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Converting Red Blood Cells to Efficient Microreactors for Blood Detoxification

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Abstract

We report a simple method to convert RBCs into efficient microreactors for important biomedical applications. A commonly used cell membrane permeation agent Triton X-100 (TX) was employed at finely tuned concentrations to punch pores in the membranes and render RBCs highly permeable to substrates while maintaining membrane integrity. Subsequently, low concentration of the fixative agent glutaraldehyde (GA) was used to stabilize the cells and avoid inactivation of the membrane proteins. Atomic force microscopy (AFM) and measurement of Young's modulus (YM) values clearly demonstrated that the reformed RBCs (Ref-RBCs) retained their size, the biconcave disk-like shape, elasticity, and mechanical flexibility comparable to natural RBCs. Furthermore, Ref-RBCs improve the blood survival time of loaded enzymes, and have high permeability to substrate molecules. By exploiting these properties of Ref-RBCs microreactors, we developed an important biomedical application of blood detoxification. When uric acid (UA) and KCN were used as endogenous and exogenous toxin models, respectively, Ref-RBCs, loaded with the corresponding detoxifying enzymes, showed much faster toxin clearance rates than the unmodified RBCs both *in vitro* and in an *in vivo* animal model. Our results suggest that these microreactors, after confirming biocompatibility, would have promising therapeutic applications, especially in blood and liver-related diseases.

Graphical abstract

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A simple method to convert RBCs into efficient microreactors was reported. Triton X-100 was employed at finely tuned concentrations to render RBCs highly permeable to substrates, while low concentration of glutaraldehyde was used to stabilize cells. Ability of these microreactors for blood detoxification was demonstrated.

Keywords

red blood cell (RBC); Triton X-100 (TX); glutaraldehyde (GA); microreactor; blood detoxification

INTRODUCTION

Mature red blood cells (RBCs) of mammals are unique as they lack the cell nucleus and most of the organelles thus providing maximum space for hemoglobin. With a hollow structure, high biocompatibility, and long blood circulation time, RBCs have significant advantages over non-natural materials as biocarriers, and can be attractive candidates to serve as microreactors for *in vivo* applications.^[1] However, several limitations must be overcome before RBCs can be used as ideal microreactors.^[2] The most critical problem is the limited and selective permeability of the RBC cell membrane that is largely attributed to the properties of the phospholipid bilayer. Together with membrane proteins, the phospholipid bilayer forms a wall-like structure and functions as a molecular filter.^[3] Only small, non-polar, lipid-soluble molecules, essential ions, nutrient molecules, and waste products can cross the cell membrane using transmembrane protein channels.^[4] However, the penetration rates of these substrates are limited as they must overcome various energy barriers. Lipid-insoluble, polar, and charged molecules and the ones with large molecular weights are blocked out because of the electrostatic repulsion effect.^[3] Also, RBCs are unstable *in vitro*, prone to lysis, and hard to store after loading external substrates. These limitations pose significant obstacles for RBCs to be used as microreactors.

In this study, we transformed RBCs into efficient microreactors and explored their biomedical applications (Scheme 1a). Experimental conditions were chosen to avoid

deactivation of membrane proteins and loss of deformability of RBCs. First, enzymes were loaded into RBCs in a hypotonic medium.^[4] After resealing, the RBCs were treated by a common membrane punching reagent, Triton X-100 (TX), to render them more permeable. Strictly controlled concentration of TX was used for maintaining the crucial pore size for free diffusion of substrate molecules while avoiding leakage of enzymes and cell lysis. Considering the fluidity of the cell membrane, which may compromise the pore sizes, we employed a carefully titrated concentration of a cell-fix reagent, glutaraldehyde (GA), to stabilize the cells following TX treatment. The reformed RBCs (Ref-RBCs) engineered in this study were highly permeable and easy to store while retaining many of the original merits of RBCs such as the shape and long circulation time and can, therefore, serve as excellent microcarriers.

Next, we explored an important biomedical application of the Ref-RBCs in blood detoxification (Scheme 1b). Endogenous or exogenous blood toxins, derived from metabolic processes or originating from the diet, pharmaceuticals, naturally occurring poisons and environmental pollutants constitute one of the most common threats to human health.^[5] Blood plays important roles in toxin absorption, transport, and circulation. Toxins accumulated in the blood may be transported to the rest of the body. Thus, blood detoxification is an important process.^[6] Generally, our bodies strive to minimize the toxin-induced damages by involving complex detoxification enzyme systems and convert toxins into non-toxic or low-toxic compounds.^[7] However, in some situations, such as serious diseases of detoxification organs (liver, kidneys, lung, intestine, *etc.*), innate defects in the expression of some enzymes, or acute intake of high amount of toxic substances, the body may fail to clear the toxins. The impaired detoxification increases the risk of many diseases including cancer, Parkinson's disease, and some chronic dysfunction syndromes^[8] and may even cause life-threatening conditions in severe situations. In these cases, there is a critical need for artificial detoxification techniques to help the body manage the toxins.

A variety of methods for artificial detoxification are currently used in the clinic, such as blood dialysis, gastric lavage, and administration of antidotes.^[9] For antidote-based detoxification, enzymes are the preferred choice because of their natural detoxification mechanism,^[10] but have disadvantages of high immunogenicity, low stability, and short circulation time resulting in poor detoxification efficiency.^[10] To overcome these limitations, nano/micro-carriers were developed to entrap or conjugate the enzymes with microreactor-like architectures.^[10–11] The materials used to construct these microreactors range from inorganic particles (silica) to organic vesicles (liposome, polymers) and may cause side effects. RBCs, on the other hand, are intrinsic components in the blood and have substantial advantages over non-natural materials.^[1b, c] The Ref-RBCs engineered in this study retained most of the features of RBCs. As shown in Scheme 1, the detoxification enzymes were loaded inside the Ref-RBC-based microreactors. We used uric acid (UA) as an *in vivo* and potassium cyanide (KCN) and UA as *in vitro* toxin models and demonstrated the high efficiency and toxin-clearance rate of Ref-RBC microreactors in an important, much needed clinical application for blood detoxification.

RESULTS AND DISCUSSION

Optimization of enzyme/protein loading conditions

We optimized a previously reported osmotic method (Figure 1a) for loading enzymes and other proteins inside the RBCs.^[1b, 4, 12] This method is based on the osmotic pressure difference between the inside and outside of the RBCs. Following separation from blood and washing with isotonic solution, the RBCs were dispersed in hypotonic solution containing enzymes and proteins of interest. Under hypotonic conditions, to maintain the osmotic balance, water enters the RBCs causing cell swelling^[13] which may be irreversible rupturing the cell or reversible when the cell has the potential to revert to the original state. In the reversible case, under optimal conditions transient pores of 200–500 Å diameters^[13b] are generated allowing a two-way exchange of materials with enzymes within this size range to enter through the pores, while intrinsic proteins, such as hemoglobin (Hb), can leak out.^[4, 13b, 14] After re-adjusting the osmolality of solution to the isotonic value, the pores are closed and the cells return to an osmotically competent, resealed state.^[4] This technique produces RBC ghosts loaded with exogenous substances, including enzymes with molecular weight up to 180 kD.^[4]

We used fluorescein isothiocyanate (FITC)-labeled bovine serum albumin (BSA) (BSA-FITC) (Figure S1) to facilitate tracking and to explore the optimum protein loading conditions.^[15] A series of loading solutions containing different volumes of PBS (0–1×) were used to obtain the optimum condition for protein loading. 0.2 × PBS showed the highest protein loading efficiency (Figure S2, S3 and Figure 1b). Bright field microscope imaging indicated that the RBCs displayed biconcave disk-like shape in PBS (Figure 1c). After protein loading, the RBCs still maintained the shape but became somewhat transparent due to the loss of Hb (Figure 1d). The successful loading of BSA-FITC into RBCs was confirmed directly by confocal fluorescence microscopy (Figure 1e, f and Figure S4). The sectional confocal fluorescence images (Figure 1f) revealed that BSA-FITC was loaded homogeneously inside the cells rather than being adsorbed on the membrane. Flow cytometric analysis (Figure 1g, Table S1) also showed that the protein-loaded RBCs still revealed strong fluorescence intensity after washing with PBS-Tween-20 (0.007%). Also, pretreatment of cells with 0.2 × PBS, resealing in 1 × PBS followed by incubation with BSA-FITC showed very weak fluorescence. Taken together these observations suggested that the BSA-FITC conjugate remained inside the cells and was not merely adsorbed on the membranes.

Enzyme loading and catalytic activity assays

Using horseradish peroxidase (HRP)^[16] as a model enzyme, the efficiency of RBC microreactors was evaluated. HRP was loaded into the cells under the optimal loading condition (0.2× PBS, with HRP concentration of 500 µg/mL). One of its common water-soluble substrates, 2, 2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) (ABTS), was chosen to monitor the activity of the enzyme by a colorimetric reaction (Figure 1h). It has been shown that in the presence of HRP, H₂O₂ can oxidize ABTS into a colored product (ABTS⁺), which has a peak absorption at 417 nm.^[16] The interference of intrinsic HRP-like activities of RBCs is insignificant here because most of the Hb, which is the main

contributor to HRP-like catalytic activities,^[17] has been removed during the loading process. As shown in Figure S5, the catalysis background is negligible. Successful loading of HRP into RBCs was demonstrated by testing the enzyme activity in the cell lysates. The loading amount of HRP was quantified to be 213 $\mu\text{g/mL}$ of RBC (Figure S6). Negligible amount of enzyme was detected if the cells were pretreated with the loading buffer and then incubated with enzymes (Figure S6), indicating that the enzyme molecules were not merely adsorbed on the surface of cells. The catalytic efficiency assays showed that the reaction activity of loaded enzymes was only 15% as that of free enzymes in the cell lysates (Figure 1i, j) indicating that most of the enzyme activity was lost after loading into RBCs. As the amount of enzymes in lysates and cells were the same, the lower efficiency was then attributed to the limited permeability of the cell membrane to substrates.

Formulation of Ref-RBCs

TX was chosen as the pore punching reagent (Figure 2a, b) and its concentration was optimized to obtain pores of proper size, which allows free diffusion of substrate molecules while avoiding leakage of loaded enzymes (Figure 2c). A series of concentrations around the critical micelle concentration (CMC) of TX (Figure 2b) were tested. The proteins Hb and BSA-FITC were used for protein release tests and the dyes trypan blue (TB) and rhodamine B (RhB) were selected to test the diffusion of small molecules into the cells. As shown in Figures 2d and e, the intrinsic or pre-loaded proteins remained inside the cells until the concentration of TX reached 0.19 mM. The membrane-impermeable molecule, TB,^[18] had very low ability to enter cells in the absence or presence of low concentrations of TX (Figure 2f). When the concentration of TX reached 0.17 mM, the TB molecules could enter the cells, with the maximum entrance capacity at 0.19 mM. However, when the concentration of TX was further increased, the amount of TB entering the cells declined again. This may be attributed to the shrinking cell volume and damaged membranes under high concentrations of TX. The membrane-permeable molecule RhB^[19] had some capacity to cross the lipid bilayer and enter the cells (Figure 2g). In the presence of TX, the crossing efficiency of RhB was improved and reached a maximum value at 0.18 mM of TX. As was the case with TB, it declined again when the concentration of TX was higher than 0.19 mM. Considering the high permeability of the RBC membrane after TX treatment, the efficiency of dye entry was analyzed by adjusting the solution to hypertonic state ($2\times$ PBS). Under the hypertonic condition, the cells were in the shrivelled state and the dyes could be sealed inside (Figure S7). The absorption/fluorescence spectra used for protein/dye monitoring were shown in Figure S8. Based on these results, 0.18 mM of TX was chosen for pore-punching in subsequent studies.

During storage, TX-treated cells had the problem of protein leakage (Figure 2h) probably due to the fluidity of cell membranes causing fusion between membrane pores and enlarging the pore size. The protein leakage was observed to some degree even in protein-loaded cells without TX treatment (Figure 2i). To stabilize the pores and thus RBCs, a common cell fixative reagent, GA (Figure 2j), was used. A high concentration of GA has previously been reported to reduce RBC biocompatibility and deformability.^[20] A gentle fixation process should therefore be used to avoid this problem. We used GA at a low concentration of 0.0005% which was reported to have a stabilizing effect on cells while having little

influence on their cytological properties or membrane proteins.^[21] As shown in Figure 2h and i, fixation with GA reduced protein leakage to 7.1% after storage for 1 week at 4° C in PBS and 9.6% for 2 days at 37° C.

Morphology characterization and biocompatibility of Ref-RBCs

As the size, shape, and mechanical flexibility play important roles in the blood circulation half-life of RBCs, we performed morphology characterization of Ref-RBCs. As shown in Figures 3a and b, after treatment with TX and GA, the loaded RBCs maintained the oval, biconcave, disk-like shape. Size distribution histograms (Figure 3c–f) revealed that after protein loading (L-RBCs), the size of the cells increased somewhat as compared with original RBCs, which might be attributed to the loss of inherent proteins that stretched the cells. The size further increased slightly following treatment with TX (L-RBC-TX), indicating even greater stretching of the cells. Subsequent treatment with GA (L-RBC-TX-GA, Ref-RBCs) caused stiffening and shrinking of the cells thereby reducing their size. The overall cell size of Ref-RBCs (6.46 μm) was similar to that of original RBCs (6.17 μm), suggesting that the cells had the potential to retain the size-dependent ability to escape phagocytosis by macrophages.^[22] Flow cytometry analyses showed that treatment with TX and GA had negligible influence on the fluorescence of the cells (Figure 3g, Table S2). This indicates that the proteins remained inside the treated cells.

The standard methyl thiazolyl tetrazolium (MTT) assay (Figure 3h, S9) revealed that the Ref-RBCs had negligible cytotoxicity even under high concentrations demonstrating their high biocompatibility. Since macrophages constitute the first line of defense in the immune system, we investigated phagocytosis by using the RAW 264.7 cells as a macrophage model. HRP, chosen as an enzyme model, was conjugated to FITC (donated as HRP-FITC) to facilitate tracking (Figure S10a–b). There were no obvious fluorescence changes when HRP-FITC loaded Ref-RBCs (HRP-FITC-Ref-RBCs, Figure S10c) were incubated with RAW 264.7 for 4 h and 12 h (Figure 3i–l, Table S3). HRP-FITC alone, on the other hand, revealed intense fluorescence which increased with the incubation time (Figure 3m–p, Table S4). Fluorescence microscopy images showed that the unwashed Ref-RBCs were dispersed outside of the RAW 246.7 cells (Figure 3q), whereas the fluorescence of HRP-FITC was clearly inside the cells (Figure 3r). These results indicated that the Ref-RBCs could evade phagocytosis by macrophages, probably attributed to the membrane proteins (CD47) as well as the large size, and therefore had the potential for prolonged blood survival time.

Mechanical characterization of Ref-RBCs

The ability of RBCs to squeeze through the microvasculature with smaller diameters than their own is crucial for their long blood circulation time. The excellent deformability of RBCs is attributed to their unique shape and superb mechanical flexibility.^[23] We used atomic force microscopy (AFM), a suitable technique for soft materials,^[24] to measure the mechanical flexibility of Ref-RBCs. The AFM height images showed that both the native (Figure 4a–d) and Ref-RBCs (L-RBC-TX-GA) (Figure 4e–h) had uniform biconcave disk-like shapes. The mean height and diameter column charts (Figure 4i, j) of treated RBCs had lower height at edges and in the center but larger diameter in the concave core, presumably due to the loss of most of the inherent proteins. To evaluate the mechanical flexibilities,

Young's modulus (YM) of RBCs before and after treatment was measured. Results (Figure 4k, l) revealed that the YM value of RBCs decreased after protein loading, suggesting that the cells became softer. Treatment by TX further softened the RBCs, whereas GA treatment slightly increased the rigidity (Figure 4m, n). The resultant Ref-RBCs thus had YM value similar to that of native RBCs (Figure 4o, p), demonstrating that the working concentrations of TX (0.18 mM) and GA (0.0005%) had little influence on the mechanical flexibility of RBCs. These results clearly indicated that the Ref-RBCs maintained the high mechanical flexibility, biconcave disk-like shape, and high elasticity, allowing them to maintain the ability to pass through microvasculature and circulate within the blood vessels.

Oxidative fragility of Ref-RBCs

During *in vivo* applications, RBCs may be exposed to both endogenous and exogenous sources of reactive oxygen species (ROS) such as superoxide and H_2O_2 ,^[25] which may cause damage to RBCs and impair their functions. The sensitivity of Ref-RBCs and control RBCs exposed to H_2O_2 (3 mM) under low level of shear stress was tested. Results (Figure S11) showed that over 70% of loaded proteins still stayed within the Ref-RBCs after 18 h, indicating that it had strong resistibility to oxidative stress.

Permeability of Ref-RBCs to small molecules

To test the permeability of Ref-RBC microreactors to small molecules, TB and RhB dyes were used as models. Dye entering efficiency was analyzed by a hyperosmosis method mentioned above (Figure S7). The analysis was performed very quickly within 10 min after dye loading. The release profiles (Figure S12) showed that more than 90% of TB and more than 85% of RhB were still within the cells confirming the high efficiency of this method. As shown in Figure 5a and b, TB had no ability to cross the intact cell membranes. There was some background diffusion when RBCs were pretreated by the loading buffer ($0.2 \times \text{PBS}$), which compromises the integrity of the membranes to some degree. Treatment with TX, however, accelerated the entry of TB into the cells rapidly achieving equilibrium within 5 min. Also, the subsequent treatment with GA did not show any change in the entry rate of TB into the modified RBCs. The diffusion rate constant (k)³¹ of TB for the Ref-RBCs was about 245 times higher than that of untreated RBCs.

The membrane permeable molecule RhB showed a moderate entry rate into RBCs achieving equilibrium within 1 h (Figure 5c, d). Similar to TB, the entry rate of RhB into TX-treated RBCs was much faster reaching equilibrium within 5 min and did not change on further treatment with GA. The diffusion rate constant (k)^[26] of RhB for the Ref-RBC was calculated to be about 21 times higher than that of untreated RBCs. Overall, the results of both TB and RhB suggested that the pores punched on the cell membranes could help both membrane impermeable and membrane permeable molecules enter the RBCs at a much faster rate. The rapid equilibrium confirms that these small dye molecules can directly diffuse into the cells through the pores. This was further confirmed by using HRP as an enzyme model and ABTS as the substrate (Figure 5g–j). The catalytic activity was about 4-fold higher in the Ref-RBCs than in the HRP-loaded native RBCs. These results clearly demonstrated that as microreactors, the Ref-RBCs are far more efficient than the non-Ref-RBCs.

We used uric acid (UA) and potassium cyanide (KCN) as models for endogenous and exogenous toxins to study the detoxification ability of the Ref-RBC microreactors. UA is a product in purine-related metabolism.^[27] High blood concentrations of UA result in hyperuricemia, which is very harmful and may lead to many diseases such as gout, diabetes, and kidney stones.^[28] Uricase (Ur), a liver enzyme that degrades uric acid, is present in many mammals but does not naturally occur in humans.^[29] Some of its recombinant versions have been approved by the Food and Drug Administration (FDA) for the treatment of hyperuricemia.^[6a] We loaded Ur inside the RBCs^[26] (with a loading efficiency of about 256 µg/mL) and evaluated its efficiency to degrade UA (Figure 6a).^[30] Since the serum concentration of UA for hyperuricemia is ~0.416 mM and ~0.357 mM for males and females, respectively,^[31] we used UA with concentrations ranging from 0.1 to 5 mM for this study. Results (Figure 6b–e) showed that the degradation of UA was more effective with Ur loaded Ref-RBCs (Ur-Ref-RBCs) than with unmodified Ur-RBCs in both PBS (Figure 6b, c) and serum (Figure 6d, e). Ur-Ref-RBCs showed over 57% activity of the Ur-RBCs lysate, while Ur-RBCs only preserved less than 15% of the activity (Figure 6c, d). As the loaded amount of Ur was the same in both RBCs and Ref-RBCs, the differences in UA degradation can be attributed to the entry rate of UA into the cells. Previous reports indicated that UA could enter RBCs at a moderate rate by a passive carrier mechanism.^[26] After pore punching, it was expected that UA would diffuse more efficiently into the cells, as observed with the dye molecules TB and RhB (see Figure 5a–d). The result for Ur-Ref-RBCs was similar to that for Ur-RBC-TX (Figure S13a, b), indicating that the treatment with GA did not reduce the permeability of cell membrane.

The detoxification assay for the exogenous toxin, KCN,^[32] was carried out by loading the corresponding detoxification enzyme rhodanase (Rh) into the RBCs (about 198 µg/mL).^[33] KCN concentrations ranging from 0.01 to 50 mM were employed. Sodium thiosulfate (Na₂S₂O₃), as the sulfur donor substrate in the presence of Ur, reacts with KCN to generate SCN[−], which can be detected by Fe³⁺ under acidic conditions to form a colored compound (Figure 6f, S14).^[6b] The KCN samples were incubated with Rh-loaded Ref-RBCs or Rh-loaded non-treated RBCs in the presence of Na₂S₂O₃ for 30 min in PBS or mouse serum. As displayed in figures 6i and j, Rh-Ref-RBCs exhibited a much higher efficiency of degrading KCN than Rh-RBCs in both PBS (Figure 6g, h) and serum (Figure 6i, j). Rh-Ref-RBCs showed over 50% activity of that of Rh-RBC lysates, while Rh-RBCs only preserved less than 13% activity (Figure 6h, j). The result for Rh-RBC-TX was similar to that for Rh-Ref-RBCs (Figure S13c, d). These results provided promising support for further application of enzyme-loaded Ref-RBCs for *in vivo* detoxification.

Pharmacokinetic properties of Ref-RBC microreactors

The pharmacokinetic properties including blood circulation time (Figure 7a) and organ distribution of Ref-RBC microreactors (Figure 7b, c) were investigated which are required for *in vivo* applications.^[34] Due to the lack of Hb in the RBC ghosts, the conventional ⁵¹Cr radiolabeling method,^[35] is not suitable here. Membrane labeling by other isotopes^[36] is not very feasible either, as the membrane of ref-RBCs has been treated with TX and GA. As we are mainly concerned about whether the ref-RBCs could increase the circulation time of loaded enzymes, we thus labeled the RBCs with luciferase (Luc). Results showed a fast

clearance rate for Luc (Figure 7a). In contrast, when loaded inside the Ref-RBCs, the blood retention of Luc was significantly improved. The survival kinetics of the Luc-Ref-RBCs was then evaluated. The survival profile of Luc-Ref-RBCs corresponded to a biphasic pharmacokinetic model, with initial phase characterized by a rapid clearance in the first 4 h followed by a slow but prolonged phase of elimination. The initial fast phase ($t_{1/2\alpha} = 0.61$ h) might be attributed to the clearance of severely damaged cells, while the less damaged cells could circulate in the blood for a longer period of time ($t_{1/2\beta} = 2.7$ days). This relatively long circulation half-life of Ref-RBCs is helpful for their detoxification function in the blood. Results (Figure 7a) also showed that the blood survival ratio of Luc-Ref-RBCs was somewhat lower than Luc-RBCs, which was attributed to TX and GA treatment, however, it was much longer than Luc-RBC-TX, which was not stable and might lyse quickly *in vivo*. It is of note that detection of Luc enzymatic activity does not necessarily accurately reflect the behavior of ref-RBC. Although Luc-loaded ref-RBC circulates longer than free Luc, this does not prove biocompatibility of the carrier ref-RBC. Our future work will explore new approaches to label ref-RBC and measure the circulation *in vivo* more objectively.

To investigate the biodistribution of Ref-RBCs, near-infrared (NIR) dye IRDye-800CW, denoted as IR-labeled BSA (BSA-IR), was loaded into the cells and used as fluorescence imaging probe. The successful conjugation of the IR dyes to BSA was confirmed by the UV-Vis and fluorescence spectra (Figure S15). The organ distribution of BSA-IR-Ref-RBCs were investigated at 4 h after i.v. injection. Free Luc, Luc-RBC, Luc-RBC-TX and Luc-RBC-GA were used as controls. The results (Figure 7b, c) showed that BSA-IR-Ref-RBCs mainly accumulated in the liver, followed by kidneys, and very little in the lung, spleen, and heart. This indicated that the Ref-RBCs were mainly cleared by the macrophages of the reticuloendothelial system (RES), especially by hepatic Kupffer cells in the liver.^[37] The biodistribution profiles were similar to those of natural RBCs. Generally, the natural RBCs do not escape from the blood circulation and extravasate into the tissues, except for hepatic sinuses and interstitium in the splenic follicles which are part of RES and responsible for the elimination of damaged RBCs. Control experiments showed that BSA-IR-RBCs and BSA-IR-RBC-GA had biodistribution similar to that of BSA-IR-Ref-RBCs but with lower liver uptake. While BSA-IR-RBC-TX showed biodistribution profiles in-between those of free BSA-IR and BSA-IR-RBC, which might be due to dye leakage from RBC-TX as it was unstable and easy to lyse. This indicated the necessity of introduction of GA. The results of complement activation assays^[38] (Figure S16) showed that the level of complement 3 (C3) in mice blood serum was increased after injecting loading buffer-treated Ref-RBC (LT-Ref-RBC) for up to 4 h, which indicated that the injection of these cells caused some immune responses but not very severe.^[39] Injection of LT-RBC without TX and GA treatment showed similar results. These results showed that the immune responses were mainly caused by the cells themselves, not TX or GA.

***In vivo* blood detoxification assays of enzyme-loaded Re-RBCs**

The potential use of the highly permeable Ref-RBC-based microreactors for the elimination of toxins *in vivo* was evaluated in mice (Figure 7d–f). UA was adopted as a toxin model for *in vivo* detoxification studies and was administered in healthy mice with doses far below the reported fatal ones.^[40] The concentration of the toxin in serum was calculated by a

previously reported colorimetric method,^[6b, 30] as illustrated in Figure 6a. It is of note that the concentration of endogenous UA molecules in serum was $13.5 \pm 1.4 \mu\text{M}$ which was negligible compared to the injected concentration of UA at about 1.5 to 2.3 mM. Control assays (Figure 7e, f) showed that UA could be continuously degraded by the healthy mice, with 38% left after 24 h, a detoxification rate that is suboptimal, especially for highly toxic compounds (*e.g.*, KCN) and endogenous toxins generated continuously from metabolism (*e.g.*, UA). In contrast, mice injected with Ur-Ref-RBC showed much faster toxin clearance rates, with only 20% of UA left after the first 4 h and more than 90% toxin degraded after 24 h. The Ur-RBCs also showed some effect on the toxin clearance which was much less compared with that of Ur-Ref-RBCs. The Ur-RBC-GA showed the lowest efficiency. Although Ur-RBC-TX showed high efficiency during the first 1 h, it decreased over time probably due to the instability of the cellular structure and fast clearance of leaked enzymes. Overall, the highly permeable Ref-RBCs showed an excellent detoxification effect making them very attractive as microreactors for blood toxin clearance and other medical applications.

CONCLUSIONS

The intrinsic features of RBCs including their longevity in blood, excellent biomechanical elasticity and flexibility, immunosuppressive ability to evade phagocytosis by the RES, and capacity to carry versatile cargos, are ideal not only for their natural *in vivo* functions but also for a variety of biomedical applications.^[1a-c] Herein, we explored one such clinical application of RBCs in blood detoxification. We used RBCs as microreactors by loading detoxification enzymes while preserving their membrane structure together with the unique attributes of shape and mechanical flexibility. Simultaneously, RBCs were rendered highly permeable to endogenous and exogenous toxins by employing an elegant and simple method. Strictly controlled concentrations of a widely used detergent TX and a cell fixative agent GA were used to first punch pores of the desired size in the cell membranes and subsequently stabilize the cells thus generating Ref-RBCs. Our study is of considerable biomedical significance and, to our knowledge, for the first time, provides a simple method to convert native RBCs into efficient microreactors for a well-defined specific clinical application of blood detoxification. Endogenous toxins or those originating from the diet, pharmaceuticals, and environment underpin a variety of diseases including gout, neurodegenerative diseases, inflammation, and cancer.^[5, 41] Ref-RBC can be tailored to specific conditions with the inherent advantages of longevity in blood circulation and minimum immunogenicity. Besides detoxification in blood-, liver-, and kidney-related diseases, engineered RBCs can have limitless biomedical applications. Most significantly, RBCs represent natural drug delivery vehicles and can also be used for targeted drug delivery by tethering the desired ligands on the cell membranes.^[1a-c] Furthermore, the capability to encapsulate a variety of nanoparticles, imaging agents, and/or therapeutic payloads provides new prospects of developing RBCs into novel theranostic biocarriers for simultaneously treating and monitoring diseases in the most natural fashion.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Protein loading and enzyme activity tests. (a) Enzyme loading into RBCs in a hypotonic solution followed by resealing of the membrane in an isotonic solution. (b) Effect of PBS concentration on the uptake of BSA-FITC by RBCs. (c) Bright field microscopy images of original RBCs and (d) BSA-FITC loaded RBCs. (e) Confocal fluorescence microscope images of BSA-FITC loaded RBCs. (f) Sectional confocal fluorescence images of BSA-FITC-RBC. Images at top and bottom are from different sections. The membranes were stained by red fluorescence dye DiI. Left: FITC channel; middle: DiI channel; right: merged. (g) Flow cytometry analyses of BSA-FITC loaded RBCs (L-RBC) after washing 7 times with PBS (1), L-RBC after washing 3 times with Tween-20-PBS (2), and RBCs pretreated with 0.2x PBS loading buffer and then incubated with BSA-FITC (3). Geometric means of the fluorescence intensities are shown in Table S1. (h) Illustration of HRP catalyzed ABTS-H₂O₂ reaction system. (i) UV-Vis absorption spectra of ABTS-H₂O₂ reaction system after addition of HRP loaded RBC (HRP-RBC) and lysates of HRP-RBC. (j) Relative catalytic activities of HRP-RBC and lysates of HRP-RBC. (inset) Photographs of ABTS-H₂O₂ reaction system after addition of HRP-RBC or lysates of HRP-RBC. All cell samples for analyses were dispersed in 1 × PBS buffer.

Figure 2. Permeabilization and stabilization of RBC bioreactors. (a) Chemical structure of TX. (b) Concentration-dependent dynamics of TX. Below CMC, TX molecules are in a monomer state. When the concentration reaches CMC or is greater, TX molecules aggregate to form micelles. (c) The pores allow the substrates/products diffuse freely in and out while entrapping the enzymes and proteins inside. (d & e) Structures of Hb and BSA (top left) and the UV-Vis profiles of Hb and BSA-FITC (top right) and photographs (bottom) for supernatant solutions of Hb- and BSA-FITC-loaded RBCs following treatment with different concentrations of TX. (f & g) Chemical structures of TB and RhB (top left). UV-Vis or fluorescence profiles (top right) and photographs for lysates of RBCs after incubation with TB and RhB (bottom). The RBCs were pretreated with different concentrations of TX before incubation. (h & i) The BSA-FITC release profiles (top) for L-RBC, L-RBC-TX, and L-RBC-TX-GA at 37 °C in PBS. Photographs were taken at 48 h and at 7 days. (j) Stabilization of pores of RBCs with glutaraldehyde (GA).

Figure 3. Morphology characterization and macrophage endocytosis assays of Ref-RBC. Confocal microscope images (FITC channel) of (a) L-RBC-TX and (b) L-RBC-TX-GA. The size distribution histograms of (c) RBC, (d) L-RBC, (e) L-RBC-TX and (f) L-RBC-TX-GA. (g) Flow cytometry histograms of FITC fluorescence intensities of L-RBC-TX and L-RBC-TX-GA. The geometric means are presented in Table S2. (h) *In vitro* viability of 293T (human embryonic kidney 293) cells after incubation with various concentrations of Ref-RBC for 48 h. Flow cytometry analyses of (i) untreated Raw 264.7 (Control) and Raw 264.7 co-incubated with HRP-FITC-Ref-RBC for (j) 4 h and (k) 12 h. (l) Histograms of FITC fluorescence intensities of untreated Raw 264.7 (Control), Raw 264.7 co-incubated with HRP-FITC-Ref-RBC for 4 h and 12 h. The geometric means are shown in Table S3. Flow cytometry analysis of (m) untreated Raw 264.7 (Control), RAW 264.7 co-incubated with HRP-FITC for (n) 4 h and (o) 12 h. (p) Histograms of FITC fluorescence intensities of untreated Raw 264.7 (Control), Raw 264.7 co-incubated with HRP-FITC for 4 h and 12 h.

The geometric means are presented in Table S4. Fluorescence microscopy images of Raw 264.7 cells after incubation with (q) HRP-FITC-Ref-RBC and (r) HRP-FITC for 4 h.

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Figure 4.

AFM characterization and mechanical measurement of Ref-RBC. (a–c) 2D and (d) 3D height morphology images of RBCs. (e–g) 2D and (h) 3D height morphology images of BSA loaded RBC after treatment with TX and GA (L-RBC-TX-GA). Column charts of the mean values of (i) heights and (j) diameters of RBC and L-RBC-TX-GA at the edge and in the center. The histograms of Young's modulus and Gaussian fit curves of (k) RBC, (l) L-RBC, (m) L-RBC-TX and (n) L-RBC-TX-GA. The column charts of the (o) mean and (p) major values of Young's modulus of various cells are as indicated. Mean value: the average value. Major value: the most distributed value in statistics.

Figure 5.

Molecule entry rates and enzyme catalytic efficiencies. (a) Intracellular concentrations (C_i) of TB at different times. (b) Kinetics of uptake of TB, where k is the diffusion rate, C_o is the concentration of TB outside the cell. (c) Microscopy images taken in 2x PBS of different cells after incubation with TB for indicated times (d) Intracellular concentrations (C_i) of RhB at different times. (e) Kinetics of uptake of RhB, where k is the diffusion rate, C_o is the concentration of RhB outside the cell. (f) Fluorescence microscopy images of different cells after incubation with RhB for various times taken in 2x PBS. (g) Illustration of the enzyme-catalyzed reaction inside the RBCs. (h) Photographs of ABTS- H_2O_2 reaction system catalyzed by different RBCs. (i) UV-Vis absorption spectra of products of ABTS- H_2O_2 reaction system following catalysis by different RBCs for 10 min. (j) Column chart of relative activities of different RBCs where the activity of lysates of HRP-RBC ($A_{\text{HRP-RBC Lysates}}$) is defined as 1 and $A/A_{\text{HRP-RBC Lysates}}$ indicates the relative activity.

Figure 6.

In vitro detoxification assays. (a) Illustration for UA-HRP cascade reactions to evaluate the UA degradation efficiency. UA reacts with water and oxygen in the presence of the enzyme Ur to produce allantoin and H₂O₂. In the presence of HRP, H₂O₂ can oxidize the substrate ABTS into a color product (ABTS⁺). The absorption changes at 417 nm, Abs (417), can be used to evaluate the degraded UA level. (b, d) photographs and Abs (417) to visualize the UA degradation level catalyzed by Ur-Re-RBC or Ur-RBC (b, c) in PBS or (d, e) in fresh mouse serum. Activity of lysates of Ur-RBC was also taken as control. (f) Rh-catalyzed and Fe³⁺ colorimetric reaction to evaluate the KCN degradation efficiency. Rh can catalyze the KCN to react with S₂O₃²⁻ and produce SCN⁻, which could form a brown complex with Fe³⁺ under acidic condition. The absorption changes at 460 nm, Abs (460), can be used to evaluate the degraded KCN level. (g, i) Photographs and (h, j) Abs (460) to evaluate the KCN degradation level catalyzed by Rh-Re-RBC or Rh-RBC (g, h) in PBS buffer or (i, j) in mouse serum. Activity of lysates of Rh-RBC was also taken as background control.

Figure 7. Pharmacokinetic properties and *in vivo* blood detoxification assays of Ref-RBC bioreactors. (a) Blood survival profiles of Luc loaded Ref-RBC (Luc-Ref-RBC). Free Luc, Luc loaded RBC (Luc-RBC), Luc-RBC-TX and Luc-RBC-GA were used as controls. ID: injected doses. Data are the mean $\pm$ S.E.M., $n = 5$ mice. (b) Column charts and (c) fluorescence images of *in vivo* distribution of BSA-IR-Ref-RBC in various organs. BSA-IR, BSA-IR-RBC, BSA-IR-RBC-TX and BSA-IR-RBC-GA were used as controls. He, heart; Li, liver; Sp, spleen; Lu, lung; Ki, kidney (d) Illustration for *in vivo* blood detoxification in a mouse model system.(e) *In vivo* UA degradation profiles in intoxicated mice by Ur loaded Ref-RBC (Ur-Ref-RBC). Control: Native RBCs without the loaded enzymes. Ur-RBC, Ur-RBC-TX, Ur-RBC-GA were also used as controls. Data are the mean $\pm$ S.E.M., $n = 5$ mice. (f) Photographs of serum UA analyses.

Scheme 1.

Conversion of native RBCs into bioreactors and their application in blood detoxification. (a) Conversion of native RBCs loaded with detoxification enzymes into highly permeable Ref-RBC bioreactors by treatment with TX followed by GA. (b) Endogenous or exogenous toxins in the blood can enter into Ref-RBC and are converted to non-toxic or low-toxic molecules by the detoxification enzymes loaded inside the Ref-RBC.