accomplished previously by covalently attaching PEG to the RBC membrane. With sufficient PEG coverage, RBCs exhibit decreased rouleaux formation and increased settling time (19, 30), indicative of decreased cell-cell interactions. PEGylation of RBCs dramatically reduced clearance of RBC-bound particles (Fig. 6). The half-life of PEGylated RBC-particle complexes was over 24 hrs, and about 3% of particles remained in circulation even after 1 week.

Discussion

Sustained release of drugs in circulation poses a significant challenge in the field of drug delivery. Previous attempts to tackle this challenge have made use of RBCs or nanoparticles separately. RBCs offer excellent candidates for the systemic delivery of drugs due to their abundance and long *in vivo* circulation lifetimes. Previous attempts to use RBCs have been based on encapsulation of drugs in RBCs (31, 32) or anchorage of drugs to RBCs (33–35). However, encapsulation of drugs in RBCs offers poor control over drug release and is inherently limited to *ex vivo* modification of cells. Anchoring drugs to the RBC membrane has been shown to increase their circulation lifetime (34, 35). This methodology is applicable to drugs that are effective while still attached to the RBC, although slow release from the RBC surface should be feasible in principle.

Nanoparticles potentially offer control over the release rates of the therapeutic agents. However, the use of nanoparticles for intravascular drug delivery is limited by their rapid clearance from systemic circulation. Surface modification of nanoparticles by PEG and other hydrophilic polymers has shown improved circulation times (6, 23, 36). However, surface modification strategies have been employed for very small nanoparticles. Further, recent data indicate that the protective function of surface coating is reduced with repeated injections (5, 24, 37).

Attachment of polymeric nanoparticles to RBCs combines the advantages of the long circulation of RBCs and the robustness of polymeric nanoparticles. Although the particles are eventually removed from the circulation, extended half-lives are achieved without affecting the circulation of RBCs themselves. Use of RBCs to extend the circulation of the particles offers several advantages. Importantly, it avoids the need to modify the surface chemistry of the entire particle, thus leaving opportunities open to attach chemicals to the exposed surface for targeting applications.

The duration of particle circulation depends on the strength of binding to the RBC. The half-lives of nanoparticles attached to RBCs are comparable to those previously reported for surface-modified liposomes and nanoparticles. Particles eventually detach from the RBC, possibly due to shear forces, cell-cell interactions, and cell–vessel wall interactions. Once detached, the particles are cleared primarily in the liver and to some extent in the spleen. Particle attachment does not affect circulation of the RBC to which it was attached. By improving the binding between the particles and the RBCs it may be possible to keep the particles in circulation for longer periods of time, theoretically up to the circulation lifetime of the RBC (120 days). It is possible that particles that detach from RBCs in circulation can adhere to other blood cells. However, we anticipate this effect to be minimal for nonphagocytic cells, since polystyrene particles showed minimal adhesion to RBCs in presence of plasma proteins.

In this study, particle attachment to RBCs was accomplished through electrostatic and hydrophobic interactions for plain, carboxyl-modified, and PEI-modified particles, as well as specific interactions for aldehyde-modified particles. It may be possible to further improve the binding strength using covalent attachment or high-affinity ligands. In the future, it may also be possible to specifically target the particles to the RBC surface using blood group antibodies. This would not only potentially increase the binding strength but also allow direct *in vivo* attachment of particles to RBC membranes, thus enabling a single injection therapy based on long-circulating nanoparticles. Note that this study did not employ drug-loaded nano-

particles. Drug-loaded nanoparticles may have different binding and circulation properties than polystyrene particles, depending on their chemistry.

The methodology described here may be useful for delivery of drugs. In addition to drug delivery applications, RBC-bound particles may be used as circulating bioreactors. The exposed surface of the particles could be used to immobilize enzymes and improve their *in vivo* circulation lifetime. Immobilization could be accomplished using hydrophobic interactions with the particle surface, avoiding covalent reactions that may damage the RBC. The enzyme would have direct access to plasma in the systemic circulation. RBC-mediated prolonged circulation may also be applied to gene delivery applications in which extended circulation times are difficult to achieve. Synthetic gene delivery vectors, such as PEI, suffer from rapid clearance by the RES restricting transfection to the liver and lung (38, 39). RBC attachment of gene vectors may provide a long-circulating depot, thereby increasing their residence time in blood. Further studies should focus on exploring these opportunities.

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