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Drug delivery by red blood cells: vascular carriers designed by mother nature

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Importance of the field: Vascular delivery of several classes of therapeutic agents may benefit from carriage by red blood cells (RBC), for example, drugs that require delivery into phagocytic cells and those that must act within the vascular lumen. The fact that several protocols of infusion of RBC-encapsulated drugs are now being explored in patients illustrates a high biomedical importance for the field.

Areas covered by this review: Two strategies for RBC drug delivery are discussed: encapsulation into isolated RBC ex vivo followed by infusion in compatible recipients and coupling therapeutics to the surface of RBC. Studies of pharmacokinetics and effects in animal models and in human studies of diverse therapeutic enzymes, antibiotics and other drugs encapsulated in RBC are described and critically analyzed. Coupling to RBC surface of compounds regulating immune response and complement, affinity ligands, polyethylene glycol alleviating immune response to donor RBC and fibrinolytic plasminogen activators are described. Also described is a new, translation-prone approach for RBC drug delivery by injection of therapeutics conjugated with fragments of antibodies providing safe anchoring of cargoes to circulating RBC, without need for ex vivo modification and infusion of RBC.

What the reader will gain: Readers will gain historical perspective, current status, challenges and perspectives of medical applications of RBC for drug delivery.

Take home message: RBC represent naturally designed carriers for intravascular drug delivery, characterized by unique longevity in the bloodstream, biocompatibility and safe physiological mechanisms for metabolism. New approaches for encapsulating drugs into RBC and coupling to RBC surface provide promising avenues for safe and widely useful improvement of drug delivery in the vascular system.

Keywords: carrier, drug delivery, intravascular, red blood cells

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1. Introduction: red blood cells as carriers for drug delivery

The optimization of vascular delivery of drugs is an important biomedical problem. This problem is especially acute in the case of delivery of potent and specific, yet labile and complex biotherapeutic agents, including enzymes, which in most cases require precise localization in the target site. One means to achieve this goal is coupling drugs to carriers, such as synthetic or natural polymer structures of diverse geometries, phospholipid liposomes, albumin, antibodies or other biological molecules [1]. The use of drug carriers promises to enhance the specificity, effectiveness and safety of therapeutic, diagnostic or prophylactic interventions. Functions of drug

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Article highlights.

- RBC is naturally designed as a biocompatible carrier for vascular drug delivery, with prolonged lifetime in circulation and safe mechanisms for eventual elimination, including fixation of complement and uptake by macrophages. Drug encapsulation into RBC and coupling to RBC surface represent two approaches for RBC drug delivery.
- Drugs can be encapsulated into isolated RBC using methods including hypotonic dialysis and resealing. RBC-encapsulated drugs are separated from blood, but can exert their activities in circulation if they or their substrates diffuse through RBC membrane. Unintentional damage to RBC in the course of encapsulation compromises their biocompatibility and negatively impacts drug delivery, except cases when macrophages are the target. Undamaging methods for RBC encapsulation provide clinically testable drug delivery systems that markedly prolong circulation time, bioavailability and effect of cargoes. Delivery of RBC-encapsulated anti-inflammatory drugs into phagocytic cells and enhanced bioavailability of detoxifying enzymes in the bloodstream represent attractive examples of this strategy.
- Drugs can be coupled to the RBC surface using a variety of covalent and non-covalent crosslinkers, as well as anchored onto circulating naive RBC using recombinant fusion proteins with specific affinity to RBC. Animal studies show that surface coupling to RBC can be used for the improvement of antigen delivery, masking of RBC antigens, clearance of pathogens from blood and intravascular delivery of therapeutics that are supposed to act within the vascular lumen. In particular, coupling of fibrinolytic plasminogen activators to RBC provides the basis for a paradigm-shifting prophylactic thrombolysis, an approach that showed very promising results in animal models of peripheral, pulmonary and cerebral thromboses.
- RBC represents a versatile, safe and widely useful carrier for optimization of drug delivery in the vascular system. Several RBC-drug delivery strategies are posed for the translation into industrial development and clinical testing and use.

This box summarizes key points contained in the article.

carriers include: i) providing the optimal drug half-life in circulation and clearance mechanism; ii) restriction of unintended drug uptake by and effects in non-target tissues; iii) targeting to the intended therapeutic site; and iv) providing optimal timing of the action, that is, its timely initiation and termination.

Among other carriers, erythrocytes (red blood cells [RBC], non-nuclear biconcave discs with a diameter of ~ 7 μm, thickness ~ 2 μm and plasma membrane surface area ~ 160 μm^2) represent a potentially attractive and, in some aspects, unique carrier for drug delivery (**Table 1**). One microliter of human blood contains ~ 5 million RBC and the total number of RBC, the most abundant cellular constituent of the blood (> 99%), in the human body approaches 30 trillion. Human erythrocytes normally have a lifespan of 100 – 120 days (of note, mouse RBC lifetime is about a third of human's counterpart), travel ~ 250 km through the cardiovascular system and function as natural carriers for oxygen. RBC lifespan and size markedly exceed those of other drug delivery systems (e.g., < 10 h and < 100 nm for PEG-modified 'stealth' liposomes). Thus, RBC are attractive carriers for intravascular delivery, for example, for prolonging drug circulation and restricting unintended extravasation [2-4].

Injected substances interact with RBC, which partition and metabolize > 50 known drugs (e.g., captopril, sulfanilamide, testosterone, insulin, catecholamines, tacrolimus, antibiotics and other agents) [5]. Therefore, RBC may unintentionally serve as a natural blood compartment participating in biodistribution, pharmacokinetics, slow release, metabolism and action of diverse drugs, including antitumor agents [6] and genetic materials [7]. However, the intentional use of RBC as drug carriers is arguably of even greater interest. This area of research began in the early 1970s, when it was proposed that effects of certain drugs might benefit from encapsulation into autologous or immunologically compatible donor RBC that can be safely injected in the host, thereby improving circulation of a protected drug and its delivery to certain targets in the body [8]. A prolonged lifetime in the circulation, availability, considerable surface and volume (mean corpuscular volume of human and mouse RBC is ~ 90 and ~ 50 μm^3, respectively), high biocompatibility and natural mechanisms for safe elimination of RBC represent attractive features of this tentative drug carrier. Erythrocytes are champions among other drug delivery systems from the standpoint of longevity of circulation, biocompatibility and restriction of unintended permeation of drugs into extravascular compartments. Hydrodynamic forces driving RBC to the main bloodstream in the circulation and endothelial glycocalyx minimize RBC interaction with vascular walls [9]. Normally, RBC do not undergo extravasation from the circulation into tissues except hepatic sinuses and interstitium in the spleenic follicles, that is, open to circulation sites of RBC genesis and elimination, part of the reticuloendothelial system (RES). RES macrophages in the spleen and liver rapidly and effectively take up senescent, damaged and modified RBC by means of phagocytosis, leading to their lysosomal degradation.

RBC transfer many substances in the bloodstream. Oxygen-carrying hemoglobin is the most important cargo among other molecules naturally encapsulated in the interior volume provided by erythrocyte plasma membrane. RBC membrane is supported from within by a complex cytoskeleton comprising a hexagonal lattice of actin-spectrin network interconnected with anchoring integral plasmalemmal proteins by means of numerous structural and connector proteins. Dynamic regulation and remodeling of this cytoskeleton maintained via activity of small GTPases asserts morphological stability, plasticity and deformability of RBC necessary for millions of repeat travels through tight capillaries [10]. As in other cell types, RBC plasma membrane represents an asymmetrical phospholipid-cholesterol-composed bilayer,

Table 1. Comparison of erythrocytes with other drug delivery systems*.

	Size (nm)	Shape	Half-life in blood	Diffusion in tissues
RBC	5000 – 7000	Biconcave disc	10 – 15 days	RES openings only
PEG-liposomes	50 – 500	Spheres	3 – 6 h	Tumors (EPR), endocytosis
Polymersomes	50 – 500	Spheres	10 – 20 h	Tumors (EPR), endocytosis
Filomicelles	40 × 20,000	Filaments	1 – 3 days	Unknown, possibly EPR
Polymer micelles	20 – 300	Spheres	0.1 – 6 h	Tumors (EPR), endocytosis
Proteins and conjugates	5 – 5000	Irregular spheres	10 min – 6 h	Diffusion and endocytosis

*Note that half-life in circulation is shown for mice for the sake of consistency in the comparison (data for humans are not available for many drug delivery systems), whereas in humans RBC half-life is close to 60 days. Highly asymmetrical filomicelles have a diameter of 40 nm and length ranging from 1 to 30 μm. Size, shape and half-life in circulation vary immensely for proteins and protein conjugates. For example, protein molecules such as fibrinolytic plasminogen activators are small (< 5 nm) and have very short half-life in the bloodstream (5 – 20 min), whereas immunoglobulins are slightly larger (10 nm) and circulate for a long time (half-life a few hours in mice and up to a few days in humans). There is no direct relationships between protein size and circulation time, for example, albumin, a molecule of about the same size as plasminogen activators, circulates for a much longer time (half-life of a few hours). Protein conjugates can vary in size from 2 to 3 nm (fusion proteins) to micrometers (multimolecular protein complexes and polyplexes). Proteins and protein conjugates are the least restricted to the bloodstream, which they can leave (i.e., extravasate) by means of diverse pathways including diffusion between endothelial cells and endocytosis via endothelium. Submicrometer liposomes and other carriers can extravasate in pathological tissues owing to the EPR effect, and undergo endocytosis in endothelial cells. RBC carriers are the most restricted to the bloodstream; normally they can extravasate only in openings of the reticuloendothelial system (e.g., hepatic sinuses and spleenic follicles).
EPR: Enhanced permeation and retention.

that is, phosphatidylserine is enriched in the inner leaflet, whereas the outer surface is slightly negatively charged, mostly due to anionic components of the glycocalyx extended from integral and surface glycoproteins associated with RBC plasmalemma playing the role of transporting (i.e., ion exchangers and channels), structural and protective elements. Glycophorin A and band 3 represent two major integral glycoproteins among > 300 proteins found in RBC plasma membrane [11]. Some of these proteins are expressed at relatively low and variable levels throughout the human population, yet exert key functions of protecting RBC from damage and elimination from the bloodstream by the immune system (see below).

Owing to these unique natural transporting features, RBC may be an optimal type of carrier for drugs that either need to be delivered into the RBC-eliminating cells, such as RES macrophages, or are meant to work in the bloodstream (**Figure 1**). In the last three decades, significant efforts have been invested in order to prove the validity of this paradigm and establish clinically applicable strategies for RBC carriage of drugs. RBC have been tested for drug delivery in numerous animal [4,12-14] and human studies [14]. Certain challenges and limitations of using RBC as drug carriers have been identified in these studies. Thus, the use of RBC modified *ex vivo* (e.g., using donor or autologous blood) limits treatment options to hemotransfusion settings, technically challenging for widespread use. Further, unintentional reduction of biocompatibility of modified RBC represents a serious potential problem. However, from the results of recent animal studies, approaches were devised to solve or circumvent these problems and boost confidence that RBC-based drug delivery systems will find medical utility relatively soon. Indeed, several RBC-drug delivery approaches are now undergoing clinical testing and industrial development. This article reviews two strategies for drug delivery by carrier RBC: loading into RBC and coupling to the RBC surface.

2. Vascular delivery of drugs encapsulated into carrier red blood cells

From a grossly oversimplified practical viewpoint, an RBC resembles a durable sac-voyage made of elastic material. This might help explain why the exploration of RBC transporting capacities in the drug delivery field started with drug encapsulation inside RBC ghosts. Since the early 1970s, this area of research has received substantial attention and produced the first clinically tested RBC-based drug delivery systems. More than 200 papers, monographs and proceedings chapters have been published on diverse aspects of drug encapsulation into carrier RBC; readers are referred to the previous inclusive reviews for more complete literature references as well as a more specific description of methods for encapsulation of drugs into carrier RBC [15-17]. Several excellent reviews outlining the ideology, methodology and outcomes of animal and human studies have been published on this subject, including a recent article in this journal by one of the leading experts, Mauro Magnani [16-20]. It would be impossible to cover in sufficient depth all aspects of this strategy in a reasonably concise article; hence, a rather cursory overview is provided of the field with a focus on the most recent results and clinical studies.

2.1 Drug loading into carrier red blood cells and elimination of modified red blood cells

Originally, RBC carriage was proposed to improve delivery of cargoes into target cells [8,21]. The methods of electrical insertion and hypotonic RBC loading followed by resealing provided encapsulation of diverse agents, including

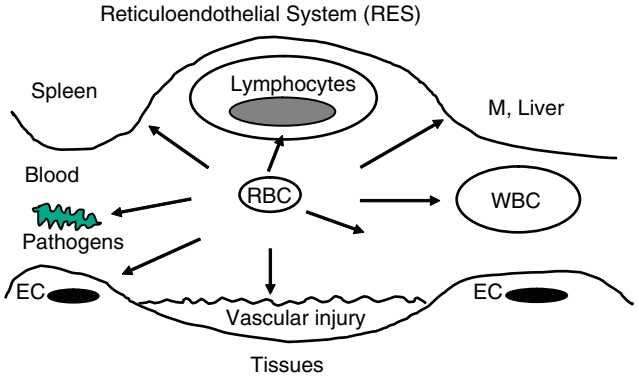

Figure 1. Examples of therapeutic sites and targets accessible for intravascular drug delivery using RBC carriers. Large, long-circulating RBC have an access and can deliver their cargo to targets in blood, including other blood cells (lymphocytes, white blood cells [WBC]), and pathogenic agents such as bacteria and toxins, to sites of vascular injury and endothelial cells lining the vascular lumen (EC), and to diverse components of the reticuloendothelial system (RES), such as macrophages in liver and spleen.

antibiotics, steroids, antimicrobial agents, proteins and genetic materials, into RBC, with loading efficacy ranging from reasonable to fair and to excellent (10 to 30% and to 70%, respectively) of drugs retaining their functional activity [22-24]. *In vitro* studies showed that encapsulated agents can be released from RBC either slowly (e.g., by means of diffusion through RBC plasma membrane [25] and/or its eventual degradation) [13,26] or rapidly (e.g., by means of lysis of carrier RBC by plasma complement) [27,28]. Early studies also showed that RBC carriers facilitated intracellular delivery of encapsulated agents including proteins and DNA into cells in culture [21].

Examples of pharmacological and imaging agents encapsulated into carrier RBC include diverse enzymes [29,30], fluorescent labels [30], erythropoietin for sustained stimulation of hemopoietic potential [31], hemoglobin cofactor inositol hexaphosphate for enhancing RBC oxygen carrying capacity [32,33], antithrombotic drugs heparin [34] and thrombolytics [35], amikacin for antiparasite therapy [36,37], insulin [38], fluoro-AMP [25] and methotrexate for cancer chemotherapy [35], isotopes for imaging of blood pool using gamma-scintigraphy and MRI [39-42], antisense oligonucleotides for gene knockouts [43] and antiviral interventions [44], plasmid DNA for gene therapy [45] and, more recently, magnetic nanoparticles [46].

Organ distribution, pharmacokinetics and effects of some of these drug delivery systems have been tested in studies in lab animal species [32,43] and, in a more fragmentary fashion, in human patients [42,47-49]. Some of these studies provided rather mixed results, perhaps owing to unintended damage inflicted to carrier RBC during drug loading that compromised their biocompatibility, or variability and heterogeneity of RBC loading (noted even within the same batch in the early studies) [29,30]. For example, RBC-encapsulated and free erythropoietin showed similar pharmacological properties in mice and its activity in plasma was essentially gone within < 1 day after injection despite the fact that tracing of ^{51}Cr-label showed relatively prolonged circulation of drug-loaded RBC, with half-life in blood ~ 6 days versus 7 days for intact RBC [50]. However, intravenous injection in mice of antisense oligonucleotides encapsulated into opsonized RBC enhanced hepatic delivery of the cargo [43], whereas injection of RBC-encapsulated plasmid DNA resulted in a relatively prolonged transfection of the transgene in phagocytic cells in the RES and blood [45]. A relatively stable MRI signal was detected in blood samples taken from mice during the week post-intraperitoneal injection of RBC-encapsulated magnetic nanoparticles [46].

RES macrophages and other professional phagocytes eliminating senescent and damaged RBC represent a natural target for drugs encapsulated in RBC. In fact, it is rather difficult to avoid uptake of carrier RBC by phagocytes and divert delivery to other targets of interest. Drug loading into RBC inevitably causes some damage to its membrane and, in some cases, internal content (e.g., depletion of RBC systems for storage and utilization of energy [51] and nitric oxide transport and metabolism [52]). To avoid these complications, major efforts have been dedicated to studies of mechanisms of RBC biocompatibility, its damage on RBC modification, and means for its preservation for drug delivery applications [53-58].

Conformational changes and abnormal clustering of membrane glycoproteins including major components glycophorin A and band 3, caused by crosslinking agents and RBC membrane damage by osmotic stress during drug loading [53], lead to cytoskeletal dysfunctions (loss of RBC plasticity and mechanical stability [55]), sensitize RBC to lysis caused by oxidants [59] and provoke fixation of immunoglobulins that naturally present in plasma [60]. Exposure of phosphatidylserine from the inner leaflet of the RBC plasma membrane [61] and other components normally absent on the RBC surface augments these processes and predisposes RBC to adhesion to endothelium [62]. Unintentional inactivation of specific RBC surface glycoproteins inhibiting complement (e.g., Decay Acceleration Factor [DAF] and CD59) [56]) and masking RBC from recognition by phagocytes (e.g., CD47 [63] and SHPS-1 [64]) compromises RBC biocompatibility further.

These adverse effects reduce RBC plasticity and resistance to osmotic and mechanical damage, lead to RBC opsonization by immunoglobulins and complement promoting phagocytosis and directly destroying RBC, which collectively may lead to RBC lysis, aggregation, immune reactions, cellular uptake, adhesion to vascular endothelium and rapid elimination by means of phagocytosis and entrapment in the microvasculature [30,65-67]. These adverse effects compromise drug delivery and may cause serious damage typical of intravascular hemolysis, including acute vascular, renal and immune reactions to free hemoglobin, hypoxia and toxic effects of RBC stroma

towards RES macrophages [68]. On the other hand, rapid phagocytosis of altered RBC offers a direct and effective way for intracellular delivery, on condition that RBC have not been destroyed by complement *en route* in the circulation.

Major technological advances were made about a decade ago, that is, design of devices allowing semiautomatic high-throughput blood filtration and hypotonic dialysis-based encapsulation into human RBC of drugs, including steroids dexamethasone (DEX) and prednisolone, as well as oligonucleotides and peptides, producing amounts of loaded RBC sufficient for use in human patients [69,70]. Based on these developments, several companies embarked on industrial development of scaled-up production of drug-loaded human RBC and are now pursuing clinical studies. Below, a brief overview is given of a few examples of delivery of specific types of drug encapsulated in carrier RBC in animal and human studies. Most of these strategies pursue drug delivery to phagocytic cells in blood and the RES (first of all hepatic and splenic macrophages), or prolonged circulation of RBC-encapsulated enzymes detoxifying various toxic substances in the bloodstream (**Figure 1**).

2.2 Red blood cell-mediated drug delivery to macrophages and other cell types

As expected, modifications that altered RBC surface antigens and membrane plasticity (e.g., treatment with crosslinking agents) resulted in their rapid phagocytosis by macrophages in the RES [71,72], providing a mechanism to deliver encapsulated drugs into lysosomes in these and other cell types with active internalization processes, including tumor cells (see below) [26,73]. Pilot studies showed that microparticles made from RBC ghosts facilitate delivery of cytotoxic agents to malignant cells [74].

2.2.1 *Enzyme replacement therapies for lysosomal storage diseases*

Genetic deficiency of lysosomal acidic hydrolytic enzymes results in pathological accumulation of their substrates in the cellular vesicles, leading to lysosomal storage disorders [LSD], manifested by inflammation, neurological, vascular, hepatic and pulmonary disorders [75]. Macrophages and other cells with active lysosomal metabolism represent the prime therapeutic target in LSD management. Pending the development of effective and safe gene therapy, the only viable and clinically used option for therapeutic management of LSD is periodic infusion of deficient recombinant enzymes [76], that is, enzyme replacement therapy (ERT) [77]. However, inactivation *en route*, poor pharmacokinetics and uptake by the target cells compromise this approach: only a minute fraction of these protein drugs gets into the lysosomes, hence there is an acute need to improve delivery using drug carriers [78].

Effective uptake and lysosomal addressing of drug-loaded RBC destined for fast phagocytosis due to membrane modification offers a natural mechanism for ERT delivery [4,22]. In fact, initial studies of RBC as carriers for drug delivery were focused on RBC loading with enzymes for ERT. Hypotonic exchange (i.e., reversible mild poring of RBC membrane in an enzyme suspension) was the most widely accepted and effective among methods for encapsulation in RBC enzymes used as ERT for treatment of Gaucher's disease, including galactosidase [8], glucuronidase [79] and glucocerebrosidase [4,22]. Efficacy of enzyme loading was reversely proportional to the molecular mass, yet even 180 kDa proteins have been encapsulated in RBC using hypotonic exchange [8]. RBC-encapsulated enzymes show stable activity [22] and deliver enzyme cargoes into cells *in vitro* [4]. Animal studies revealed relatively more effective lysosomal delivery of RBC-encapsulated versus free enzymes, despite (or, perhaps, rather in agreement with) very limited circulating capacity of the former formulation [79]. Limited clinical studies in the late 1970s showed no overt toxicity of RBC-encapsulated glucocerebosidase in a patient with advanced adult type Gaucher's disease, yet the therapeutic effect was rather modest and transient [80]. More recently, RBC-loaded enzyme replacement therapy using thymidine phosphorylase has been tested in a patient with a genetic mitochondrial deficiency of this enzyme: treatment improved biochemical readouts, but, unfortunately, the patient's clinical condition did not improve [81].

2.2.2 *Anti-infectious drugs, antigens and toxic agents*

Phagocytosis of RBC intentionally modified in the drug loading process to be taken up by macrophages in the RES serves as a natural delivery path for agents that help to eliminate invaders residing in these cells [73,82]. For example, antiviral drug azidothymidine (AZT) was loaded in carrier RBC [83] that delivered cargo to macrophages and provided enhanced antiviral potency versus free drug in cell culture [26,84] and in a mouse model of HIV infection [85]. Encouraging results have been obtained in cells with RBC-mediated delivery of other antiviral agents [86-90], including antisense agents inhibiting HIV-1 replication in infected cells [91]. These studies provide a basis for a wide-ranging antiviral strategy [87,92,93] posed for systematic testing in animal models and human studies. It has to be noted, however, that loading of some antimicrobial drugs into RBC (i.e., antimalarial agent clotrimazole) predisposes RBC to oxidative damage [59], and oxidized RBC are taken up rapidly by hepatic RES macrophages (i.e., Kupffer cells) by means of scavenger receptor-mediated phagocytosis [94].

The antigen-presenting function of macrophages and other immune cells eliminating altered RBC also offers an attractive natural mechanism for delivery of antigens to boost the immune response [95]. In addition to optimized delivery of antigens, carrier RBC plays the role of the adjuvant, by presenting multiple copies of antigens and stimulating nonspecific immune response [96]. Further, encapsulation of cytokines including interleukins and interferon into RBC facilitates delivery of these agents to macrophages [97], resulting in stimulation of the immune response in animal models [98].

Alternatively, RBC encapsulated with toxic agents, such as ricin toxin, kill target cells, first of all, phagocytes [99]. Injection of a toxin-loaded RBC leads to macrophage depletion [100], providing anti-inflammatory effect and alleviation of acute graft injury after transplantation [101]. In theory, delivery of toxic agents by RBC to immune cells may be used for anti-inflammatory interventions, as well as for induction of antigen tolerance.

2.2.3 Anti-inflammatory agents

Delivery of RBC-loaded anti-inflammatory drugs such as glucocorticoids including DEX to pro-inflammatory cells such as macrophages represents a further extension of the previous approach. It promises to optimize bioavailability of these poorly soluble drugs and selectivity of their delivery to active phagocytes in the RES and other components of the immune system. Initial studies *in vitro* developed loading of DEX derivatives (e.g., more soluble DEX-phosphate) into RBC; because this procedure predisposes RBC to opsonization by complement facilitating phagocytosis, RBC-loaded DEX was taken up by cultured macrophages and inhibited their pro-inflammatory response to agonists [70].

In a recent series of studies by Magnani's team, this approach is tested in human patients with several disease conditions associated with and aggravated by inflammation. For example, reinfusion of RBC-loaded DEX in 10 patients suffering from chronic obstructive pulmonary disease (COPD) has apparently been well tolerated and resulted in a sustained elevation of blood DEX level after a single injection [14]. In another lung disease with a major inflammatory component, cystic fibrosis, reinfusions in the patients of autologous RBC-loaded DEX at monthly intervals provided a sustained low level of DEX for 28 days in the bloodstream, were well tolerated and provided significant reduction of inflammatory reactions [102]. A similar approach has been tested in patients with inflammatory bowel disease (IBD, such as Crohn's disease), and in this study three repetitive reinfusions of autologous RBC loaded with DEX (every 4 weeks) also provided a prolonged elevation of DEX blood level, permitting the patients to withdraw previously prescribed poorly tolerated steroids and still achieve remission, which was maintained for several months [103]. Similar encouraging results have been observed in a study involving 18 pediatric patients treated with monthly infusions of autologous RBC-loaded DEX for 2 years [104]. Finally, a very recent randomized controlled study of this treatment in 40 IBD patients refractory to conventional steroids showed that by 8 weeks of treatment 75% of 20 patients who received RBC-loaded DEX were in clinical remission (versus 80% in the group that received prednisolone and 10% in the control group, respectively), but no adverse effects were detected in the RBC-DEX-treated group, in contrast to 80% of patients showing adverse effects of prednisolone [105]. Certainly, the number of patients enrolled in these studies (10 – 15) was very small on the scale of regular clinical trials. However, these encouraging pilot results imply that RBC-mediated delivery of anti-inflammatory agents may find utility in the treatment of acute and chronic inflammation.

2.2.4 Anticancer agents

Loading anticancer drugs into carriers restricts their toxicity to the body and improves their delivery to tumors, and relies on several mechanisms, both specific (e.g., antibody targeting) and less specific (e.g., enhanced permeation and retention effect [EPR], typical of solid tumors). Liposomes, linear polymers and polymer micelles represent the most popular carriers for anticancer drugs. However, RBC carriers may find a niche in tumor treatment, for example, by providing formulations with prolonged circulation. In support of this notion, loading a hydrophobic antitumor agent dequalinium into mouse RBC provided much longer half-life in circulation than PEG-liposomal formulation (5 – 6 days versus 4 h) [106].

Anticancer drugs such as antibiotic doxorubicin have been encapsulated into carrier RBC using diverse loading schemas, including glutaraldehyde crosslinking of RBC membrane [13,107]. This modification greatly enhances RBC uptake by macrophages and other cells exerting active phagocytosis [71]. Accordingly, doxorubicin-loaded RBC delivered the cargo into macrophages in culture [108] and accumulated in the liver (predominantly in macrophages) after intravenous injection in animals, including dog [109].

This intervention provided encouraging efficacy in the treatment of lymphoid tumor in dogs without marked cardiac toxicity (a hallmark adverse effect of doxorubicin), yet inflicted unexpected substantial chronic suppression of myeloid cells [110]. Nevertheless, a formulation of human autologous RBC encapsulated with a related anthracycline antibiotic daunorubicin prepared using a methodology avoiding RBC membrane crosslinking has been injected into patients with acute leukemia and showed a more prolonged drug level in plasma and fewer side effects than after injection of free drug [111]. Similarly, doxorubicin-loaded autologous RBC reinfused in patients with lymphomas provided reduction of peak level and extension of drug level in plasma of patients, resulting in significant elevation of the area under the curve and reduction of side effects compared with free drug [112].

Delivery of drug carriers to solid tumors relies in major part on the EPR effect mediated by abnormally high permeability of tumor vasculature and lack of effective lymphatic drainage. In the context of vascular permeability and tumor extravasation by means of the EPR effect, large RBC represent a less effective delivery platform than submicrometer carriers such as liposomes. To address this challenge, antitumor drugs (daunorubicin) have been conjugated with small vesicles (average diameter 100 nm) formed from RBC plasma membranes [74]. Soon after intravenous administration (< 30 min) these RBC-based nanovesicles are eliminated from the circulation mainly by the RES in liver and spleen [113]. However, injection of this formulation produced more potent antitumor

effects than free drug in mouse models [114], most probably owing to slower drug release in the vicinity of tumor cells [115], as these vesicles apparently do not enter target cells [116].

2.2.5 *Genetic materials*

Finally, RBC ghosts have long been studied as a means for intracellular delivery of genetic materials. Most efforts in this area have been focused on cytoplasmic delivery of small oligonucleotides for interference in protein synthesis. Early works demonstrated proof of principle in cell cultures and provided initial comparison of this and liposomal antisense delivery systems [44]. More recent studies confirmed intracellular delivery of oligonucleotides [70] and other means of genetic interference, such as peptide nucleic acid inactivating viral RNA replication in culture of human macrophages infected with HIV [91]. Several recent studies took this direction in animal studies. Thus, injection of RBC-encapsulated DNA plasmid led to a prolonged (up to 3 days post-injection) expression of a transgene in blood mononuclear leukocytes [45]. Of note, no transgene expression has been reported at this time in organs, including hepatic Kupffer cells, which in most animal studies take the lion's share of injected materials. The nature of targeting to blood leukocytes versus RES macrophages indirectly inferred by this outcome remains enigmatic. By contrast, a very recent study showed that antisense-loaded RBC being opsonized in plasma deliver their cargo to the liver, consistent with known destination of RBC to this organ [43].

2.3 Loading of detoxifying enzymes in RBC

The delivery of RBC-encapsulated drugs discussed above is based on uptake of modified RBC by macrophages, immune and tumor cells. By contrast, in theory, minimally altered RBC loaded with drugs degrading diffusible toxic compounds could circulate for a prolonged time, avoiding rapid uptake by RES, thereby providing sustained antidotes to toxins.

The design of non-traumatic loading schemas and use of extra precautions (e.g., elimination of subpopulation of senescent RBC from the mixture) yielded formulations of drug-loaded resealed RBC showing biocompatibility and pharmacokinetics similar to those of unloaded RBC in animal studies [50,66]. This provided marked prolongation of half-life in the bloodstream of RBC-encapsulated agents versus free counterpart injected by means of the same route, including alcohol oxidase [117], erythropoietin [50], carbonic anhydrase [30] and other agents [30,46]. Isotope-loaded RBC have been used to visualize blood pool imaging by gamma-scintigraphy and MRI [41].

Loading capacity of RBC is limited; hence, highly potent and specific enzymes represent more effective cargo for this approach than non-enzymatic antidotes such as glutathione [118]. More than 10 different detoxifying enzymes have been encapsulated into carrier RBC to test this hypothesis, including urikase to eliminate uric acid [119], thiosulfate-cyanide sulfutransferase (AKA rhodanese) converting cyanides into less toxic thiocyanates [120-122], phosphothioesterase to antagonize organophosphorus compounds including toxin paraoxon [123-125], alcohol oxidase and alcohol dehydrogenase for elimination of methanol and other toxic alcohols [117,126,127], L-asparaginase [48,49,128], adenosine aminase and other enzymes (**Table 2**). Derivatives of some of these enzymes modified with polyethylene glycol (PEG-enzymes, partially masked from the immune system) have also been encapsulated in mouse, human and sheep RBC [129-131].

In most of these studies, encapsulated enzymes retained activity and degraded their substrates *in vitro*. Studies of circulation, biodistribution and *in vivo* stability of enzyme-loaded RBC were rather fragmentary and, in many cases, revealed reduction of circulation time compared with normal RBC [132]. Nevertheless, even this suboptimal approach afforded more prolonged blood level, as well as more potent and sustained detoxifying effects compared with free enzymes, including rhodanese [132] and alcohol oxidase [117]. Infusion of RBC-encapsulated phosphotriesterase protected mice against lethal dose of a toxin paraoxon [125]. In some cases, encapsulation of enzymes with cofactor molecules facilitated substrate influx and enzymatic conversion, for example, the kinetics of RBC-encapsulated rhodanese has been improved by co-loading of organic thiosulfonates [122,133]. Such a binary detoxification system provided significant reduction of cyanide level [132] and protected against lethal dose of cyanide in mice [134], showing promising efficacy in a mouse model of cyanide poisoning [135]. Injection of homologous RBC loaded with PEG-urease/PEG-aldehyde dehydrogenase in sheep provided more prolonged elevation of the enzyme activities in blood than injection of free PEG-modified enzymes (6 versus 2 days, respectively) [136].

Several formulations of RBC-encapsulated detoxifying enzymes have been tested in primates and human patients. In an early study, RBC-encapsulated asparaginase showed more prolonged circulation (in the range of several days) and more prolonged reduction of asparagine plasma level than free enzyme injected in monkeys [128]. In mice, ^{51}Cr-labeled RBC-encapsulated asparaginase circulated as a complex showing the same half-life for RBC carrier and the drug (although it was shorter than the half-life of control RBC) [137]. In humans, pharmacokinetics of RBC-loaded asparaginase showed considerable variability between 13 tested patients, yet provided a significant prolongation of half-life in the plasma versus free enzyme (27 – 29 days versus 8 – 24 h, respectively) and a more profound and prolonged reduction of asparagine level in plasma [48]. Based on this encouraging outcome, Kravtzoff and co-workers studied tolerance of RBC-asparaginase in human patients and did not find any overt harmful effects or antibodies in plasma after a single injection [49]. Separation of marginal subpopulations of drug-loaded RBC (e.g., senescent or unintentionally damaged RBC) using gradient centrifugation helped to minimize dosing variability between and within the same batches [138]. New methods for asparaginase loading into RBC based on enzyme modification by low-molecular-mass protamine have been

Table 2. Examples of therapeutic enzymes encapsulated in carrier RBC.

Enzyme and ref.	Size	Function	Condition to treat	Stage of development
Galactosidase [8]	464 kDa (tetramer)	Degrades galactosides (sugars, lipids, proteins)	LSD	Studies in primates
Glucuronidase [79]	80 kDa	Degrades heparin and mucopolysaccharides	LSD (Gaucher's disease)	Studies in rodents
Glucocerebrosidase [4,22]	60 kDa	Degrades β-glycosides	LSD (Gaucher's disease)	Testing in humans
Thymidine phosphorylase [81]	100 kDa	Converts thymidine and phosphate into thymine and 2-deoxy-α-D-ribose 1-phosphate	Genetic mitochondrial deficiency	Testing in humans
Carbonic anhydrase [30]	30 kDa	Converts CO_2 into bicarbonate	Detoxification of CO_2	Studies *in vitro*
Uricase [119]	33 kDa	Oxidizes uric acid	Uric acid detoxification	Studies in mice
Thiosulfate-cyanide sulfurtransferase [120-122]	37 kDa	Converts cyanides into thiocyanate	Cyanide detoxification	Studies in animals
Phosphothioesterase [123-125]	15 – 35 kDa	Thioester hydrolysis	Detoxification of organophosphorus compounds	Studies in mice
Alcohol oxidase [117,126,127]	140 – 600 kDa	Converts alcohols into aldehydes	Detoxification of alcohols	Studies in mice
Alcohol dehydrogenase	80 kDa	Converts alcohols into ketones and aldehydes	Detoxification of alcohols	Studies in mice
L-asparaginase [48,49,128]	80 kDa	Hydrolyses asparagine into aspartic acid	Eradication of asparagine-dependent tumors	Studies in mice [137], monkeys [128] and humans [48]
Glutamine synthase [140]	Variable	Forms glutamine from glutamate and ammonia	Ammonia detoxification	Animal studies
Adenosine deaminase [143]	47 kDa	Degrades adenosine	Elimination deoxiadenosine that inhibits immune cells in patients with reduced ADA	Human studies [144]

LSD: Lysosomal storage diseases.

proposed recently, yielding formulations with longer half-life in mice than RBC-loaded asparaginase produced by a hypotonic dialysis method and showing promising therapeutic effect in a mouse model of leukemia dependent on exogenous asparagines [139].

Other detoxifying enzymes encapsulated in carrier RBC include glutamine synthase for ammonia detoxification, providing circulation of detectable enzymatic activity for 48 h and reduction of ammonium level by 50% in mouse blood [140]. Alcohol, glutamate and aldehyde dehydrogenases have been encapsulated into mouse RBC with variable yield and high resultant enzymatic activities (e.g., RBC/GDH effectively metabolized high quantities of ammonia) using electroporation [127] and hypotonic dialysis [141]. These loading schemas reduced RBC stability, yet injection of RBC-encapsulated GDH alleviated ammonia toxicity in mice [141]. Isotope tracing of ^{51}Cr-RBC showed that in contrast to control RBC, a major fraction (> 50%) of electroporated RBC with encapsulated dehydrogenases was eliminated from the bloodstream within several hours, yet the kinetics of second phase of RBC/ADH removal was only slightly faster than control RBC ($t_{1/2\beta}$ approached 4.5 versus 5.3 days, respectively) [142].

Native and PEG-modified adenosine deaminase (ADA) has been encapsulated in human RBC to achieve sustainable elimination of non-metabolized deoxiadenosine that accumulates in and inhibits immune cells in the patients with reduced adenosine deaminase level in blood [143]. Human studies showed that PEG-ADA-loaded RBC circulate better than native ADA-loaded RBC, although both formulations have a fairly short half-life (20 versus 12 days, respectively, which still was longer than that of PEG-ADA itself, 3 – 6 days) [47]. Clinical studies performed by this group involve a very limited numbers of patients (1 – 2 per study), yet a recent publication reported a patient who had been treated by RBC-loaded PEG-ADA for correction of a genetic deficiency for 9 years with a total of 225 infusions every 2 – 3 weeks without overt adverse effects and with significant, although transient, improvement

in lymphocyte numbers, sustained level of blood ADA activity and immunoglobulin level, and clinical improvements [144].

3. Coupling therapeutics to the RBC surface

Coupling therapeutics to the surface of carrier RBC represents an alternative to the encapsulation strategies considered above. The RBC membrane provides an extended surface area that may be used for anchoring multiple copies of protein or other therapeutic molecules. Lack of isolation of a drug from blood *en route* to the therapeutic site represents an obvious downside of surface coupling versus encapsulation. This concern, however, does not seem to be acute for drugs that are supposed to work in the bloodstream. Furthermore, using pro-drug formulations resistant to plasma inhibitors holds promise to resolve issues associated with premature inactivation and side effects *en route*. On the other hand, surface coupling strategies avoid damaging encapsulation procedures and therefore offer theoretical advantages of drug loading without compromising RBC biocompatibility. In addition, coupling of drugs to the RBC surface circumvents issues related to drug release (approaches to trigger drug release by using controlled lysis by complement have been suggested, yet practically useful controlled release from carrier RBC remains an elusive goal) [28]. Of note, coupling to the RBC surface resolves diffusional limitations: even enzymes that react with small, membrane-permeable substrate are more active when bound to the RBC surface than when incorporated within the cell [145]. Further, surface coupling offers a unique option to load drugs on circulating RBC without the technically and logistically cumbersome need for their extraction necessary for drug encapsulation and reinfusion.

Generally speaking, techniques for coupling diverse molecules to RBC were designed in the 1950s, in the process of developing reagents for immunological reactions of agglutination. Numerous crosslinking agents and procedures were applied to conjugate proteins and other antigens and biological molecules to RBC of different animal species, including humans. Subsequent studies revealed that these conjugation methods grossly damage RBC membrane, reducing RBC plasticity, resistance to lytic agents and biocompatibility. Nevertheless, reliable methods of biocompatible conjugation molecules to RBC have been designed recently (see below).

A theoretical paper of mid-1990s provided a speculative yet stimulating analysis of hypothetical RBC membrane anchors and methods of coupling of therapeutics using chemical conjugation to tentative affinity ligands and gave a few examples of experimental studies in this area that were available at the time [20]. Several practical strategies for coupling therapeutics to carrier RBC surface have evolved and have been tested *in vitro* and *in vivo* in the last two decades. These strategies can be divided into three wide categories, described in further detail in the following sections: i) chemical coupling of agents to the RBC surface (either covalent or non-covalent); ii) coupling to the RBC membrane of a receptor that binds a therapeutic agent (and, in some cases, augments its functions); and iii) conjugation of therapeutics or their receptors with affinity ligands (e.g., antibodies or their fragments) that bind to RBC thereby anchoring cargoes on RBC (**Figure 2**). This latter strategy permits loading drugs on the RBC surface by injecting these conjugates into the bloodstream. These strategies provide coupling of antibodies, antigens, enzymes, cytokines and other biologically active cargoes to RBC and have been explored for vascular delivery of several classes of therapeutics, including, more recently, model polymer nanocarriers anchored to RBC either nonspecifically [146,147] or by means of affinity peptides [148]. Hereafter, surface coupling to RBC is considered for delivery of the following classes of biotherapeutics: i) antibodies and antigens; ii) affinity ligands for coupling and eliminating circulating pathogens; iii) proteins regulating complement system; and iv) proteins controlling formation and dissolution of blood clots.

3.1 Coupling of antigens and antibodies to the surface of carrier RBC

In theory, RBC carrying antibodies or antigens on their surface can be used to deliver diverse cargoes to intravascular targets [149] or agents modulating immune response [95], respectively. Since the early 1980s, many methods have been tested for coupling protein molecules to RBC for *in vitro* applications, including the use of nonspecific chemical crosslinkers such as tannic acid [150] and chromium chloride [151,152]. These methods have been surpassed by more specific crosslinkers, for example, a biotin–avidin pair offering modular anchoring of diverse biotinylated chemicals including proteins and nucleic acids to defined reactive groups on the RBC membrane, that is, amino acids [153-155], sulfhydryl groups [156], sugars [157] and lipids [158,159]. In particular, controlled biotinylation of RBC lysine residues using NHS esters of biotin has become arguably one of the most popular means for conjugation cargoes to the RBC surface for a wide variety of applications *in vitro* and *in vivo* [69,160-164].

One such application, namely, isolation and tracing of subpopulations of RBC in circulation [165,166] as well as monitoring their survival [167] and volume [168], has found use in animal [169,170] and human studies involving injection of normal, senescent and sickle-cell biotinylated RBC [171,172]. Furthermore, methods for direct biotinylation of RBC in the bloodstream using intravascular injection of biotin esters have been explored (this method probably yielded promiscuous modification of diverse blood cells and endothelium) [170,173]. On a cautionary note, an excessive biotinylation may reduce RBC biocompatibility and alter RBC antigens [164], and repetitive injections of biotinylated RBC elicit an immune response [174]. It was also found in the late 1980s that excessive crosslinking of biotinylated RBC membrane by avidin, streptavidin or neutravidin, all of which have four high-affinity binding sites for biotin and thereby serve for coupling biotinylated molecules to RBC, causes RBC lysis by

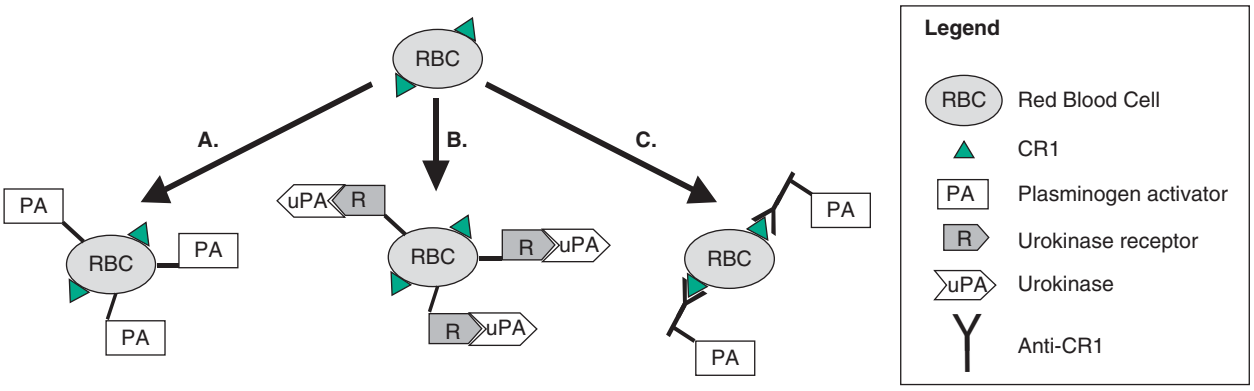

Figure 2. Strategies for coupling therapeutic agents to RBC surface. This schema uses plasminogen activators (PA, see Section 3.5) to illustrate methods for coupling drugs to RBC, yet these principles are applicable to a wide variety of therapeutics. **A.** Direct coupling of drugs (PA) to RBC using covalent or non-covalent crosslinking agents is a prototype paradigm that involves modification of isolated or donor RBC followed by infusion in a patient. This paradigm is especially attractive for pursuing a PEG-stealth RBC approach (Section 3.2). **B.** Non-covalent binding of a drug to its receptor conjugated to RBC may provide a more physiological anchorage for some biotherapeutics and provide an extra modality for functional regulation of their activity (see an example of coupling urokinase uPA to its receptor suPAR coupled to RBC in Section 3.5.3). **C.** Conjugation of a drug with fragments of antibodies binding to RBC avoids *ex vivo* manipulations with RBC, and eases administration and dosing, enhancing clinical feasibility (Sections 3.4 and 3.5.3).

complement activated via the alternative pathway [27,175], leading to intravascular hemolysis and rapid elimination of modified RBC [176]. Studies of the molecular mechanisms of this phenomenon revealed that modification of RBC membrane glycoproteins DAF and CD59 (which suppress complement activation and assembly of membrane-attacking complex in RBC membrane, respectively) is responsible for this unwanted side effect [56,177,178]. This allowed design of non-damaging means for coupling of molecules to RBC by means of biotin–avidin crosslinker without loss of RBC biocompatibility [159,179]; thus RBC carrying up to 10^5 molecules of protein cargo conjugated via streptavidin in a biocompatible manner circulate in animals similarly to naive RBC, without enhanced clearance, lysis or organ uptake [180,181].

Several labs explored the use of RBC coated with antigens and cytokines for stimulation of immune response *in vitro* [182,183] and, on a more limited scale, *in vivo* [184]. Antibody-guided RBC formulations have also been tested for delivery to populations of lymphocytes to achieve selective modulation of immune response [152,185]. Further, model studies demonstrating proof of principle for targeting antibody-carrying RBC to selected sites in the vasculature have also been published [3,149,151]. However, the use of antibody-coated RBC for targeted drug delivery in the vascular system has not been actively pursued in the last decade. The need for *ex vivo* modification of RBC for antibody conjugation and drug loading as well as lack of practically adequate means for controlled release of drugs encapsulated into the carrier RBC represented a daunting technical challenge. In addition, safety issues associated with hemotransfusion inevitably involved in this strategy were viewed unfavorably by the industrial sector in the age of HIV and other blood-transmitted infections. As a result, synthetic carriers including liposomes and polymer particles surpassed drug targeting strategies using antibody-carrying RBC.

3.2 Polyethylene glycol conjugation to red blood cells: an approach to universal donor blood

Conjugation of highly hydrophilic PEG with chain length in the range molecular mass 3 – 10 kDa has evolved since the 1970s as a universal 'stealth' technology, prolonging circulation and masking from defense systems in the body of liposomes, polymer nanocarriers, proteins and other drug carriers and drugs themselves. The goal of producing PEGylated 'stealth' RBC, proposed by Scott and co-workers a decade ago, is to obtain a 'universal' donor blood for hemotransfusion by chemically camouflaging RBC antigens [186-188]. Indeed, PEG-coated RBC are less effectively opsonized, taken up by phagocytes and recognized by antibodies to RBC antigens [189-191]. Masking against immune reaction to mismatched ABO antigens is the most challenging goal; in this setting, PEG-coating did not offer any protection and rather aggravated hemolysis in the initial studies [191]. Further studies using alternative PEG-coupling techniques and combinations of PEG with different chain length produced encouraging results in masking ABO and Rh(D) blood group antigens [192-194]. More recently, even masking of xeno-antigens on RBC has been attempted, yet the outcome was not too encouraging [195].

Covalent coupling of cyanuric chloride-activated PEG derivatives to amino acids is the most popular method for PEG conjugation to RBC [191,196]. PEG coupling using sulfo-group chemistry [192,194], insertion of lipid derivatives of PEG [197] and other methods has also been

developed [186,198,199]. The extent of 'stealth' feature or masking increases proportionally with PEG length in the range 5 – 35 kDa, and with branching and surface density [200]. Studies of RBC electrophoresis showed that PEG conjugation increases apparent length and density of RBC glycocalyx [201] and that long and especially branched PEG polymers work more effectively in this respect [202]. Similar results were obtained when RBC was modified by Pluronic, a tri-block copolymer combining two PEG chains at the ends of a less hydrophilic moiety [203]. Of note, RBC modification using PEG length and surface density that does not mask complement receptor type 1 (CR1) against antibodies profoundly decreased binding of immune complexes to RBC, indicating that even relatively small steric hindrance may obstruct multivalent interactions of immune complexes with CR1 [204]. This observation is important in the context of strategies for CR1-mediated clearance of pathogens discussed below.

PEG modification at the extent that effectively masks RBC from recognition by the immune system does not affect functions of RBC membrane glycoproteins (e.g., ion exchange function of band 3), or RBC stability, plasticity and biocompatibility [190]. Thus, PEG-RBC circulate with half-life similar to that of non-modified RBC in syngenic animals [186,190]. Further fractionation of PEG-coated RBC provides the PEG-RBC subpopulation that is most effectively masked, less damaged and circulates equally to native RBC [205]. Furthermore, PEG-coated RBC are less susceptible to parasite infection, including malaria *Plasmodium* [206], owing to masking their receptors [196]. In addition, PEG-modified RBC demonstrate reduced tendency to aggregation [203,207] and improved rheological properties: reduced low shear flow viscosity [189] and enhanced thickness of membrane protective layer and plasticity necessary to endure hydrodynamic stress in circulation [196,208,209]. In the last decade, PEG-RBC has been an active area of research, promising to alleviate acute problems of shortage of matched donor blood (see reviews by Garratty on this subject [210,211]).

3.3 Elimination of circulating pathogens using red blood cell-coupled antibody heteropolymers

It was found several decades ago that one of the RBC transmembrane glycoproteins, CR1, binds C3b component of activated complement and immune complexes containing this protein and that macrophages in the RES safely detach such immune complexes, including antibody–pathogen complexes from circulating RBC, without damaging the cell [212]. Two decades ago, Taylor *et al.* postulated that this natural function, that is, immune clearance of pathogens from the bloodstream, could be performed as a controlled medical procedure that would not require complement activation, by injecting artificial immune complexes called heteropolymers that consist of a monoclonal antibody to CR1 conjugated with an antibody to a pathogen or toxin [213]. This strategy was emulated later by a strategy for a decoy-type clearance of HIV-1 by RBC carrying electro-inserted CD4 in their plasma membrane that showed initial promise in *in vitro* studies using cell cultures of blood mononuclear cells from HIV-1 patients [214].

In primates and humans, > 90% of CR1 is expressed on RBC at levels of ~ 500 – 1500 copies per cell, providing an anchorage site for CR1 antibodies and compounds conjugated with anti-CR1 (i.e., heteropolymers) injected in blood [215-218]. Loading of such heteropolymers on RBC provided effective binding of model antigens and bacteria *in vitro* [213,219,220] and in non-human primates [221] (note, rodents do not express CR1, hence all *in vivo* studies of CR1-mediated blood clearance were performed in monkeys until recent production of a transgenic mouse expressing human CR1 [222]). CR1 represents an immunologically privileged site for antibody binding to RBC: isotope tracing and fluorescent imaging studies revealed that binding of neither anti-CR1 nor anti-CR1 conjugates targets carrier RBC for phagocytosis [223], yet phagocytes recognize pathogens and immune complexes bound to CR1 by means of either C3b or anti-CR1 [224], cleave off extracellular domain of CR1 [225] and take up the pathological 'cargo' from the RBC surface [226-228]. Studies in primates demonstrate that diverse ligands can be coupled to circulating RBC using anti-CR1 conjugates and eliminated by phagocytes without damage or shortening RBC survival [213,215,229,230].

This concept has been tested in primates and showed promising effectiveness of elimination of diverse bacterial [231] and viral [232-235] pathogens using CR1 heteropolymers targeted to these pathogens, thereby alleviating the infection [235,236]. Further, coupling to RBC of anti-CR1 conjugated with DNA, a common antigen for autoantibodies in lupus, afforded clearance of such autoantibodies in monkeys [230,237,238]; the same principle of CR1-targeted heteropolymers has also been explored for elimination of cytokines as anti-inflammatory intervention [239], indicating potentially wide biomedical application of this concept [215].

3.4 Coupling inhibitors of complement to red blood cells and transfer of red blood cell-anchored proteins to endothelium

Complement system, consisting of > 20 regulatory and executing proteins in blood plasma and cellular membranes, exerts key functions of innate immunity, including opsonization and destruction of pathogens, tumor and foreign cells, and activation of pro-inflammatory cascades in the sites of invasion. However, in some pathological conditions, host cells suffer injury inflicted by overzealous or misguided activation of complement. Vascular endothelium and RBC represent the most common and vulnerable target for adverse effects of autologous complement activation, especially in situations of functional deficiency of membrane glycoproteins inhibiting complement: DAF (CD55 or DAF), CD59 and CR1 (CD35, represented in mice by an analogue called Crry). DAF and CR1 inhibit early stages of complement activation, thereby protecting cells from lysis and reducing

the generation of pro-inflammatory mediators, whereas CD59 blocks the formation of hemolytic pores in the plasmalemma. Complement-mediated hemolysis of RBC deficient in DAF and CD59 is involved in the mechanism of paroxysmal nocturnal hemoglobinuria (PNH), whereas endothelial damage by complement is involved in acute vascular injury in the ischemia-reperfusion syndrome, such as in organ transplantation.

Several groups pursued targeting complement inhibitors to RBC, to protect them from hemolysis and inhibit complement activation. Of note, natural DAF and CD59 are anchored on the luminal surface of RBC membrane by means of a lipid anchor, glycosylphosphatidylinositol (GPI). Accordingly, experimental methods have been designed for the insertion of DAF, CD59 and other GPI-linked proteins into RBC plasmalemma using the lipid anchor [240,241]. Insertion of lipid-anchored CD59 in RBC plasmalemma protects against complement-mediated hemolysis [242], providing a proof of principle for a new approach for PNH therapy.

The plasma membrane domains that harbor artificially inserted GPI-linked proteins have not been fully elucidated [243], yet they differ from cholesterol-rich domains that tightly retain natural GPI-anchored proteins [244]. As a result, GPI-anchored proteins artificially inserted in membrane ('painted') release more easily from RBC [241]. Perhaps this is one of the reasons why RBC transfer artificially 'painted' GPI-linked proteins to other cells *in vitro* [245] and *in vivo*, where endothelium represents the preferential acceptor of GPI-inserted proteins from RBC [246]. As a result, injection of RBC carrying GPI-linked DAF and CD59 artificially inserted using gene therapy means alleviates acute ischemia-reperfusion injury in a swine-to-primate model of xenograft transplantation [247].

Note, chemical conjugation to RBC plasmalemma is undesirable for coupling of complement inhibitors because this approach would make coupling irreversible, which is not desirable for protein transfer from RBC to endothelium. In addition, it would require RBC isolation, modification and reinfusion, which inevitably reduce the practical utility of the approach and RBC resistance to complement. Conjugation with antibodies also represents a suboptimal means for binding complement-regulating proteins to RBC, as crosslinking RBC membrane by multivalent conjugates can damage RBC. From the standpoint of safety, as well as industrial and clinical translation, small monovalent antigen-binding fragments of antibodies, that is, recombinant single chain variable fragments (scFv), represent the preferable targeting moiety. Using modular gene engineering biotechnology methods, these recombinant proteins can be fused by means of short connecting peptides with a variety of executing proteins. Thus, recombinant scFv fusions targeted to RBC hold the promise of providing a series clinically useful for loading biotherapeutics onto circulating RBC without the need for RBC isolation, modification and hemotransfusion.

Expression of scFv in diverse vectors enables large-scale, GMP-quality production of homogeneous monovalent scFv fusion proteins [248,249]. Advantages of scFv include: i) lack of Fc-mediated side effects; ii) lack of crosslinking of anchoring sites on RBC and RBC aggregation; iii) owing to its smaller size (~ 50 kDa), scFv/PA can be injected intramuscularly, as other recombinant protein therapies (e.g., insulin); iv) established techniques for humanization and reduction of immunogenicity of scFv further help to minimize the likelihood of eliciting immune reactions; and v) modular recombinant format of scFv fusion supports synthesis of diverse fusions [250].

For example, a recombinant fusion protein combining scFv directed to Rh(D) blood group antigen with human CR1 binds to CR1-deficient RBC and restores RBC ability to bind immune complexes [251]. This *in vitro* study implies that this approach may eventually be used either to replenish CR1 functions (i.e., immune clearance and complement inhibition) in CR1-deficient patients (~ 10% of humans are CR1-negative), or to boost CR1 immune clearance in a manner performed by heteropolymers described in the previous section. Recently, Spitzer and co-workers produced a scFv of a monoclonal antibody TER-119 recognizing a mouse analogue of human glycophorin A [252] (GPA), fused it with DAF [253] and Crry [254], and demonstrated that this monovalent fusion construct binds to RBC after intravascular injection in mice without damaging RBC and, furthermore, enhances RBC resistance to lysis by complement *in vivo* [253,254]. Furthermore, recently this team performed a neonatal *in vivo* gene transfer of TER-119 scFv/Crry in mice using retroviral gene transfer vector and demonstrated that prolonged synthesis of this fusion protein in mice leading to its sustained coupling to circulating RBC restores protection against excessive complement lysis in genetically deficient mice [255]. These studies, showing proof of principle for *in vivo* delivery to RBC of therapeutic fusion proteins (injected or encoded by gene therapy means), open exciting applications, some of which are discussed in the next section.

3.5 RBC carriage of agents regulating formation and dissolution of blood clots

An idea of intravascular delivery of agents controlling blood clotting and clot dissolution using carrier RBC was proposed a quarter of century ago, as illustrated by an early work reporting conjugation of fibrinolytic drug streptokinase to RBC targeted to collagen, a thrombogenic component of extracellular matrix that is exposed to blood in sites of vascular injury [3]. In theory, coupling drugs to RBC surface may favorably alter their pharmacokinetics (i.e., prolong lifetime in circulation) and optimize interaction with components of blood coagulation and fibrinolytic systems accessible from the RBC surface. Attempts in this direction included conjugation of heparin to RBC to enhance potency of anticoagulant thromboprophylaxis in patients predisposed

to thrombosis [256] and conjugation of pro-thrombotic RGD-containing peptide to RBC to design a substitute for platelet infusion in patients predisposed to hemorrhagic disorders. [27]

RBC carrying conjugated drugs reported in these studies exerted good functional activities *in vitro* [2,3,256]. However, until recently no attempts to test these drug delivery systems in animal models had been reported, presumably because conjugation of drugs grossly compromised RBC biocompatibility. In particular, inactivation of complement inhibitors DAF and CD59 in RBC membrane [257] led to hemolysis, phagocytosis and rapid elimination of RBC [27,175-178,258]. Eventually, methods to couple up to ~ 10^5 copies of therapeutic proteins per RBC without RBC damage were devised [150,159,163,179,180]. *In vivo* studies using radioisotope tracing showed that ^{51}Cr-RBC/^{125}I-drug complexes circulate similarly to control RBC for several days after injection in rats [259] and mice [180,181]. This success brought back on the agenda attempts to couple fibrinolytic agents to RBC and test the benefit/risk ratio of this approach in animal models of thrombosis.

3.5.1 Need for a safer and more effective management of thrombosis

Sealing of damaged blood vessels by mural clots prevents bleeding, whereas pathological vessel occlusion by intravascular clots (thrombosis) causes tissue ischemia and damage, leading to acute myocardial infarction (AMI), ischemic stroke, pulmonary embolism and peripheral vascular disease, among other complications. Thrombosis is the leading cause of mortality and disability in the US [260,261]. Thrombi are prone to recur within hours to days after an AMI or stroke and the risk is great after transient ischemic attack or pulmonary embolism and in immobilized patients [262-265]. Thrombosis and embolism are also common and dangerous complications of surgery that are especially difficult to manage owing to the risk of acute bleeding at the operative site. Invasive interventions (e.g., angioplasty, carotid endarterectomy) may be complicated by the formation of clots that embolize to the brain and cause neurological disorders. Thus, situations in which patients are at highest short-term risk for occurrence or recurrence of thrombosis are known. However, antiplatelet and anticoagulant agents provide limited protection and pose considerable risk of bleeding [266-269], especially soon after the surgery; in addition, some anticoagulants (e.g., Warfarin) require many hours to develop an effect, which is inadequate for acute short-term thromboprophylaxis.

Emergency therapy of thrombosis uses injection of plasminogen activators including tissue type (tPA) and urokinase (uPA), middle-size proteases (molecular mass 30 – 60 kDa) that generate plasmin, which cleave fibrin clots and thus restore perfusion [249,270]. However, inadequate delivery (blood clearance within < 15 min [271]), inactivation by plasma inhibitors such as PAI-1 [272] and impermeability of occlusive clots [273] restrict the effectiveness of therapeutic fibrinolysis by PA. Minutes after infusion of mega-doses of fibrinolytics (e.g., ~ 100 mg of tPA) needed to overcome its inefficiency and achieve fibrinolysis locally, excess drug diffuses into pre-existing hemostatic mural clots predisposed to bleeding and into tissues such as the CNS [274], where it may cause cerebral hemorrhage, damage to the blood–brain barrier (BBB) and toxicity within the brain [275]. Owing to the danger of bleeding and the collateral CNS damage, fibrinolytics cannot be used in > 95% of stroke patients and in the postoperative period other than as an emergent potentially life-saving intervention. A more ideal thromboprophylactic agent would prevent occlusive thrombi from forming without lysing hemostatic mural (e.g., postsurgical) clots and causing extravascular toxicity.

Attempts to improve tPA delivery and its benefit/risk ratio have not yielded decisively better outcomes [276-279]. Ironically, the higher affinity of newly developed PA variants for clots promotes drug retention on the clot surface, impeding permeation into the occluding thrombi. The only current use of tPA, that is, post-thrombosis, is also marred by inevitable delays (time needed for diagnosis, injection and the lysis proper, slowed by clot impermeability), increasing the risk of ischemia-reperfusion (I/R) injury, which worsens outcome. Prophylactic delivery of a fibrinolytic into the interior of growing, early nascent thrombi will arrest clot propagation and cause rapid clot lysis. Lysis of clots from within will be more homogeneous and complete than 'therapeutic' external fibrinolysis from the clot surface. This would minimize the incidence of the secondary embolism and rethrombosis. Prophylactic use of tPA in patients at high risk of imminent primary or recurrent thrombosis will lessen the formation of impermeable occlusive clots resulting from delays in fibrinolysis. However, fibrinolytics are not used for prophylaxis because of their rapid clearance and side effects. Newly designed mutant plasminogen activators with enhanced potency and fewer side effects may improve therapy [279-284], but their diffusion into occluding clots has yet to be tested and no fibrinolytic has been designed for prophylactic use. All existing plasminogen activators are short-lived (< 30 min, insufficient for prophylaxis) and small (< 10 nm) agents, capable of diffusing into hemostatic clots (bleeding) and into the CNS (collateral damage). None is suited for use as prophylaxis in postsurgical or other patients at imminent risk of thrombosis.

3.5.2 Coupling plasminogen activators to red blood cells: a new strategy for short-term thromboprophylaxis

Recent animal studies showed that biocompatible coupling to RBC converts plasminogen activators from problematic therapeutic agents into effective and safe agents for intravascular thromboprophylaxis. Hemodynamic factors are favorable, propelling RBC towards the blood mainstream [285-287],

restricting drug contact with vascular walls and mural clots. Infusion of RBC/tPA complex in rats and mice:

1. does not cause complement activation, hemolysis, phagocytosis or accelerated clearance of RBC carrying up to 10^5 tPA molecules stably coupled per RBC [288];
2. blocks tPA diffusion into hemostatic clots [288] – in fact, RBC/tPA does not affect normal hemostasis or cause bleeding from fresh postsurgical hemostatic clots susceptible to free tPA [289];
3. RBC glycocalyx protects tPA from plasma inhibitors [290] while preserving its fibrin affinity [291] and activation by fibrin [291], which facilitates dissolution of clots.
4. prolongs tPA circulation by many orders of magnitude [291], permitting its use as prophylaxis [288] – in rats and mice < 20% of RBC/tPA is cleared within 3 h, which projects to a half-life of ~ 2 – 3 days; the half-life of RBC is ~ 3 – 4 weeks in mice versus 3 – 4 months in humans, thus the expected prophylactic window of RBC/PA in humans may vary from hours to many days and weeks depending on dose.
5. delivers tPA into the interior of intravascular venous and arterial nascent clots, which are then lysed from within during clot extension in settings where even a 10-fold higher dose of soluble tPA is ineffective [288], which is Trojan Horse lysis [291,292].

Furthermore, most recent animal studies showed that RBC/tPA provides effective and safe thromboprophylaxis in the most challenging and dangerous vascular bed, that is, cerebral circulation, thereby preventing deleterious consequences of brain ischemia and stroke in mice [293]. This study showed that prophylactic RBC/tPA injection in mice provides dissolution of subsequently formed occlusive cerebrovascular thrombi, leading to rapid and stable reperfusion, marked alleviation of ischemic brain injury and improved survival, not attainable by even 10-fold higher doses of soluble tPA (which in fact greatly enhanced mortality). In contrast to free tPA, RBC/tPA did not aggravate brain edema, injury and hemorrhage in mice with cerebral ischemia and a rat model of post-thrombotic intracranial hemorrhage. Moreover, either prophylactic or post-insult injection of RBC/tPA attenuated disorders of vascular regulation and CNS tissue injury in the brain of pigs subjected to global ischemia/hypoxia, whereas free tPA grossly aggravated these pathological changes [294].

3.5.3 *Advanced approaches for coupling plasminogen activators with red blood cells*

Encouraging outcomes of animal studies using injection of RBC/tPA complexes preformed *ex vivo* using chemical crosslinking paved the way for the most recent attempts to advance coupling techniques. Thus, in addition to coupling to RBC via streptavidin crosslinker used as a prototype method in all previous studies of RBC/tPA, urokinase has been coupled to RBC via urokinase receptor chemically conjugated to RBC without loss of biocompatibility [295]. This approach reduced undesirable interactions of urokinase with its vascular receptors and allowed trigger fibrinolysis, because interaction with RBC-coupled receptor activated urokinase [295].

Preformed RBC/tPA conjugates have properties suitable for prophylactic use in settings where transfusion is common, but would be less practical in other settings and challenging to develop commercially. To avoid the need to couple tPA to isolated RBC *ex vivo* followed by transfusion, ways to target tPA to circulating RBC directly have also been designed. **Figure 3** illustrates this strategy for coupling plasminogen activators to carrier RBC.

The first approach capitalized on CR1 anchoring strategy, as agents conjugated with monoclonal antibodies to CR1 [296] bind to circulating RBC after intravenous injection in animals including primates, circulate with RBC, and bind ligands *in vivo* without RBC damage [213,221,297]. Animal studies showed that tPA conjugated to a CR1 monoclonal antibody binds without harm to circulating RBC in mice and affords safe and effective prophylactic thrombolysis [289] comparable to that provided by infusion of RBC/tPA [288]. To alleviate challenges associated with translation and application of chemically produced antibody/tPA conjugates and achieve predictable loading of RBC over a wide range of drug concentrations, a new recombinant tPA mutant fused to an antigen-binding scFv of TER-119 monoclonal antibody to glycophorin A (anti-GPA scFv) [298,299] was designed very recently; pilot animal studies showed that this approach allows to load up to 20,000 copies of anti-GPA scFv/tPA fusion per RBC after a single intravenous injection of the fusion without detectable changes in behavior of circulating RBC. On an encouraging note, injection of scFv/tPA in mice provided swift dissolution of subsequently formed intravascular clots, which were impervious to injections of equimolar doses of soluble tPA [300].

4. Expert opinion

Erythrocytes represent a unique and promising, yet rather underdeveloped platform for drug delivery. After an initial burst of interest and research effort three decades ago, drug delivery by RBC was overshadowed by artificial carriers offering a wider range of applications, as well as better control over formulation, storage and utilization of the drug delivery system than original RBC-based concepts. Thus, RBC is a good carrier for drug delivery to intravascular and RES targets, but many other important therapeutic targets (e.g., solid tumors, extravascular tissue components, CNS) are normally inaccessible to RBC. As the drug delivery field originated and developed predominantly in the context of targeted delivery of toxic anticancer drugs, such limitation of the spectrum of suitable targets RBC negatively impacted the development of this drug delivery platform.

Studies of RBC drug delivery logically started with *in vitro* experimentation using isolated RBC and within two decades

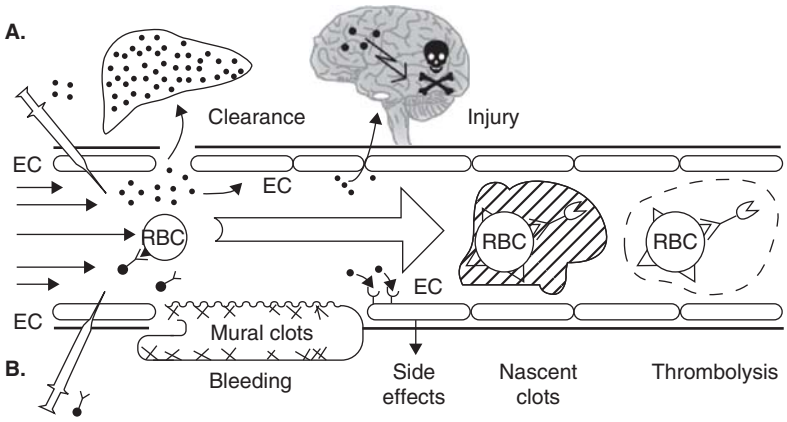

Figure 3. A concept of prophylactic fibrinolysis by scFv/tPA targeting to RBC. A. Plasminogen activators (dots) are relatively ineffective, in part owing to rapid uptake by liver, and unsafe owing to bleeding (indiscriminate lysis of hemostatic mural clots), vascular side effects (e.g., activation of receptors on endothelial cells [EC]) and injurious effects of tPA diffusing into the CNS. **B.** Coupling to RBC will dramatically prolong the longevity of scFv/tPA variant. RBC will restrain scFv/tPA binding to cellular receptors, and restrict its access into mural hemostatic clots and the CNS. Propulsion of RBC towards the mainstream will further offset interactions of the pro-drug with hemostatic clots and vascular walls. RBC-bound scFv/tPA will have virtually unlimited access to the interior of nascent pathological thrombi and thereby will dissolve pathological intravascular clots and prevent vascular occlusion.

progressed to extensive animal studies, a necessary step for validation of the viability, efficacy and safety of this drug delivery system, including immune response to RBC-coupled proteins in settings similar to immunization procedures. Of course, certain specific aspects of the RBC drug delivery (e.g., immune response, subtle effects in the CNS and tolerability of RBC/drug complexes by specific patient populations) can be addressed only in clinical studies. The fact that several clinical trials of RBC drug delivery systems have recently been initiated instills hope that these translational barriers will eventually be overcome.

Among potentially useful biomedical applications, RBC-mediated intracellular delivery of drugs including replacement therapies, imaging probes and toxic agents into RES macrophages and other cells exerting robust endocytic uptake seems to be a reasonably promising goal. Delivery of antimicrobial, immunogenic or toxic agents into defense cells can be used for eradication of intracellular parasites, stimulation or suppression of immune reactions, respectively. It remains to be understood better, however, which particular advantages are offered by RBC in this context versus other drug delivery systems, all of which are effectively taken up by RES macrophages and other defense cells (it is more difficult to avoid addressing this). Further, to translate this concept into the clinical domain, mechanisms of action of RBC-loaded drugs must be more fully understood and optimized. The pharmacokinetics, stability and rate of release of drugs in plasma have to be systematically characterized and effects on diverse populations of cells in the body defined. This drug delivery system is also a bit promiscuous in terms of selectivity of targeting: many cell types in the body can take up RBC and their remnants. For example, phagocytes, immune and tumor cells all may be targets of antibiotic-loaded RBC. It is conceivable that more precise targeting towards some of these populations may greatly optimize therapeutic effect. It would be undesirable, however, if efforts in this direction were to provide formulations that were very complex, and difficult to produce and utilize in medical practice (to be fair, this concern is generally applicable to most previous and current drug delivery carriers).

Strategies using RBC carriage to augment systemic effects of drugs in circulation such as sustained effects of detoxifying enzymes require no targeting and represent one of the unique areas of RBC drug delivery that offers clear potential advantages over other carriers, owing to unique longevity of RBC. Maintenance of maximal biocompatibility of loaded RBC, yield of enzyme loading and kinetics of transmembrane transport of their substrates and products, as well as controlled release and metabolism of encapsulated cargoes, represent challenging, yet theoretically surmountable aspects of this approach.

The need for *ex vivo* manipulations with RBC, a relatively limited shelf-life and concerns related to the safety of donor matching and blood-borne infections are recognized potential downsides of carriage drugs attained by *ex vivo* RBC loading and reinfusion. These concerns are shared by all types of hemotransfusion, cell replacement and stem cell therapies. Note that transfusion of blood and blood products is a very widely used and generally safe therapeutic intervention worldwide, the safest and most effective type of cell transplantation strategy. Use of autologous blood (reinfusion) minimizes the safety concerns.

Drug delivery systems targeted to anchor molecules on the RBC surface (e.g., scFv-fusion proteins) and therefore

allowing safe and technically simple loading of circulating RBC hold a promise to solve completely this issue and greatly expand general applicability of RBC carriers in medicine. In particular, the design of intravascular drug delivery platforms using coupling to circulating RBC agents that control the immune system, eliminate pathogens and toxins from the bloodstream and correct pathological aspects of thrombosis and hemostasis seem most exciting and unique utilities of RBC drug delivery, promising timely translation into industrial development and clinical practice.

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Declaration of interest

The author states no conflicts of interest and has received no payment in the preparation of this manuscript.

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