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Erythrocytes as carriers for drug delivery in blood transfusion and beyond

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Abstract

Red blood cells (RBCs) are innate carriers that can also be engineered to improve the pharmacokinetics and pharmacodynamics of many drugs, particularly bio-therapeutics. Successful loading of drugs, both internally and on the external surface of RBCs, has been demonstrated for many drugs including anti-inflammatory, anti-microbial, and anti-thrombotic agents. Methods for internal loading of drugs within RBCs are now entering clinical use. While internal loading can result in membrane disruption that may compromise biocompatibility, surface loading using either affinity or chemical ligands offers a diverse set of approaches for the production of RBC drug carriers. A wide range of surface determinants is potentially available for this approach, although there remains a need to characterize the effects of coupling agents to these surface proteins. Somewhat surprisingly, recent data also suggest that red cell mediated delivery may confer tolerogenic immune effects. Questions remaining before widespread application of these technologies include determining the optimal loading protocol, source of RBCs, and production logistics, as well as addressing regulatory hurdles. RBC drug carriers, after many decades of progress, are now poised to enter the clinic and broaden the potential application of RBCs in blood transfusion.

Keywords

red blood cells; drug delivery; pharmacokinetics; biocompatibility; immunogenicity

Introduction

Inadequate pharmacokinetics and bio-distribution hinder the delivery and efficacy of many drugs. Conjugation with polymers (e.g., polyethylene glycol, PEG)[1], loading within synthetic carriers (liposomes, polymers, nanoparticles, etc)[2], coupling with natural carrier

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molecules[3], and other approaches have been devised to reduce elimination and optimize delivery of chemical drugs, biotherapeutics, and imaging probes. In addition to these "artificial" delivery systems, blood elements (red blood cells[4], white blood cells[5], platelets[6]) have special biological features that might enable them to serve as "smart" natural carriers for vascular drug delivery.

Red blood cells (RBCs) are biocompatible carriers that circulate for three months, encounter diverse intravascular cells and select tissues, and exert numerous vital functions[4, 7, 8], including important roles in homeostasis. The collective surface of the plasma membrane of RBCs is the most extended cellular surface accessible to blood (Figure 1) which provides a dynamic, multifunctional interface that regulates cascades of coagulation[9], and complement[10, 11], among others. RBCs also participate in host defense via carriage of antigens to immune cells and clearance of pathogens from the blood.

RBC-carriage of drugs often greatly prolongs their circulation and can act upon a variety of vascular targets to correct hemostasis[12], eliminate pathogens and microorganisms[13-15], mitigate toxins[16], and clear other pathological mediators[17, 18] and malignant cells[19]. Some extravascular sites including hepatic sinuses and splenic follicles are also normally accessible to RBCs. RBCs can also be exploited to deliver drugs to extravascular targets that become accessible in the context of the pathological process, for example at sites of vascular damage and bleeding.

Recent findings imply that RBC can serve as "super-carriers" for synthetic nanocarriers of drugs and imaging probes by altering their pharmacokinetics through prolonging their circulation, or engineering their bio-distribution via transfer from the surface of carrier RBC to the pulmonary endothelium[20] and other cell surfaces. These features of RBC carriage provide an impetus for understanding mechanisms underlying redistribution of agents coupled initially to their cell membranes[21]. In addition to altering the pharmacokinetics and biodistribution of a drug cargo, RBC carriage may also alter its functionality. For example, the RBC glycocalyx may protect attached biotherapeutics from plasma inhibitors and mask attached synthetic nanocarriers from clearance mechanisms[22].

Pioneering studies in the nineteen-seventies first explored the idea of RBC drug delivery[23]. However, in the subsequent decades, few groups persistently worked in this area; RBC delivery was generally viewed pessimistically in a drug delivery community focused on artificial carriers. Nonetheless, efforts to understand and utilize RBC drug delivery have evolved into a flourishing direction of biomedical research. Currently, a growing number of academic and industrial groups are actively pursuing use of RBCs for vascular drug delivery, both experimentally and translationally[24].

The RBC drug delivery literature has grown extensively in the last decade, and a number of reviews on the subject has been recently published[4, 7, 24-26]. Here, we focus on aspects of RBC drug delivery most pertinent in the context of transfusion: drug loading, manufacture and delivery regimens, immunological aspects of RBC drug delivery, and the effect of cargoes on the RBC itself.

Drug loading of carrier RBCs

Drugs can either be encapsulated within RBCs or coupled to their surface. Both approaches have been studied for several decades and evolved from early experimental, but clinically unsuitable prototypes, into translationally feasible protocols that yield clinically useful drug carriers. These approaches (Figure 2) are summarized herein, and sources and methods of loading are summarized in Tables 1 and 2, respectively.

Encapsulation of biological and chemical agents into RBCs has been explored for almost half a century[27]. Agents tested for encapsulation in RBC include enzyme replacement therapies[23, 28], antimicrobial and anti-viral agents[29], antigens to modulate immune response[30, 31], and anti-inflammatory drugs[32, 33]. The activity of drugs encapsulated within RBC can be altered by binding to hemoglobin, oxidation catalyzed by iron, interaction with NO-donors, or other catalytic reactions. Enzymes encapsulated inside carrier RBCs can be used to detoxify or neutralize substrates that diffuse through the cell membrane[34]. Multiple studies using a drug encapsulation approach have affirmed the feasibility and promise of infusing drug-loaded RBCs[25, 26, 32, 35-42].

Loss of RBC biocompatibility is a challenging aspect of drug encapsulation[43, 44]. Protocols based on transient osmotic shock and membrane resealing can provide clinically acceptable biocompatible encapsulation of drugs partially freed of hemoglobin [23, 29, 45, 46]. RBCs loaded with small molecules and therapeutic proteins, such as encapsulated dexamethasone and enzymes, have been successfully tested in animal models[46, 47] and in clinical trials[28, 48-51].

Methods to couple therapeutics to the RBC surface have also been explored, including covalent conjugation via amino acids[52, 53], thiol groups[54, 55], sugars[56] and lipids[19, 57]. Examples of RBC surface loading include coupling of antigens and cytokines to stimulate the immune response[58] and antibodies that target RBC-loaded cargoes to their therapeutic sites[55, 59, 60]. Specific examples involving conjugating anti-thrombotic agents, ligands to capture pathological agents and inhibitors of complement are described below. Recently, insertion of lipid-modified biomolecules into the RBC membrane has been used for targeting to phagocytes[19].

As in the case of encapsulation, early methods of surface loading compromised the biocompatibility of RBCs, provoking lysis and elimination via activation of complement and other pathways[17, 61, 62]. Development of methods for RBC surface loading that avoided these adverse effects allowed effective surface loading of RBC and permitted injection in animals without overt shortening of their circulation or other toxicities[60, 63, 64].

Surface loading on RBCs is well suited to deliver agents that modulate hemostasis, including ligands of fibrin[12], heparin[65], and fibrinolytics[66]. For example, RBC carriage prolongs circulation of plasminogen activators (PAs) by orders of magnitude[67], restricts their diffusion into the CNS and pre-existing hemostatic clots, abrogates undesirable interactions with vascular cells[68], and delivers drug to the interior of nascent thrombi[69]. These features accelerate thrombus dissolution and enable thromboprophylaxis in settings where the risk of thrombosis and bleeding are both high[70]. In addition to improving the

pharmacokinetics, RBC carriage favorably alters several important properties of PAs: i) switches them from activating pro-inflammatory receptors in the CNS parenchyma to protective signaling via intravascular receptors; ii) accelerates dissolution of cerebral thrombi in animals providing reperfusion, protection of brain tissue and improved survival; iii) attenuates brain damage in rats with intracranial hemorrhage and blunt trauma; and iv) protects brain function in pigs with cerebral thrombosis[68, 71-75]. In stark contrast, unconjugated PAs cause CNS bleeding, neurotoxicity, and lethality in these settings.

Targeting drugs to RBCs

Drug encapsulation within allogeneic or autologous RBCs and conjugation to the RBC surface typically require multi-step manipulations involving cell isolation, numerous incubations, washings, and repacking. However, they are technically challenging procedures that may limit translational prospects. Further, multistep extracorporeal drug loading hinders the utility of RBC drug delivery in some relevant clinical settings, such as emergency medicine.

This problem might be resolved with the advent of drugs or drug carriers endowed with specific affinity to RBCs. Such non-covalent surface RBC loading offers the potential advantage of not only one-step extracorporeal loading, but even *in vivo* loading of circulating RBCs, bypassing the need for extracorporeal manipulations. To achieve this, drugs or nanocarriers have been conjugated chemically or genetically to ligands that bind safely to RBCs. For example, biotherapeutics including fibrinolytics conjugated with antibody to complement receptor type 1 (CR1, Knops antigen) bind safely to circulating RBCs in mice[76, 77]. This approach capitalizes on the immune-privileged nature of CR1 ligands, both physiological such as C3b-containing immune complexes involved in immune clearance, and artificial, such as anti-CR1 conjugated agents capturing pathogens.

A more general approach involves fusing biotherapeutics to a single chain variable region fragment (scFv) of antibodies that bind to RBCs[78]. In contrast to antibody-based conjugates, monovalent scFv-fusions do not cause cross-linking of RBC acceptor molecules or Fc-mediated side effects[79]. Recombinant technology enables large-scale, GMP-quality production of homogeneous monovalent scFv fusion proteins. A fusion of soluble CR1 with scFv directed to Rh(D) blood group antigens has been used to restore RBC CR1 function in genetically deficient mice[80].

Several groups successfully devised recombinant biotherapeutics fused with the scFv fragment of TER-119, a monoclonal antibody to an epitope associated with the mouse analogue of human glycophorin A (GPA)[81]. TER-119 scFv fused to complement-regulating proteins including DAF and CR1 enhanced RBC resistance to lysis by complement[78, 80, 82]. Neonatal gene transfer of TER-119 scFv/CR1 using a retroviral gene transfer vector prolonged synthesis, sustained coupling to RBCs, and restored protection against complement mediated lysis in genetically deficient mice[83]. TER-119 scFv fused with fibrinolytics provided thromboprophylaxis in mice[84, 85]. TER119 scFv fused with the recombinant endothelial anticoagulant and anti-inflammatory glycoprotein thrombomodulin (TM), had a half-life in the bloodstream ~100-fold longer than soluble TM and proved to be more effective in preventing arterial and venous thrombosis[86].

As illustrated by TER119 scFv fusions, the modular molecular format enables diversification of therapeutic cargoes. In theory, therapeutic features can also be diversified by regulating binding and on- and off-rates using in vitro affinity maturation or fusing cargoes with scFv fragments derived from different antibodies directed to the same antigen. This is projected to enhance control over intravascular residence time and the rate of detachment from RBC, offering the potential for either rapid or protracted drug delivery. From a drug delivery standpoint, it is important to take into account factors such as the number of antigens that may be targeted, their distribution on the RBC surface, their physiological functions in the membrane, their role in membrane integrity, and even potential signaling. Both the nature of the RBC surface anchoring epitope and the properties of the drug cargo portion of the RBC-targeted fusions are important in terms of potential adverse effects including immune response, cellular signaling, and adhesion.

The roster of RBC determinants potentially useful for this purpose is fairly extensive and ranges from high copy number targets such as glycophorin A and band 3 protein to low copy number targets such as complement receptor 1 (CR1) (Table 3)[87]. In theory, many RBC membrane proteins may be amenable to safe ligand coupling and some may prove to offer unique advantages for specific therapeutic purposes, e.g. spatial availability for activation and regulation.

As one successful example, CR1, which clears C3b-containing immune complexes, has been targeted with bispecific antibody constructs to remove pathogens from the blood in primate models[15, 88] and CR1-targeted agents have been used to protect against diverse pathologic agents including bacteria, cytokines, viruses, and pathogenic autoantibodies[89-91].

The success of targeting of diverse TER-119 fusions in mice without detectable adverse effects implies a potential utility for high-copy loading using this glycophorin A associated epitope[81], but direct translation from mouse to human RBCs is hindered by lack of TER-119 cross-reactivity with human RBC. Coupling of proteins and nanoparticles to mouse and rat erythrocytes on glycophorin A has also been achieved with a 12-mer peptide ligand known as ERY1, although this ligand also lacks cross-reactivity with human RBC[92, 93]. While ligands to glycophorin A and band 3 on human RBCs have been characterized to some extent, these may significantly alter membrane deformability[94], and little is known about the coupling of biomolecules to most human RBC membrane proteins.

Effects of drugs on RBCs

Adverse effects of drug loading on RBC behavior in the circulation may mirror pathological changes in the plasma membrane and cytoskeleton as occurs in hereditary spherocytosis and elliptocytosis. Alterations associated with RBC aging, disease, and drug loading typically hinder RBC elasticity, durability and biocompatibility, leading to accelerated elimination and potentially lysis[95].

All drug loading approaches - encapsulation, surface conjugation and targeting - can harm RBCs. Although it seems logical that RBC damage would be proportional to the dose as

well as the composition of the drug load, there is a lack of systematic side-by-side comparative studies of these issues. Non-covalent surface coupling using affinity ligands would be expected to be more benign than invasive protocols such as numerous washings, hypotonic opening, and chemical modification, but again there is a need for detailed investigation.

Usually, the initial phase of RBC clearance is most profoundly affected by drug loading due to rapid hepatic and splenic elimination of the cells most severely altered. Increased uptake does not necessarily compromise the objectives of drug delivery and if often not associated with evidence of organ dysfunction or marked biochemical changes. In contrast, even a modest increase in pulmonary uptake is a reason for concern, as it usually reflects RBC aggregation and retention in the microvasculature, which can impair oxygen exchange. Similarly, intravascular hemolysis with the risk of renal and other organ damage is a potentially serious dose-limiting toxicity.

Encapsulation is arguably more invasive than surface anchoring. The need to generate even transient pores in the RBC membrane for gradient-driven import of drugs inevitably challenges its integrity. This can lead to at least partial loss of essential mechanical properties of the cell membrane that maintain reversible deformability and resistance to damage by hemodynamic stress and squeezing through the microvasculature. However, even surface loading may alter the cytoskeleton and expose phosphatidyl serine (PS), which serves as a cofactor for assembly and activation of coagulation and complement reactions. Alterations of cell shape, loss of glycocalyx, depletion of energy sources, and loss of ions or water can lead to frank loss of RBCs during drug encapsulation (which may exceed 50% in protocols currently undergoing clinical testing) and shorten the intravascular survival of the remaining drug-loaded RBCs.

Non-covalent affinity targeting using monovalent scFv-fusions is less damaging in principal, but unguided anchoring of drugs to the RBC's surface may nonetheless impede biocompatibility and alter behavior in vivo through reorganization or blocking of protective components (e.g. DAF, CD59, CR1) or inhibition of CD47 that confers RBCs with “self” or “do not eat me” signals to phagocytes[96]. Extensive coverage of the RBC membrane may lead to alterations in membrane plasticity and other disruptive side effects commonly seen after encapsulation as discussed below.

Cross-linking of membrane components can cause potentially harmful side effects, especially when multivalent ligands are employed. However, even monovalent ligands can alter RBCs, for example, by reducing deformability[94] through conformational changes in the anchoring protein[97]. These effects are target protein dependent as ligands appended to some targets can increase deformability, as has been observed with CR1[98], while several others decrease deformability, as seen with glycophorin A and band 3[97, 99, 100]. Ligand binding to glycophorin A was also shown to result in increased intracellular reactive oxygen species[100]. The extent to which such effects are dependent on multi-valency is uncertain. Ligands binding to different epitopes on the same anchoring molecule differ in their effects depending on their size, affinity, charge and proximity of the targeted epitope to RBC surface. Ligand-induced changes may cause overt hemolysis and anemia (as in some

antibody-mediated transfusion reactions), or more subtle (but more important in the context of drug delivery) changes, such as abnormal flow dynamics and cellular distribution in the blood due to changes in membrane rigidity[101, 102].

Binding of ligands may also induce RBC membrane vesiculation[103] and release of microparticles causing inflammation and thrombosis, as seen in hemolytic RBC disorders[104-106]. Exposure of PS, a typical “symptom” of RBC damage, transforms the RBC surface into pro-coagulant interface favoring assembly of and activation of clotting factors, as mentioned above[9, 107] as well as promoting adhesion to the endothelium[108]. Hemoglobin and other internal RBC components released through “leaky” membranes can cause inflammation and toxic reactions such as acute renal damage[109]. The potential for deleterious effects of RBC modification will likely depend on the pathophysiologic context. For example, although a reduction in RBC deformability might be deleterious in some settings, it has also been shown to inhibit malarial parasite invasion[110].

Modification of RBCs may even enhance biocompatibility in some cases. For example, ‘universal’ RBCs generated by enzymatic treatment to convert them to type O have been transfused successfully into healthy recipients[111]. Also, targeting of certain drugs such as scFv-TER-119 fusions carrying complement inhibitors have been demonstrated to alleviate damage to RBCs deficient in endogenous complement inhibitors, as described above.

Immunological considerations

Drug loading to RBCs also may alter immunological features of the resultant complex. One might expect that anchoring foreign biological objects (molecules, their fragments and complexes, microorganisms) to the RBC surface would induce immune responses, as evidenced by drug-related autoimmune RBC antibodies. In some cases these antibodies can be neutralized by free drug but frequently they only bind to the drug/membrane complex[112, 113]. Whether induced or preformed, such antibodies can cause severe, life-threatening hemolytic anemia.

Enzymes released by certain pathogens, particularly during severe infection, can alter the RBC surface causing normally cryptic structures to be exposed. This is generally referred to as “T-activation” wherein various T antigen carbohydrates are expressed during the time of infection[114]. Naturally occurring antibodies to these normally cryptic structures that are present in all normal serum (except newborns) makes T-activated cells “polyagglutinable” and can causes the RBCs to lyse when patients are transfused with plasma.

T-activation and drug-induced antibodies point to the sensitivity of the immune system to seemingly minor perturbations of surface structures on RBCs. Indeed, transfusion of incompatible RBCs based on a single amino acid difference in a single membrane protein (e.g. Jk(a) vs. Jk(b) antigens) can induce polyclonal IgG and life-threatening hemolysis or hemolytic disease of the newborn. However, despite the relatively high degree of polymorphism in RBC surface proteins, only a subset of transfusion recipients ultimately develop alloantibodies[115]. The mechanisms by which these antibodies are generated is a subject of extensive investigation given their relevance to allogeneic transfusion[116, 117]. Recently developed models[118] have demonstrated some unique aspects of how RBCs

interface with the host immune system, including “antigen modulation” by C3 on the RBC surface[119], and resistance of a subset of allogeneic RBCs to antibody mediated clearance[120]. Importantly, erythroid-specific expression of otherwise strongly immunogenic antigens, was able to induce humoral tolerance in mouse models[121].

In this context, in an intriguing and somewhat counterintuitive finding, coupling antigens to RBCs by fusion to Ter-119 induced T cell deletion and conferred immunological tolerance, including in clinically relevant models of autoimmune type 1 diabetes[122]. The authors speculated that surface-loaded carrier RBCs undergo apoptosis to yield non-immunogenic remnants conferring stealth immunological features onto the appended cargoes. Similarly, tolerance to L-asparaginase, a therapeutic for leukemia which is, in part, limited by drug-neutralizing antibodies, can be induced by RBC-mediated delivery[123]. RBC-mediated delivery may induce tolerance through the induction of regulatory T cells[124]. Reduced immunogenicity of therapeutic enzymes by encapsulation within RBCs[49, 125] has also been attributed to separation of the cargo from immune system by the RBC plasma membrane. Nevertheless, the consequences of exposing RBC components normally hidden from the immune system caused by loading and potential immunogenicity of a fraction of the drug cargo retained on the RBC surface must be carefully examined for each intended use.

A patient's immune and disease status may also modulate response to drug-RBC therapeutics. Not all transfusion recipients make detectable alloantibodies to foreign RBC antigens (“non-responders”). In the absence of inflammation, mice transfused with RBCs expressing transgenic human glycophorin A (hGPA) antigen did not produce detectable anti-hGPA immunoglobulins in contrast to the same protocol in the setting of inflammation[126], perhaps due to changes in the subset of antigen presenting cells that phagocytose RBCs[127]. In fact, these mice demonstrated evidence of true immunologic tolerance in that later challenge with hGPA in the context of inflammation still did not result in an immune response. These data suggest that transfusion of RBCs (potentially including some RBC drug-carriers) might induce tolerance rather than provoking a harmful host response in the absence of inflammation. It remains to be demonstrated how anti-inflammatory agents bound to RBC might alter this balance of tolerigenicity and immunogenicity. Overall, this area of RBC drug delivery research and its effect on the immune response is in further need of systematic and detailed mechanistic studies.

Regimens and dosing of drug delivery by RBC

Drug encapsulation and surface loading *ex vivo* and targeting to circulating RBC *in vivo* each have pros and cons that vary in part with the therapeutic goal (Figure 2).

Vascular infusion provides the logical route of administering RBCs loaded ex vivo using drug encapsulation or surface binding, and portable devices suitable for sterile loading of autologous or donor RBCs using a standard automated procedure have been devised and clinically tested. Loading via encapsulation demonstrated efficacy in the delivery of dexamethasone[33], asaparaginase[35], and enzyme replacements[37], among others. As a prototypical example, a dose of dexamethasone-loaded RBCs (Erydex) is prepared from 50 mL of blood, which is approximately 1% of the total blood volume of a 70 kg adult. After

infusion of a suspension of loaded RBCs, the injected dose of the drug is carried by a small fraction of total circulating RBC.

Ex vivo RBC loading regimens involving a small fraction of RBCs theoretically pose less danger of generalized adverse effects and enable “focal” drug depots. The potential value of such “focal” modes of action has been demonstrated in studies of spatiotemporal parameters of dissolution of fibrin clots by RBC-bound fibrinolytics. RBC-bound fibrinolytics quickly formed focal zones of expedited fibrinolysis (“cavities”) within clots, which expand due to directional force of flow, coalescing into patent channels that facilitate reperfusion prior to clot dissolution. In contrast, fibrinolytics evenly distributed throughout the clot provided reperfusion only after substantial dissolution of the clot[128, 129]. The RBC surface also provides a favorable microenvironment for therapeutic activity by way of a protective glycocalyx.

Therapeutic goals may differ in terms of the optimal distribution of drug throughout circulating RBCs (Figure 3). In some situations, a more even distribution of a drug in the bloodstream might be desirable. In theory, loading drug onto or within a larger proportion of autologous RBCs might be possible but would require larger volume phlebotomy or continuous loading via a closed extracorporeal circuit, such as an apheresis machine. A more even distribution may be attained when fusion proteins are targeted to circulating RBCs. This approach expands the versatility of the approach because the proportion of RBCs carrying the drug can be modulated by changes in dose and rate of infusion. In theory, bolus injection of a small dose of a high-affinity scFv fusion may create a situation similar to infusion of pre-loaded RBCs with few RBCs carrying the large majority of the drug, whereas slow infusion may result in more even “painting” of many circulating RBCs with drugs. In theory, the dose and duration of drug in the circulation can also be modulated by using fusions that bind to different epitopes on a target and with different affinities.

Although greatly sustained circulation is generally a favorable feature in drug design, control of this feature is an important consideration for RBC carriers particularly with respect to potential toxicities. Routes of clearance will involve either intrinsic clearance of the RBC itself (for example, via the spleen), or clearance of the appended or loaded agent as it detaches from the surface, is released from within, or is otherwise degraded. In the former case, we must consider factors that govern the rate of RBC clearance. One's own red cells are thought to circulate for approximately 120 days, and transfused units, after initial rapid clearance of a subset of RBCs (usually <10% within 24 hours, and required by the FDA to be <25% for transfused units), are thought to have similar lifespan[130]. The degree to which a portion of red cells are cleared rapidly appears to depend on several factors such as prior storage[131], and potentially even host factors related to ex vivo survival[132]. Strategies to facilitate clearance if reversal is needed could theoretically include red-cell exchange by apheresis, which would provide immediate removal from circulation. However, this approach necessitates high exposure to allogeneic units and potentially invasive procedures for adequate intravascular access, so its use may be limited to acute toxicities or with agents of very narrow therapeutic indices. Alternatively, control of release or detachment of the appended or loaded agent can also modulate delivery parameters. Incorporation of cleavable or activatable linkers has been described for many

biotherapeutics, especially antibody-drug conjugates for cancer therapy[133], and activatable zymogens have also been applied to RBC-coupled agents[85]. Such technologies offer an important handle for potential control of delivery. For internally loaded agents, rate of diffusion out of RBCs is an important parameter and can be expected to be highly dependent on loading technique and the biochemical properties of the desired agent.

Although parenteral administration (*in vivo* loading) of a recombinant fusion protein offers a more controllable and straightforward path toward the fabrication and regulatory approval of RBC surface-coupled therapeutics, one can also envision scenarios in which *ex vivo* surface loading of RBCs for subsequent transfusion of the drug-loaded RBCs is favorable. As one example, any agent that may be rapidly sequestered and cleared when soluble may be less amenable to in vivo loading. Alteration of donor RBC units in the clinical realm is already widely practiced in the form of red cell additive solutions, RBC washing, irradiation, and leukoreduction[134]. Another example of RBCs modified ex vivo for clinical application (although not for therapeutic delivery) is the tagged red blood cell scan in which RBC are loaded with isotopes for imaging to identify the source of occult lower gastrointestinal bleeding[135]. In this example, RBC manipulation is typically performed by nuclear medicine specialists using specialized, FDA-approved commercial kits.

Transfusion aspects of RBC drug delivery

In the transfusion medicine or blood banking realm, modification of RBCs results in the designation of blood banks as manufacturers of drugs and thus many hospital blood banks are regulated as such by the FDA and are subject to strict inspection and accreditation requirements[136]. A common characteristic of RBC manipulations in the clinical setting is the preferred maintenance of closed, sterile connection systems whenever possible to limit contamination. Access of RBC units in an ‘open’ system is possible, although it typically results in products that must be administered within 24 hours. Such a limitation may be tolerable for particular RBC carriers.

The stability of RBC-drug complexes for in vitro storage requires characterization for each drug and method of loading. With present loading technologies, RBCs have no shelf-life after drug encapsulation: i.e. they must be injected in immediately after loading. Trauma inherent in cell isolation, washing, and drug loading is more likely to damage senescent or otherwise impaired RBCs. In this sense, ex vivo drug loading may fortuitously eliminate weak carriers and select for RBCs that enhance delivery, albeit at the cost of reduced yield.

If transfusable, drug-loaded RBCs are to become a clinical reality, the source of the RBCs must be carefully considered. Autologous donation mitigates the need for immunological matching with recipients as well as many concerns about infection, but this approach may be limited by health requirements for autologous donation[134], depending on the volume of red cells that will be needed to manufacture a particular therapeutic and potential for anemia. For example, although dexamethasone-loaded RBCs currently in clinical trial require only about 50 mL of whole blood for manufacture, a typical whole blood collection is 400-500 mL, a volume that may be problematic for treatment of diseases in which recipients are not likely to be eligible for autologous collection. Conversely, allogeneic RBC units represent a large, well-established, and safe potential pool for manufacture of surface loaded RBC

carriers and have already been used in trials of asparaginase-loaded RBCs[35]. However, issues of alloimmunization, infectious diseases, and transfusion reactions (allergic, febrile, etc) cannot be definitively eliminated. Finally, ex vivo culture of mature RBCs that can be surface modified[137], while demonstrating promise in very early pre-clinical settings (and unmodified cells in healthy human recipients[138]) remains far from widespread clinical availability and questions of allogeneic versus autologous sources would remain.

The schedule for production and administration of *ex vivo* RBC-loaded or RBC-coupled drugs must also be taken into consideration. Most commonly used additive solutions result in RBC products that can be stored up to 42 days[132]. However, once a RBC unit is modified (for example, following irradiation), this 'shelf-life' can be significantly reduced, going down to at most 24 hours when an 'open' system must be used. Possibilities for carrier RBCs may include loading of drugs into fresh RBCs for immediate administration (as is currently practiced for RBC-loaded drugs in trial and tagged RBC scans), but also loading or coupling of drugs into/onto stored units (both autologous and allogeneic). In addition, further storage of drug-loaded RBCs may be possible post-loading or post-coupling (for example, to simplify central manufacture and subsequent distribution), but concerns about the potential adverse effects of storage, such as changes in membrane properties and accumulation of 'storage lesion' byproducts, would be introduced with such an approach[139-142], While the results of recent trials suggest that stored RBCs are not inferior in a variety of patient populations[143-146], the relevant endpoints and risk/benefit profiles are likely to be significantly different in the design of RBC-coupled/loaded drugs for prophylaxis or for treatment of chronic diseases.

Conclusion

Three general approaches to drug delivery – natural, synthetic, and hybrid carriers– have distinct potential utilities, benefit/risk ratios, and investment/cost ratios that vary among pathological conditions, clinical settings, and even among individual patients. RBC carriers incorporate facets of each of these approaches, and, from this perspective, conditions that typically involve blood transfusion or cellular therapy represent a very attractive area for their application. Technical, organizational, financial, and safety issues inherent to RBC carriers are manageable as illustrated by early successes in drug-loaded RBCs and engineered cellular immunotherapies. Thus, loading RBCs with drugs seems a natural extension of treatment of diseases that currently include or rely upon transfusion. Surgical and other types of trauma, sickle cell disease, anemia and other cytopenias, and acute lung injury are scenarios in which combining the effects of transfusion with pharmacotherapy delivered by RBCs may provide added benefit.

On the other hand, the roster of pathophysiology that might benefit from RBC-mediated drug delivery broadens the potential application of red cell transfusion and cellular therapies to include oncological, neurological, cardiovascular, metabolic, infectious, and other diseases, as well as intoxications with endogenous and exogenous noxious agents. RBC-carried imaging and diagnostic agents may find additional utilities in these and other conditions. One can hope that decisive improvement of therapeutic or diagnostic outcomes

provided by RBC-mediated delivery in such disorders will eventually expand the general utility of blood transfusion.

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Highlights

- Red blood cells (RBCs) are promising carriers for bio-therapeutics to enhance their PK and control their site and mechanism of action.
- RBC drug carriers include those loaded internally or externally and may be prepared *ex vivo* or *in vivo* from allogeneic or autologous units.
- Surface coupling onto membrane antigens offers a biocompatible, flexible platform for loading of drugs.
- The optimal epitopes for coupling onto RBCs remain to be definitively characterized.
- Drug loading onto RBCs may alter immune responses, including potential induction of tolerance.

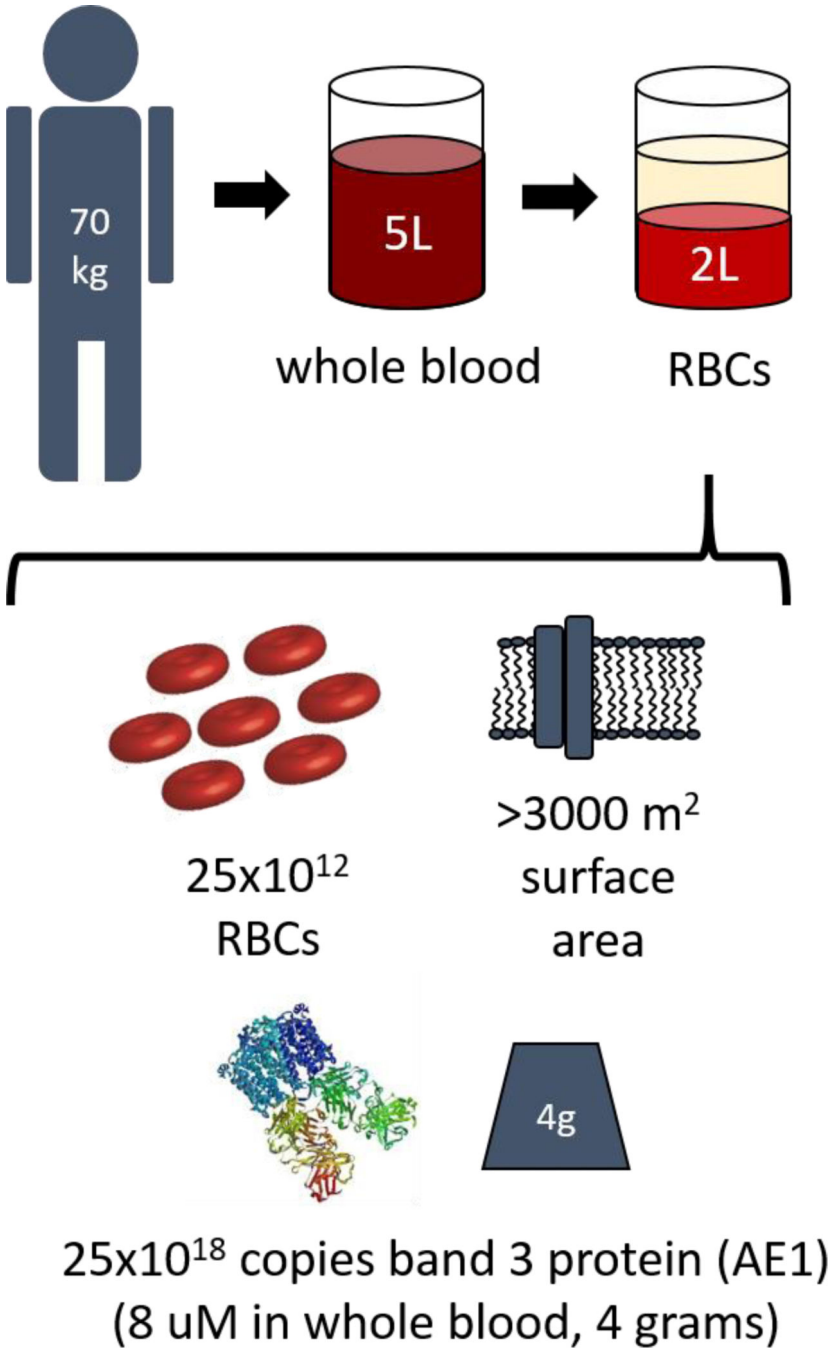

Figure 1.
Dimensional analysis of RBCs within a typical adult.

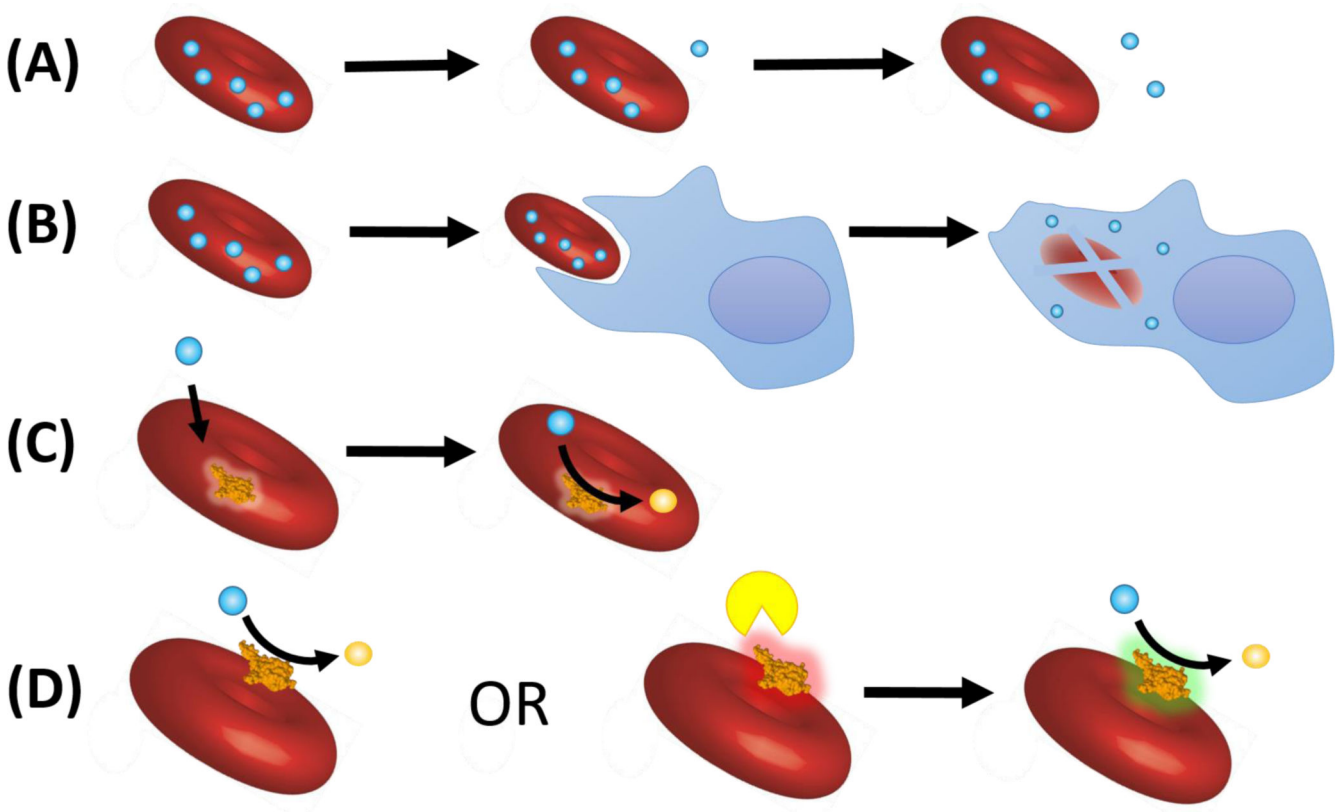

Figure 2. General approaches to RBC drug carriers

(A) Slow drug release from drugs encapsulated within RBCs provides sustained delivery (B) Drug-loaded RBCs are phagocytosed and deliver drugs to cells of the reticuloendothelial system (such as macrophages) (C) Encapsulated enzymes act upon diffusible substrates (D) Surface loaded enzymes act on extracellular targets either constitutively or after activation by other proteins/enzymes to an active state (green), which may provide enhanced specificity.

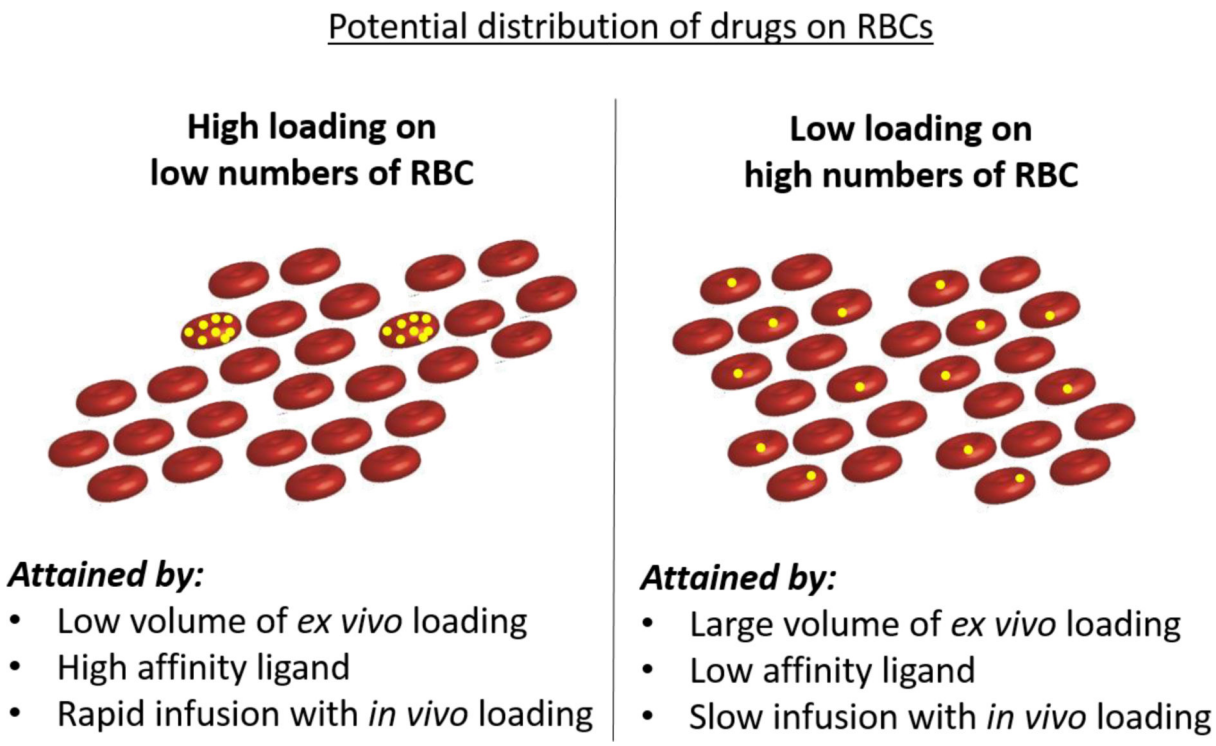

Figure 3.
Potential distribution of drugs on or within RBCs and the approaches by which they may be attained.

Table 1

Pros and cons of the various sources of RBCs for drug-loading

RBC source	Pros	Cons
Autologous	• Lessened infectious disease risk • No allo-immunization	• Donor RBC may be pathologically altered • Larger volume phlebotomy may limit donor eligibility in disease states
Allogeneic	• Does not necessitate phlebotomy of patients • Facilitates central manufacture • Existing infrastructure for unit collection and distribution	• Allo-immunization potential • Infectious disease risks
Fresh	• Better circulation/survival • No accumulation of ‘storage lesion’ byproducts	• Necessitates ‘on-demand’ production • Logistical hurdles to deliver units/drugs to some centers
Stored	• Ease of central manufacture • Facilitates production timing	• ‘Storage lesion’ byproducts and cellular changes may compromise efficacy • May alter interaction with drug cargo or appended ligands

Table 2

Pros and cons of the types of loading of RBC drug carriers

Loading Type	Pros	Cons
Ex vivo	• Greater control over surface coupling and degree of loading • More complex loading methods can be performed on isolated RBC	• Potential compromise of RBC viability and circulation • Complex regulatory, manufacture, and distribution concerns • Sterility risks with open system
In vivo	• More reproducible manufacture and facile distribution • Single-entity compound possible • Better defined regulatory structure	• Must reach target before clearance or degradation • Likely limited to surface-loading
External/surface	• Less complex to manufacture • Can engineer degree of loading by using different surface determinants • Affinity changes can be engineered to control drug release	• May alter membrane rigidity or plasticity • Requires identification and characterization of affinity ligand • Drug often not in native free form
Internal	• Protects drug from extracellular clearance, inactivation, and immune recognition • Drug may be encapsulated in free form	• Compromised membrane integrity • Lessened circulatory survival • Usually requires drug release, or diffusion of substrate into RBC

Table 3

Potential target antigens for coupling therapeutics to RBCs, their non-RBC distribution, likely function, and relative numbers per cell

RBC Antigen (Blood group designation)	Other Tissue Expression	Function	Est. Sites per RBC
Band 3 Protein (Diego)	Distal and collecting tubules of the kidney	Anion exchange, structural integrity	10^6
Glycophorin A (MNS)	Renal epithelium and endothelium	Chaperone for band 3 transport to membrane, major sialoglycoprotein contributing to negatively charged glycocalyx	
GLUT1 (none)	Endothelial cells of barrier tissues	Glucose transport	
Glycophorin B (MNS)	Renal epithelium and endothelium	Minor sialoglycoprotein contributing to negatively charged glycocalyx	
RHCE and RHD (Rhesus)	Largely erythroid specific	Membrane structure and integrity, homology with ammonium transporters	10^5
CD59/Protectin (undefined)	Widely expressed	GPI-linked complement regulatory protein, inhibits membrane attack complex	
Kell (Kell)	Myeloid tissues, marrow, brain, muscle	Endothelin-3 converting enzyme	10^4
CD55/Decay accelerating factor (Cromer)	White blood cells, platelets, lung	GPI-linked complement regulatory protein, accelerates decay of C3 and C5 convertases	
ICAM-4 (LW)	Relatively erythroid specific	Intercellular adhesion molecule, ligand for integrins	10^3
Complement Receptor 1 (Knops)	White blood cells, glomerulus, follicular dendritic cells	Complement regulatory protein, processing of immune complexes	