RESEARCH ARTICLE

The Effect of Polymeric Nanoparticles on Biocompatibility of Carrier Red Blood Cells

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Abstract

Red blood cells (RBCs) can be used for vascular delivery of encapsulated or surface-bound drugs and carriers. Coupling to RBC prolongs circulation of nanoparticles (NP, 200 nm spheres, a conventional model of polymeric drug delivery carrier) enabling their transfer to the pulmonary vasculature without provoking overt RBC elimination. However, little is known about more subtle and potentially harmful effects of drugs and drug carriers on RBCs. Here we devised high-throughput in vitro assays to determine the sensitivity of loaded RBCs to osmotic stress and other damaging insults that they may encounter in vivo (e.g., mechanical, oxidative and complement insults). Sensitivity of these tests is inversely proportional to RBC concentration in suspension and our results suggest that mouse RBCs are more sensitive to damaging factors than human RBCs. Loading RBCs by NP at 1:50 ratio did not affect RBCs, while 10–50 fold higher NP load accentuated RBC damage by mechanical, osmotic and oxidative stress. This extensive loading of RBC by NP also leads to RBCs agglutination in buffer; however, addition of albumin diminished this effect. These results provide a template for analyses of the effects of diverse cargoes loaded on carrier RBCs and indicate that: i) RBCs can tolerate carriage of NP at doses providing loading of millions of nanoparticles per microliter of blood; ii) tests using protein-free buffers and mouse RBCs may overestimate adversity that may be encountered in humans.

Introduction

Red blood cells (RBCs, bi-concave discoid cells filled with hemoglobin and lacking organelles including the nucleus) represent the most abundant cellular constituent of the blood (>99%) and play an important role in drug delivery. Drug delivery systems circulating in blood encounter RBCs, which may lead to unintended effects of drug on RBCs, and vice versa. Interaction of drug delivery systems (e.g., polymeric nanocarriers) with RBCs and its consequences have a high significance, both mechanistic and translational [1–12].

As carriers, RBCs offer a multitude of advantages including bioavailability, biocompatibility and longevity in circulation (~45 and ~120 days in mice and humans, respectively), which makes them a highly attractive vehicle for vascular delivery of drugs and nanocarriers.
Recently, several groups reported interesting characteristics of behavior in vitro and in animal models of nanoparticles (NP) non-covalently attached to RBC surfaces [3,13–16]. To summarize these exciting studies, RBC-coupled nanoparticles showed prolonged circulation in mice, reduced uptake in clearance organs (e.g. liver and spleen), and enhanced targeting and delivery to the lungs, enabled by the transient transfer of nanoparticles from carrier RBCs to the endothelium.

Coupling nanocarriers to the surfaces of mouse RBCs at 50:1 ratio, as was done in these previous studies, did not induce negative effects in RBCs in vitro or alter their overall performance in vivo. However, little is known about the effect that attached nanocarriers have on RBCs, either as a result of intentional RBC carriage, such as in the above examples, or from unintentional interactions. In this study, we designed a set of high-throughput in vitro assays characterizing sensitivity of nanocarrier-carrying RBCs to biologically relevant insults often encountered in circulation; specifically osmotic, mechanical, oxidative and complement stress, as well as RBC agglutination. These tests can provide a pre-screening for selection of formulations exhibiting the least sensitivity to these biological insults, and likely enhanced performance, prior to in vivo testing in both preclinical and clinical settings.

**Materials and Methods**

**Ethics Statement**

All animal studies were carried out in strict accordance with Guide for the Care and Use of Laboratory Animals as adopted by National Institute of Health, approved by University of Pennsylvania IACUC under protocol 805013. Mice were anesthetized with ketamine/xylazine/acepromazine. Mice were bled at one time point, alternating eyes to bleed from. At the end, all animals were euthanized by cervical dislocation and confirmed by detection of no heart beat.

All studies involving human subjects were approved by the University of Pennsylvania Institutional Review Board under protocol 822534. Written informed consent from donors was obtained for the use of blood samples in this study. Blood samples were destroyed after the study. Names and any personal information about individual participants were not taken.

**Blood collection**

CJ7BL/6J male mice were purchased from the Jackson Laboratory (Bar Harbor, ME). All mice were housed in a temperature and humidity controlled environment (18–23°C with 40–60% humidity under a 12-hour light-dark cycle) with ad libitum access to food (Labdiet 5010 autoclavable rodent diet, Brentwood, MO) and water.

Blood donation of human voluntary donors took place at the University of Pennsylvania. A volume of 4mL of whole blood was collected in either a vial containing ~ 3.2% Na citrate (BD Vacutainer) or Na heparin 75 USP Units (BD Vacutainer). Blood collected in Na Heparin was used to avoid calcium depletion for complement assays. In addition, a volume of 4mL of whole blood was collected in serum collecting tubes (BD Vacutainer). Blood from CJ7BL/6J mice, which also took place at the University of Pennsylvania, was collected in 20% Na heparin. Both human and murine blood was spun at 1000g for 10 min at 4°C to isolate plasma or serum. Serum was stored at 4°C for 3h until use. Plasma was discarded. A buffy layer containing white blood cells, observed in the interface between plasma and erythrocytes, was also removed and discarded.

Isolated erythrocytes (RBCs) were washed by adding ice cold 1x Dulbecco’s-Phosphate-Buffered-Saline (DPBS) pH 7.4 up to 12mL total volume and pipetting gently up and down to mix buffer with RBC extensively. RBC suspension was centrifuged again (451g, 15 min, 4°C) and supernatant was discarded. This wash step was repeated for a total of 3 times.
Attachment of Nanoparticles to RBCs

Briefly, 200nm carboxylated polystyrene particles (Polysciences) were washed in water, centrifuged at 15,000g for 20 min and later suspended in 4% sodium citrate pH 7.4. NPs were incubated with either murine or human RBCs at a ratio of 50:1; 200:1; or 2000:1 for 1 h under constant rotation at 4°C. NP-RBC solution was washed with ice cold DPBS three times at 100g for 8 min to remove unattached NPs.

RBC Osmotic Fragility

Osmotic fragility assay was performed on freshly obtained erythrocytes in the presence of an anticoagulant. RBCs and RBC-NP suspensions were washed three times with ice cold DPBS and re-suspended in ice cold DPBS at 10% hematocrit, followed by incubation with different ratios of NPs to RBCs for 60 min at 4°C. RBCs were washed three times with ice cold 4% DPBS to wash off unbound NPs. RBC lysis was monitored by the release of hemoglobin upon incubation of cells with salt concentrations ranging from 0mM to 150mM for ~0 min (e.g. immediately removed from salt solution) at 37°C. RBC suspensions were centrifuged at 13,400g for 4 min and the absorbance of the supernatant was recorded at 540nm by TECAN Infinite M200 plate reader. Each sample in water was taken as 100% RBC lysis. All RBC suspensions in the following fragility assays followed this same procedure.

RBC Mechanical Fragility

Mechanical fragility assay was performed on freshly obtained erythrocytes in the presence of an anticoagulant. All erythrocytes samples were subjected simultaneously to the same mechanical stress. RBC and RBC-NP suspensions of 1.0% hematocrit along with 8x4mm glass beads (Pyrex) in DPBS were rotated 360° for different time periods at 24 rpm at 37°C. The control samples were not subjected to the glass beads. The hemoglobin released from the RBCs during rotation was immediately assayed, as was the free hemoglobin in the control samples. Free hemoglobin was transferred into a new tube and was centrifuged at 13,400g for 4 min.

RBC Oxidative Fragility

Hydrogen peroxide-induced lysis was performed on freshly obtained erythrocytes in the presence of an anticoagulant. All erythrocytes samples were subjected simultaneously to the same oxidative stress. RBC and RBC-NP suspensions of 1.0% hematocrit with the addition of 3mM H$_2$O$_2$ in DPBS were rotated 360° for different time periods at 24 rpm at 37°C. The control samples were not subjected to H$_2$O$_2$. The hemoglobin released from the RBCs during rotation was immediately assayed, as was the free hemoglobin in the control samples.

RBC Complement Lysis

Complement lysis assay was performed as previously described [17–20]. Streptavidin (SA) and 6-biotinylaminocaproic acid N-hydroxysuccinimide ester (long arm biotin ester, BxNHS) were purchased from Sigma. Briefly, fresh isolated erythrocytes from heparinized blood were washed with three times with cold DPBS before the addition of biotin. BxNHS, dissolved in DMF, was added to RBC suspension of 10% hematocrit with the addition of 1mg/mL in DPBS. Streptavidin (1mg/mL in DPBS) was then added to 10% biotinylated RBC and later incubated at room temperature for 30 min. Unbound streptavidin was removed by centrifugation. RBC, RBC-NP, and RBC-B-SA suspensions of 1.0% hematocrit with the addition of the complement obtained from fresh homologous serum, diluted ½ in DPBS containing Ca$^{2+}$ and Mg$^{2+}$ were rotated...
360° for 4h at 24 rpm at 37°C. The addition of biotin-streptavidin (B-SA) to RBC suspension was used as a control. The hemoglobin released from the RBCs during rotation was immediately assayed for lysis, as was the free hemoglobin in the control samples. Each sample in water was taken as 100% RBC lysis.

**RBC Agglutination assay**

Agglutination assay was performed on freshly obtained erythrocytes in the presence of an anticoagulant. RBC and RBC-NP suspensions of 1.0% hematocrit were dispensed onto a 96 well U-shaped plates. The results were visually accessed for agglutination after 1 hour at 37°C after the RBC suspension adsorbed had fully sedimented. RBC suspension in dog serum was used as a positive control of agglutination process. To confirm RBC agglutination, RBC and RBC-NP suspensions were observed using a 25x objective lenses on a Micromaster microscope (Fisher Scientific) equipped with micro-camera.

**Results**

**Characterization of the test system effects of RBC hematocrit and animal species**

Naïve RBCs were first investigated alone, without attachment of nanocarriers, so as to establish a baseline with regards to RBC sensitivity and resistance against a variety of biological insults. RBC concentration (% hematocrit) was shown to be reversely proportional to hemolysis during an osmotic fragility test. At intermediate levels of osmotic stress, in this case caused by hypotonic buffer, the percent of hemolysis increased as RBC concentration decreased (S1 Fig). This finding agrees well with previous studies [17]. To avoid very low readings of optical density, typical of highly diluted RBC samples, 1% RBC suspensions were used throughout the study. Additionally, mouse RBCs are dramatically more sensitive than human RBCs to osmotic, mechanical and oxidative stresses/insults (S2 Fig), which highlights the need to be cautious when correlating potential human efficacy in mouse models that, in some cases, are less durable.

**Sensitivity of NP-RBC to osmotic stress**

Attachment of NP to RBCs at NP/RBC ratios 50:1 and 200:1 did not alter hemolysis of mouse and human RBCs in sub-physiological osmotic conditions (e.g. 73mM NaCl), whereas NP loading at NP/RBC ratio of 2000:1 aggravated osmotic lysis of mouse but not human RBCs (Fig 1). Osmotic lysis occurred rapidly, in these cases the percentage of RBC hemolysis does not increase significantly from ~0 min to 3h of both mouse and human RBCs within tested range of NP:RBC ratios.

**Sensitivity of RBC-NPs to low level shear stress**

RBC resistance to mechanical stress is one of the key physiological features that contribute to RBC longevity in the bloodstream. Hemodynamic stress can cause mechanical damage to RBCs, resulting in rupture of the cell’s membrane which leads to hemolysis [21–24]. To investigate how NP-carriage effects RBC resistance to mechanical stress, we first tested RBC sensitivity to a relatively prolonged low level shear stress (rotation at 24rpm at 37°C for 46h), similar to what RBCs repetitively encounter in the microcirculation.

As shown in Fig 2A, naive mouse RBCs showed low levels of hemolysis at 8h (3% lysis); increased rotation times resulted in an increase in hemolysis from 15% after 24h to 80% at 46h. Within the time interval at which mouse RBCs withstand mechanical stress (8 hours and
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**A.**

![Graph A](image)

- Naïve RBC
- NP:RBC (50:1)
- NP:RBC (200:1)
- NP:RBC (2000:1)

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**B.**

![Graph B](image)

- Naïve RBC
- NP:RBC (50:1)
- NP:RBC (200:1)
- NP:RBC (2000:1)

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**C.**

![Graph C](image)

- Naïve
- NP:RBC (50:1)
- NP:RBC (200:1)
- NP:RBC (2000:1)

- Mouse RBC
- Human RBC
partially 24 h), NP loading to RBC at ratios of 50:1 and 200:1 did not sensitize RBCs. Loading at NP/RBC ratio of 2000:1 markedly aggravated the hemolysis induced by mechanical stress (15% lysis after 8h; 60% lysis after 24h). However, as was the case with osmotic insults, human RBCs were also more resistant to mechanical stresses for all NP/RBC ratios. As shown in Fig 2B, NP loading to human RBCs at 200:1 ratio slightly aggravated hemolysis, whereas loading at 2000:1 ratio markedly aggravated RBC damage caused by mechanical stress.

Sensitivity of RBC-NP to vigorous mechanical insult

In the heart chambers and aorta, RBCs encounter excessively high levels of shear stress from the damaging effects of vigorous collisions with the valves and vascular walls. To model severe mechanical stress, glass beads were added to the RBC suspension and additionally subjected to the rotational shear stress. Even short (15 min) incubation under these conditions caused ~20% and ~5% hemolysis of naive mouse and human RBCs, respectively (S2 Fig). This hemolysis was modestly increased for RBC-NPs after 30 min exposure for mouse RBCs at high levels of NP load (Fig 3A & 3B), however, this was not the case for human RBCs even at the highest NP load for 2 hours of exposure (Fig 3C & 3D).

Sensitivity of RBC-NPs to oxidative insult combined with low level shear stress

Damage to RBCs in vivo may be enhanced under pathological conditions. For example, oxidative stress is a common component of many human maladies including inflammation and ischemia-reperfusion, where H₂O₂ is one of the key reactive oxygen species (ROS) released from activated phagocytes in inflammation and ischemia-reperfusion. RBC exposure to ROS is likely to occur in the microvasculature, where mild hemodynamic conditions permit accumulation of leukocytes and ROS.

Accordingly, we assessed the sensitivity of RBCs and NP/RBCs exposed to 3mM H₂O₂ at low level of shear stress. The results (Fig 4A–4C) show that at these conditions ROS aggravates hemolysis of mouse RBC in a time-dependent manner and NP loading at any tested ratio, including NP/RBC 50:1. It should be noted that manifestation of H₂O₂ aggravation requires several hours and becomes especially pronounced after 24 hours. As such, since human RBCs have continued to outperform mouse RBCs in regards to insult resistance, and given that microvasculature is the important common site of oxidative stress (see "Discussion"), longer-timepoints were investigated and perhaps not surprisingly, the attachment of NPs to human RBCs at any ratio did not enhance oxidative hemolysis (Fig 4D and 4E).

Sensitivity of NP/RBCs to complement lysis under low level shear stress

Complement is the key component of the innate defense system that recognizes, destroys and marks microorganisms and altered (e.g., damaged) host cells for phagocytosis. Senescent and modified RBCs are sensitive to complement lysis and do not have membrane-repairing enzymes employed by nucleated cells. We examined whether the attachment of NPs onto RBCs increases the RBC susceptibility to complement-mediated lysis via alternative pathway that relies on direct, antibody-bypassing attack of predisposed RBCs [18].
Fig 2. Effect of low stress over time on red blood cells with 200nm nanoparticles adsorbed onto their surface at 1.0% hematocrit. Hemolytic curves for mouse (A) and (B) human RBC/NP:RBCs were obtained.
after constant rotation at 24 rpm at 37°C for up to 46h. Curves were determined for NP:RBC ratio (50:1; 200:1; 2000:1). Percent hemolysis of different NP:RBC ratios at 24h (C). Values are means (n = 5) ± SD. (***, P<0.001 vs naive mouse RBC) (###, P<0.001 vs naive human RBC).

Fig 3. Mechanical fragility of red blood cells with 200nm nanoparticles adsorbed onto their surface at 1.0% hematocrit. Percent hemolysis of mouse NP:RBCs (A) and human NP:RBCs (C) (50:1; 200:1; and 2000:1) were obtained after constant rotation with glass beads at 24 rpm at 37°C for up to 8h and 24h, respectively. Percent hemolysis of different NP:RBC ratios for mouse RBC at 0.5h (B) and human RBC at 2h (D). Values are means (n = 4–5) ± SD. Please note that some deviation bars are too small to be evident.
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For the positive control, we attached streptavidin to biotinylated RBC which leads to RBC hemolysis via the alternative complement pathway due to inactivation of the RBC membrane glycoproteins DAF and CD59, which normally control homologous complement [18–20, 25–29]. Here, human RBCs were exclusively investigated given their enhanced insult resistance. Human RBCs were not lysed at any concentration of analogous serum, whereas streptavidin attachment to biotinylated RBC led to nearly complete hemolysis in the analogous serum (Fig 5). NP loading at NP/RBC ratios of 200:1 and 400:1 did not aggragate complement lysis further. However, at NP/RBC ratio of 2000:1, nearly a 3-fold increase of lysis was detected.

Sensitivity of NP/RBCs to agglutination

To further evaluate other possible effects of NP on RBCs, agglutination processes were analyzed. This phenomenon reflects abnormal changes in RBC plasticity, adhesiveness and propensity to absorb plasma agglutinins, all of which occur in a number of pathologies. For example, the formation of rouleaux, where RBCs appear as stacked coins and individual RBCs cannot be distinguished (aggregates), are associated with hematomal and other diseases.

A standard dilution assay in a V-shape titration micro-plate revealed that NP loading on RBCs even at low NP/RBC ratio of 50:1 led to detectable RBCs agglutination in buffer (Fig 6A and 6C). The agglutination induced by the NPs was confirmed by microscopy showing large clumps of NP-loaded, but not naive RBCs (dog serum was used as a positive control of RBC agglutination) (Fig 6E and 6G). However, agglutination process was inhibited in the presence of BSA, even at a NP/RBC ratio as high as 200:1 (Fig 6B and 6D). No RBC clumps were observed by light microscope (Fig 6F and 6H).
Fig 6. Agglutination of red blood cells with 200nm nanoparticles adsorbed onto their surface at 1.0% and 10% hematocrit in buffer with and without BSA. Agglutination was visualized on a U-shaped bottom plate for mouse (A,B) and human (C,D) NP:RBCs (50:1 and 200:1). The deposition of RBC on the bottom as a dot indicates a negative agglutination reaction. Microscopic imaging of RBC agglutination. Agglutination was also visualized with light microscope at 250x magnification for both mouse (E,F) and human (G,H) at 10% Hematocrit. Dog serum served as a positive control. Data shown are representative of three independent experiments.

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Discussion

Effect on cells and tissues encountered on the route to therapeutic target site is an important factor of any drug delivery system from a performance and safety viewpoint. As such, reliable and sensitive tests are needed for pre-screening of multiple iterations of nanocarriers prior to expensive and low-throughput animal experiments. Red blood cells (RBCs), encountered by all circulating delivery systems offer a relatively high-throughput, reproducible and biologically relevant model system for testing unintended effects of carriers. RBCs are arguably the most available cellular material from any species including humans, which helps to explain expansion of studies characterizing adversity of drug delivery systems using relatively simple readouts of RBC agglutination and hemolysis [30].

On the other hand, RBCs have a potential as drug carriers. In particular, RBCs may help in optimizing nanocarrier circulation, circumventing uptake by the reticuloendothelial system (RES) [1–3, 12, 31]. Other methods, such as coating nanocarriers by PEG, or using elongated and elastic nanocarriers have been shown to decelerate carrier’s clearance [32–33]. In theory, this goal can also be accomplished by coupling nanocarriers onto the surface of RBCs, given their ability to naturally circulate for long-times [1–4]. In addition to prolongation of carrier’s lifetime in the bloodstream, RBC offers mechanisms for masking attached cargo compounds by the glycocalyx [5–11] and for their delivery to targets accessible from blood—thrombi, immune cells, macrophages, endothelial cells and the RES sinuses, to name a few [2,7,9,34–37].

Recently, several labs embarked on exploration of RBC-mediated carriage of nanocarriers [3–4,13, 38]. In particular, a non-covalent absorption of polymeric nanoparticles (NPs, 200 nm polystyrene spheres used as model drug delivery vehicles) to naive mouse RBC cardinally changed behavior of the resultant NP-RBC complexes injected in the bloodstream of mice. Somewhat expected, circulation time of RBC-bound NP was remarkably longer than that of free NP. Importantly, coupling of NP to RBC at ratios up to 50:1 did not accelerate elimination of the carrier RBC. Further, RBC carriage enabled transfer of reversibly associated NP to the pulmonary vasculature [3]. These results imply potential utility of RBCs as a "super-carrier" optimizing the PK and endothelial delivery of synthetic carriers, notwithstanding that the NP used in that study, rigid non-degradable polymeric spheres lacking PEG coating, represent the prototype model that undoubtedly will be surpassed by more advanced iterations with fully biocompatible features.

Both testing biocompatibility of diverse drug delivery systems and using RBCs themselves as drug carriers require sensitive high-throughput in vitro tests for detecting potentially harmful changes in RBCs. In this work we describe a set of relatively simple assays based on detection of RBC hemolysis and agglutination that allow analysis of multiple samples, dilutions and conditions imitating distinct factors that occur in circulation and may provoke or aggravate RBC damage, destruction and elimination.

This aspect of RBC drug delivery and modifications needs a rigorous analysis, both in vivo and in vitro. Of course, no in vitro model can fully address the complex conditions and influences experienced by RBCs in circulation. However, in vitro testing is more ethically coherent, economical, provides high-throughput analysis of numerous conditions and allows studies of human RBCs. In this context, the present study provides a template of functional tests of specific insults that may be encountered by RBCs in circulation.

Assays employing RBCs from healthy animals and subjects might underestimate potential damaging or sensitizing effects of the cargo attached to or encapsulated in RBC. Presumably, RBC-mediated carriage of drugs and nanocarriers will eventually be employed in sick patients, not healthy individuals. Pathological mechanisms may both affect RBCs predisposing them to damage, or/and lead to production of additional damaging factors and agents. For example,
oxidative stress is a prevalent pathological pathway in a plethora of human maladies. Therefore, there is a substantial probability of NP-RBC encountering an additional insult of oxidative stress including reactions with membrane lipids and proteins which produce lipid peroxidation and alter membrane structure [39–47]. Oxidative stress is involved in number of factors that contribute to RBC aging and removal from circulation [48–49]. However, RBCs possess the antioxidant defense, consisting of non-enzymatic antioxidants including glutathione and enzymatic antioxidants including catalase [50–52] and peroxiredoxin-2 [53–54]. This may help to explain delayed manifestation and relatively modest amplitude of oxidative damage of RBC loaded with NP.

The issue of relevance of in vitro assays of sensitivity of the carrier RBCs to conditions in vivo is complex. Testing effect of individual damaging factors is mechanistically informative and offers highly controlled high-throughput analyses, but it does not reflect the real situation in vivo, where several damaging mechanisms combine in their effect on RBCs in an unpredictable and uncontrolled fashion. On the other hand, in vitro assays may overestimate the damage. Using mouse RBCs illustrates this point. In addition, changes caused by isolation, energy starvation and lack of stabilizing effect of normal blood plasma all may additionally sensitize isolated RBCs versus their counterparts circulating in the bloodstream.

Of course, an ideal battery of in vitro tests would maximally adequately correspond to situation in vivo. However, in the context of design of drug delivery systems, the overestimation of danger is arguably less problematic than an opposite outcome, from the standpoint of the patient safety and early awareness of possible translational problems, prompting design of timely and effective countermeasures.

**Conclusion**

RBCs continuously undergo constant exposure to insults during their lifespan, which results in continuous biochemical, physical, and structural changes. These changes impair the ability of RBCs to transport oxygen and eventually trigger their removal from circulation by RES. Contact of RBCs with non-biological objects including nanoparticles, polymers used for masking RBC antigenic determinants, drug delivery systems targeted to RBCs, and any drug delivery system introduced intravenously may adversely alter RBCs and their functions [55–62]. The results of this study indicate that the non-covalent adsorption of model NPs to mouse and human RBCs is not detrimental at ratios of and below NP/RBC 200:1. This result is consistent with data obtained in vivo, showing minimal effect of NP on circulation of labeled carrier RBC. We used mouse RBCs and human RBCs for the sake of consistency with our previous studies in vivo in mice and gauging potential translational utility of animal data. As compared with mouse counterparts, human RBCs showed higher resistance to all challenges and NP loading doses. This result is encouraging in terms of potential translational aspects: most likely, data obtained using mouse RBCs overestimate potential adversity of RBC modifications.

**Supporting Information**

**S1 Fig. Osmotic fragility of red blood cells at different hematocrits.** (A) Hemolytic curves for freshly obtained mouse RBC at different hematocrits from 2% to 0.05% were obtained after immediate exposure to different [NaCl]. (B) % hemolysis at 73mM NaCl (solid filled bars) and at 150mM NaCl (hashed filled bars). Values are means (n = 5) ± SD. Please note that some deviation bars are too small to be evident.

(TIF)
S2 Fig. Fragility of red blood cells at 1% hematocrit from different species. (A). Osmotic fragility of freshly obtained naïve mouse and human RBC after immediate exposure to different [NaCl]. (B). Hemolytic curves for freshly obtained naïve mouse and human RBC under low stress were obtained after constant rotation at 24 rpm at 37°C for up to 46h. (C) Mechanical fragility of freshly obtained naïve mouse and human RBC were obtained with (triangles) and without (circles) glass beads at 24rpm at 37°C for up to 8h and 24h, respectively. (D) Oxidative fragility of freshly obtained naïve mouse and human RBC were obtained after rotating at 24rpm at 37°C for up to 24h and 46h, respectively, after being challenged with 3mM H2O2 (triangles). Negative controls without H2O2 treatment are also shown (circles). Values are means (n = 4–6) ± SD. Please note that some deviation bars are too small to be evident.

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Author Contributions
Conceived and designed the experiments: DP VM. Performed the experiments: DP OVM. Analyzed the data: DP VM. Contributed reagents/materials/analysis tools: BZ ACA VG MZ SM. Wrote the paper: DP VM.

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