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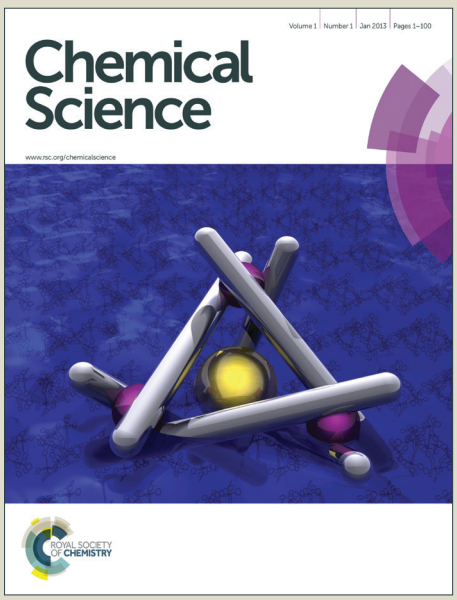

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Antigenically shielded universal red blood cells by polydopamine-based cell surface engineering

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Blood type mismatching is a critical problem in blood transfusions and it occasionally leads to severe transfusion reactions and even patients' death. Inspired by the adhesive proteins secreted by mussels, we suggest a catecholic chemistry-based strategy to shelter antigenic epitopes on red blood cells (RBCs) by using polydopamine (PDA), which can guard against coagulation reaction without other negative effects on the RBCs structure, function and viability. Both *in vitro* and *in vivo* studies confirm that the PDA-engineered RBCs (PDA-RBCs) can be applied in blood transfusion practices. The systemic assessment using murine model demonstrates that the modified RBCs have a perfect survival profile even with repeated transfusion and high transfusion rates up to around 60%. It follows that an appropriate biogenic-chemical modification can produce antigenically shielded universal RBCs and shed insight for cell transplantation by using cell surface engineering.

Introduction

Since the first successful human blood transfusion in the 17th century, modern transfusion medicine began and then developed to a classic therapeutic method with the identification of major blood groups in 1901 and subsequent use of the agglutination technique for compatibility testing in 1907.[1] Nowadays, the chronic difficulty of maintaining a blood supply is supported by the high annual requirement of RBCs of nearly 90 million units collected and donated for transfusion worldwide.[2] However, blood type mismatching is the most critical problem in transfusion medicine. Although simple blood typing (ABO/RhD) is sufficient to identify appropriate donors, the rare blood types (e.g. RhD-negative) are difficult to be found frequently.[3] The emergent situations without the matching blood type may become life-threatening, such as for pregnant women or soldiers on the battlefield.[4, 5]

There are several approaches those have been used to produce universal donor RBCs. Converting group A or B RBCs to group O RBCs is available by cleaving the terminal immune-dominant sugars from carbohydrate chains on the RBCs membrane using specific enzymes.[6, 7] Unfortunately, it is not possible to excise the immunogenic epitopes of the D antigen from RBC membranes, a protein based antigen which is closely associated with the RBC membrane.[8] Another method interferes with an antibody reaching its specific antigen on the RBCs membrane by bonding[9] or assembling[10] poly(ethylene glycol) (PEG) to the RBCs (PEG-RBCs). However, in vitro serological problems and possible reduced *in vivo* RBCs survival of 'stealth' PEG-RBCs have been found.[11] Recent findings suggest that PEG is immunogenic in animals and humans and that PEG antibodies can shorten the survival of PEG-RBCs in rabbits and pegylated proteins (e.g. PEG-asparaginase) in humans.[12-14] Thus, PEG-RBCs have vital problems for universal RBCs productions. Generation of universal RBCs by using genetically engineered hematopoietic stem cells *ex vivo* represents another appealing approach.[2] But, replacing blood transfusion in its entirety is not conceivable unless the crucial answer to the scaling-up feasibility is spring up and the cost of production can be rendered.

Alternatively, we present a catecholic chemistry-based cell engineering for preparation of universal RBCs. Dopamine, a small-molecule mimic of foot protein 5 in promiscuous fouling organisms (*Mytilus edulis*),[15] is a powerful building block for spontaneous modification on virtually any bulk material surface and that the deposited films are easily adapted under mild conditions.[16, 17] We firstly find that PDA superficial modification can mask the antigens on RBCs membranes to avoid the coagulation reaction. The structure-function analysis of PDA-RBCs shows no significant detrimental effects on the catecholic-coupled cells. It is the most important that the newly engineered and antigenically inert RBCs can share the similar *in vivo* survival profile with the native ones even with repeating and high (up to 60%) red cells transfusion.

Results and discussion

PDA-based cell surface engineering

The surface engineering process of RBCs using PDA is relatively feasible. Keeping 3~4.5% hematocrit with 2 mg/mL dopamine in an weak alkaline TBS buffer (10 mM Tris-Cl, 0.85% NaCl, pH 8.35) for 45 minutes can result in a spontaneous and effective epitopes sheltering of RBCs (Fig.

S1†). No obvious hematolysis occurs during the chemical modification. Under scanning electron microscope (SEM), the native and PDA-engineered RBCs show no difference in morphology (Figs. 1A and 1B). In order to confirm the modification on RBCs, the membrane proteins extracted from the native and derivatized cells are analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). There are distinctive shifts (Fig. 1C) in the several protein patterns of Band 3 (the anion transport channel) between native and PDA-engineered RBCs, 4.1 (the internal cytoskeletal protein) and 4.2 (an external membrane protein) and furthermore, these changes are reaction-time dependent. For example, two bands occurring in the Band 3 area show clear differences in shift when membrane ghosts from cells incubated with 30 vs. 60 minutes are compared by shifting. These phenomena imply that the dopamine molecules primarily bind to these cell-membrane proteins. A common feature of blood group antigens is their location on the outer surface of RBCs, such as the membrane or trans-membrane proteins where they are readily accessible to antibodies.[18] The dopamine sheltering can cover these antigens to interfere the macromolecule-cell or cell-cell interactions. The cell monolayers built by the native and PDA-engineered RBCs are characterized by Raman spectroscopy (Fig. 1D). The signal peaks at 1370 and 1600 cm^{-1} from modified group are attributed to the dopamine fragments cross-linked to the cell surface by PDA-derivatization (Fig. S2†). The trials from different species of blood show that this treatment is a common approach for the antigen-site sheltering on RBCs (Fig. S3†).

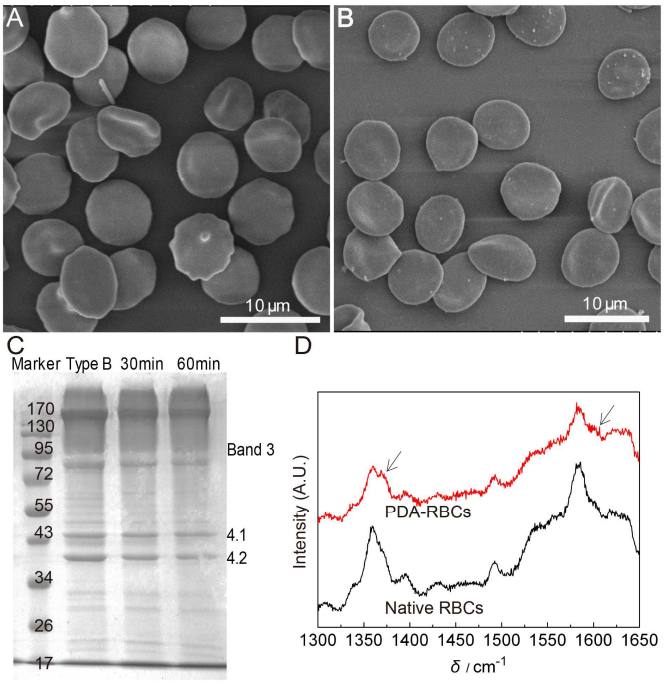

Fig. 1 Characterizations of RBCs before and after surface engineering of polydopamine. (A) SEM images of human native RBCs, and (B) PDA engineered human RBCs. (C) The fine changes in the membrane protein were analyzed by SDS-PAGE of membrane ghosts derived from native RBCs or PDA-RBCs. There are mobility shifts of proteins in the area of Bands 3, 4.1 and 4.2, confirming the binding sites. (D) Raman spectra of human type B RBCs and PDA-engineered ones for 45 min, The Raman peaks at 1370/1600 cm^{-1} indicates the PDA-modified sites on the cell surface.

Effects of PDA-engineering on antigenic recognition

To investigate the prevention effect for agglutination by the polydopamine modification, antibody mediated aggregation assay by using human blood is performed. The blood coagulation occurs definitely when the type A RBCs are mixed with their anti-type antisera such as type B sera (Fig. 2A). PDA-engineered human type A RBCs bound significantly less anti-A antibody and antibody mediated aggregation is prevented effectively in the type B antisera (Fig. 2E). Identical results are also obtained with type B and type AB RBCs in their corresponding anti-type antisera (Figs. 2B, 2C, 2D and 2F, 2G, 2H). Clearly, the surface engineering of RBCs by polydopamine achieves antigen-sheltering on the cell surface and inhibits RBCs agglutination successfully with blood type-independence.

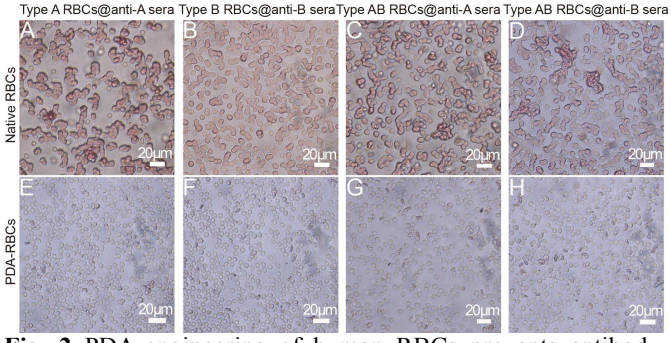

Fig. 2 PDA-engineering of human RBCs prevents antibody-mediated aggregation. Different kinds of RBCs, from type A, B, AB human RBCs can all be antigenic inert by simple PDA-modification. (A, B, C, D) The optical images of human type A, B, and AB RBCs in their corresponding anti-typing sera. (E, F, G, H) Equal concentration of type A, B and AB of PDA-RBCs was mixed with their anti-typing sera respectively. As can be seen, surface engineering of RBCs by PDA inhibits RBCs agglutination successfully with blood type-independence.

Effects of PDA-engineering on RBCs structure and functions

While PDA modification significantly interferes RBCs antigenicity, the modification of PDA has no obvious morphological, structural, or functional effects on the cells. During the treatment process, virtually no lysis is observed. The osmotic fragility curves of the native and engineered RBCs show that they share similar rupture profile. Cellular fractures of the native and PDA-RBCs are initiated at the concentration of NaCl, 0.40% (w/v) and burst completely at the concentration of 0.30% (Fig. 3A). It is also found that PDA-RBCs acquired better osmotic fragility than the native ones (Fig. S4†). The deformability of RBCs is also characterized by the maximum deformation index (DI_{max}). The results in Fig. 3B show that PDA-engineering can decrease DI_{max} of RBCs from 0.47 ± 0.04 of native ones to 0.40 ± 0.02 (p = 0.04). The surface charge of RBCs also plays an important role in affecting RBC aggregation by interaction of the electrostatic repulsive force between cells, which is determined by the zeta potential of the cell surfaces,[19] and featured by the electrophoretic mobility (EM). Compared with -0.775 ± 0.071 $\mu s^{-1}v^{-1}cm^{-1}$ of the native ones, PDA-RBCs hold the EM of -0.759 ± 0.073 $\mu s^{-1}v^{-1}cm^{-1}$ (Fig. 3C). There is no significant difference between them (p = 0.40). Furthermore, hydrophobicity of the native and modified cell membrane are the same; the contact angles of water on the substrates of the native and PDA-RBCs are 33.03 ± 4.12° and 35.19 ± 5.15°, respectively. These examination results indicate

that the modification does not change the physicochemical properties of RBCs.

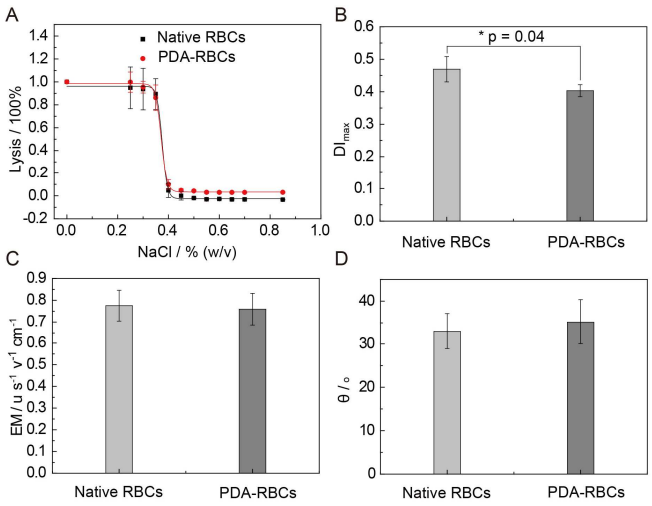

Fig. 3 Physical properties of the native and PDA-RBCs. (A) PDA engineering has no significant effect on RBCs osmotic fragility. (B) Representative profiles show that deformability of PDA-RBCs tested by ektacytometry is affected by catecholic functionality lightly. (C) Cell electrophoresis assay shows that the electrical behavior of cell surface does not change after the catecholic modification, and also for (D) Hydrophobicity characterized by contact angle of cell monolayer.

Cholesterol, acetylcholinesterase and ATPase in the cell membrane are also maintained well after the modification, and their contents and enzyme activities are preserved invariable with the native ones, as well as for 2,3-DPG and ATP contents inside cells (Table 1). These five components are critically important to ensure the stability and bioactivity of RBCs. Cholesterol is an essential structural component of the RBCs membranes that is required to establish proper membrane permeability and fluidity. Acetylcholinesterase, a prototypic acetylcholine-binding protein, attaches to the outer surface of the red cell lipid bilayer. Transmembrane ATPases import many of the metabolites necessary for cell metabolism and export toxins, wastes, and solutes. 2,3-DPG is an allosteric effecter, which enhances the oxygen releasing as requirements. 2,3-DPG and hemoglobin work together to fulfill the task of oxygen uptake. These unchanged parameters demonstrate that the dopamine modification maintains the structure and functions of RBCs. It follows that the modified RBCs can share the similar cellular deformation, stability and activity with the native ones, which are the prerequisites for the following practices.

RBCs, or erythrocytes, are the most common type of blood cell and the vertebrate organism's principal means of delivering oxygen (O_2) to the body tissues via the blood flow through the circulatory system. The cytoplasm of erythrocytes is rich in hemoglobin, an iron-containing biomolecule that can bind oxygen and is responsible for the red color of the cells. Therefore, the most important issue is that the hemoglobin oxygen affinity should be considered primary. The effects of dopamine modification on the hemoglobin oxygen affinity of treated RBCs are assessed. Oxygen equilibrium curves (obtained by a Hemox-Analyzer, TCS Scientific, CA) reveal that the oxygen affinity of PDA-RBCs is within the normal range (P_{50} = 16.3 ± 0.75 mmHg, Fig. S5†) and this value is equivalent to the untreated cells (P_{50} = 17.32 ± 0.86 mmHg). Thereby, the chemically engineered RBCs hold excellent oxygen binding and release abilities and can still perform their inherent responsibilities as the native ones.

Table 1 Structure and functional analysis of the original and PDA-treated human RBCs.

Index	Native RBCs	PDA-RBCs
Cholesterol [μmol/gHb]	41.24±2.23	44.39±3.93
Acetylcholinesterase [U/ gHb]	39.29±2.72	39.75±5.32
ATPase [U/gHb]	43.76±4.52	45.13±5.78
2, 3-DPG [μmol/gHb]	4.52±0.28	4.38±0.13
ATP [μmol/gHb]	4.49±1.54	4.76±1.58
P_{50} [mmHg]	17.32±0.86	16.3±0.75

Effects of PDA engineering on *in vivo* survival of RBCs

Systematic administration by using murine model is applied to examine the survival life span of PDA-RBCs. No significant difference in the *in vivo* survival profile of PDA-RBCs is observed during the whole lifespan of the transfused red cells. The rates of removal of the fluorescently tagged cells (Fig. S6†) in the primary transfusion were almost the same per day respectively (Fig. 4A). The lifespan of antigen-sheltering RBCs remained for above 40 days following the primary infusion, demonstrating very similar rates of clearance of native RBCs in the blood circulatory system. Furthermore, body weights and routine physical examination of the transfused mice were also investigated and the results showed no differences between the transfused mice with native and PDA-RBCs (Table S1†). *In vivo* studies provided the ultimate indication of the normality (*i.e.*, viable structure and function) of PDA-RBCs, and to support that the PDA surface engineering does not alter RBCs.

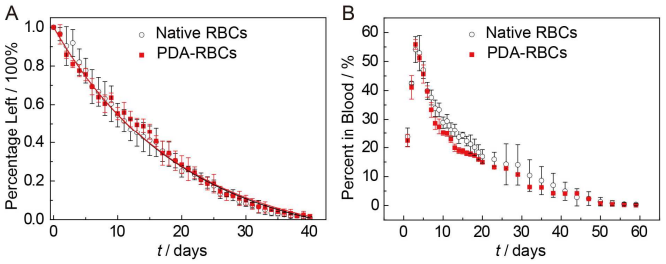

Fig. 4 *In vivo* survival of PDA-mRBCs is normal after labeled RBCs blood infusions in mouse model. Shown are the clearance rates of primary i.v. infusions of native and PDA-mRBCs (n=4, mean±SD). (A) whole retention time curve after one time of transfusion. These data are resulted in no apparent differences between groups in terms of viability or behavioral and physical activities and suggest a lack of toxicity and emphasize the *in vivo* normality of PDA-mRBCs. (B) in vivo survival profile of native and PDA-mRBCs with three transfusions, which indicates inert antigenicity of "universal red cells" based on PDA surface engineering.

To confirm the immunologic inertia of the PDA-based surface engineering, repeating transfusion tests of native and PDA-RBCs were further conducted. Our findings show that repeated transfusion of mice with PDA-RBCs does not sensitize the immunologic system to the modified cells, and these cells show normal survival even after three transfusions, and with a maximum of approximately 60% of RBCs volume consisting of engineered RBCs (Fig. 4B). Furthermore, there is no significant difference in body weight and routine physical examination of the transfused mice with control and modified RBCs (Table

S2†). Importantly, these data suggest that the superficial modification of RBCs with polydopamine does not elicit any immune response and have potentials in the practice of human red cells transfusion. In the previous approach by using PEG derivatization, the satisfactory *in vivo* survival profile can only be obtained with modified cells at low PEG concentration.[11, 20] However, the applied low PEG concentration cannot provide sufficient antigen shielding effect of RBCs to avoid agglutination reactions. The outcomes of PEG-RBCs transfusion trial are primarily due to the detrimental effects on RBCs structure by PEG modification and also the inherent immunogenicity of PEG.[21]

Because transfusion rejections are immune-mediated responses arising from the presence of antigenic proteins, glycoproteins, and glycolipids located on the RBC membrane, one possible strategy of a cellular blood substitutes lacking these surface antigens has been developed in the several decades.[22] The safety and efficacy of acellular encapsulated blood substitutes is suffering from too short circulating lifetime and secondary iron toxicity of the agents after repeated transfusions.[22, 23] Instead, biogenetic-chemical-based surficial-engineered RBC approach aims at maintaining cellular integrity, longevity, and the myriad of biofunctions of RBCs while eliminating its inherent antigenicity and immunogenicity by masking membrane antigenic sites with biogenetic and biocompatible substances in molecular level is a promising way.

Consequently, we have attempted to produce antigenically silent RBCs via the chemical modification of biogenic materials, PDA, inspired by the composition of adhesive proteins secreted by mussels. In the previous study, dopamine in the alkaline solution has been used to form thin, surface-adherent PDA films onto a wide range of inorganic and organic materials and the suggested reaction mechanisms of organic layer formation have been approved by ToF-SIMS analysis.[16] This protocol offers a facile approach from versatile particle modification,[24] to particle encapsulation which shows lower cytotoxicity and histological toxicity compared with bare ones,[25] and even to microbe cell surface modification that manipulating cell cycle.[26] Herein, we firstly utilized the chemical reactivity of dopamine toward amine and thiol functionalities for the surface modification of mammalian cells in situ. The "camouflage" effected by PDA prevents the interactions of macromolecules such as antibodies with RBCs and may as well hinder cell-cell interactions. Therefore, it is reasonable that the dopamine-modification may yield fully functional yet antigenically inert RBCs. Different from the PEG derivatization, PDA is a bionic materials and more biocompatible. The experimental results show that this chemical engineered cells do not arise the immunoreactions and antigenically inert *in vivo*. Indeed, PDA-RBCs are morphologically and structurally unaffected, exhibit inert antigenicity, and demonstrate normal *in vivo* survival profile with no immunogenicity associated with native RBCs in the circulation system in murine model.

Conclusions

In summary, our findings suggest that appropriate catecholic chemistry-based cell engineering can produce antigenically shielded "universal" RBCs and the PDA surface engineering cause negligible negative effects on the RBCs structure, functions and viability. This feasible approach may provide an attractive approach for the supply of universl red cells, especially for transfusion medicine in emergency situations with rare ABO subgroups. The analogous chemical camouflage of living cells may furthermore provide a potential methodology for cell modification to avoid alloimmunization effect.

Experimental section

Blood samples

Following informed consent, venous blood was drawn in EDTA dipotassium from healthy laboratory volunteers. Serum was collected in serum tubes. To prevent any storage artifacts, all samples were processed immediately. Care was taken to assure adequate representation of males and females and no individuals were excluded on the basis of race or gender. Statistical significance was determined by Student's t test or analysis of variance (ANOVA). All biochemicals, unless otherwise noted, were obtained from Sigma.

Surface engineering by PDA

Blood samples collected from human or animals with the hematocrit 45% were stored at 4ºC. The RBCs could be separated by centrifugation at 600 g for 5 min, and the pallet were washed twice with phosphate buffer solution (1×PBS, 10 mM, pH 7.2) or 0.85 % (w/v) NaCl. As for the modification, RBCs were re-suspending in freshly prepared 2 mg/ml dopamine in mild alkaline TBS (10 mM Tris-Cl, 0.85% NaCl, pH 8.35) with 3% hematocrit (3×10^8 cells/mL), and then stirred for 45 min. The modified RBCs cells were collected by centrifugation, and washed with 1×PBS for three times. Obtained modified RBCs were responded in 1×PBS for the following experiments.

Surface characterizations

Native and PDA-RBCs were fixed by 4% glutaraldehyde, and the images were gotten by using a S-4800 field-emission scanning electron microscope (HITACHI, Japan) at an acceleration voltage of 5 kV. Gross changes in the membrane protein pattern were analyzed by one-dimensional SDS-PAGE of membrane ghosts.[27] Briefly, membrane ghosts from the control and PDA-RBCs were prepared firstly in the presence of the protease inhibitor phenylmethylsulfonyl fluoride (100 mM). Protein concentration of the washed membranes was determined by using Coomassie® Blue (Pierce, USA). Aliquots containing 30 mg of SDS-solubilized protein were loaded on 9% poly-acrylamide gels as described. Raman spectra were obtained by accumulating 10 scans with a 514 nm Ar laser light source and a resolution of 1 cm^{-1} in the range of 1000–2000 cm^{-1} by LabRAM HR800 (Horiba Jobin Yvon, France).

Antigenic recognition assay

Antigenic camouflage was assessed by attenuation of anti-A and anti-B human antisera-mediated RBCs aggregation by microscopy (Nikon TE2000, Japan) and images were collected at 400× magnifications. Briefly, 1 μL of RBCs suspension (45% hematocrit in PBS) was placed in an aggregometer cuvette with 200 μL PBS at 37°C, with stirring, and 20 μL of anti-A and/or anti-B typing serum (or heterologous serum) was added. RBCs aggregation was then observed over time.

Structure and functions assay

Spontaneous RBC lysis was quantitated by measuring the amount of extracellular hemoglobin against the total hemoglobin concentration using the ferricyanide–cyanide (Drabkin's, Sigma) method at 540 nm. The osmotic fragility of RBCs in response to decreasing osmolality was determined as described.[28] The deformability of native and PDA-RBCs populations was measured by a laser diffraction technique, which is a sensitive method for detecting population changes in deformability based on changes in cell geometry, surface area,

cytoplasmic viscosity, and cellular hydration by using a LBY-BX2 Ektacytometer (Precil, China). In brief, Aliquots containing 12.5 μl of pelleted RBC pellets were diluted in 5 ml of 4% polyvinylpyrrolidone solution (molecular weight 360 kDa). Then, the highly diluted suspension of cells is sheared in a Couette system with a gap of 0.3 mm between 2 cylinders, one of which is able to rotate to induce shear stresses. A laser beam is passed through the suspension, and the diffraction pattern is measured at 37°C. The photometric measurement produces a signal termed the deformability index (DI). Analysis of the DI curve provides a measure of the dynamic deformability of the cell membrane as a function of the osmolality at a constant applied shear stress of 170 dynes/cm^2. The DI_{max}, quantified as the maximum value of the DI normally attained at a physiologically relevant osmolality, is related to the mean surface area of red cells. Peripheral blood controls were collected from healthy donors (n = 4). Cell electrophoresis assays were made in a cylindrical chamber of the type employing grey platinum electrodes. The basic medium used had a constant ionic strength, I = 0.15, comprising 0.144 M NaCl and 0.006 M KCl and its pH was adjusted to 7.2. Washed cells were re-suspended in the media at concentrations of 2×10^6 cells/mL, and equilibrated at 25° before placing in the electrophoresis chamber. From these data a mean mobility and standard deviation were calculated. The native and PDA-RBCs were sampled on a clean and hydrophilic silicon wafer and the sessile drop method was used for the contact angle measurements with an OCA15+ instrument (Dataphysics, Germany). The contents of 2,3-DPG, ATP in the RBCs before and after the PDA modification were quantified by ENLITEN® ATP Assay System (Promega, CA). The contents of cholesterol and the activities of acetylcholinesterase in the cell membrane were also investigated after the PDA modification (AAT Bioquest Amplite, CA), and as well for ATPase in the cell membrane were also determined by ultramicro ATPase assay kit (Nanjing Jiancheng Bioengineering Institute, China). Equilibrium oxygen binding curves at 37°C were determined by Hemox-Analyzer (TCS Scientific Corp., PA). Analyses were performed in 50mM bis-Tris buffer (pH 7.2) containing 140 mM NaCl.

***In vivo* survival assay**

In vivo survival of PDA-engineered mouse RBCs (PDA-mRBCs) was examined in ICR (Institute of Cancer Research) mice. mRBCs were obtained from multiple donor mice and pooled. Half of the RBCs were PDA-engineered as described above, and then the same volume of saline, control and treated mRBCs were labeled with PKH-26 (Sigma, USA), a fluorescent lipid that inserts into the membrane. 200 μL labeled mRBCs (10% in total blood volume) were injected intravenously (*i.v.*) into recipient mice. Cell survival was followed up to 50 days until no signals were detected by measuring PKH-26 fluorescence on a Cytomics FC 500 MCL flow cytometer (Beckman Coulter, CA). Repeating transfusion test was conducted by three times. Firstly, 600 μL whole blood was drawn out and then the same quantity of saline, fluorescently labeled control or PDA-mRBCs were transfused (n = 4 in each group). Routine physical examinations were conducted before and 4 hours after transfusion. Body weights were collected and recorded in the whole course.

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Notes and references

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† Electronic Supplementary Information (ESI) available: Several detailed experimental information, characterization data, and some control experiments are included in the supporting information. See DOI: 10.1039/b000000x/

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