Drug delivery by erythrocytes: "*Primum non nocere*"

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Abstract

Red blood cells (RBCs) are naturally capable of transporting diverse cargoes throughout the circulatory system, both loaded to their surface or within their inner volume. Starting largely from the 1970s, diverse approaches for encapsulation into, and surface coupling onto, RBCs have been investigated as potential drug delivery systems. In the last decade, these efforts have yielded diverse strategies to load drugs and nanocarriers to RBCs, and to optimize their pharmacokinetics, distribution, and effects in the body. Several formulations of donor RBCs encapsulated with enzymes and drugs are currently undergoing clinical trials for treatment of oncologic and neurologic conditions. Newer approaches include design of drugs with an affinity to circulating RBCs, encapsulation into RBCs using membrane permeating compounds, and design of hybrid drug delivery systems combining synthetic components with fragments of RBC membranes. Notwithstanding the growing enthusiasm and optimism in RBC drug delivery, in this article we discuss potentially problematic issues of this biomedical concept, especially impairment of biocompatibility of the carrier RBCs, and other adverse and unintended effects. Rigorous and systematic analysis of the cautionary aspects described in this article should be further developed and extended in order to soberly gauge the risk/benefit balance of any given RBC-based drug delivery application. While there is little doubt that RBC drug delivery will ultimately flourish, focusing research efforts on approaches that are unlikely to cause adverse effects in patients will help to sooner bring this day.

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1. Introduction

Blood and its cellular components, particularly red blood cells, are considered to be among the most useful natural carriers for drug delivery [1–6]. In fact, red blood cells (RBCs) have been explored in this context since the 1960s. More recently, leukocytes and platelets have also attracted attention as alternative carriers [7,8], offering theoretical opportunities to co-opt their natural functions such as tropism to sites of action and ability to transform and release their contents in response to local mediators. Although RBCs lack these functions, they are not necessarily a bad choice as a carrier in view of their apparent functional simplicity and high availability, as well as possessing several inherent advantages as a drug carrier, particularly their abundance as long-circulating blood cells that can be "hitchhiked" for carriage of therapeutic agents.

However, despite the apparent simplicity and obviousness of this strategy, RBC drug delivery is a highly challenging task. Many aspects of production, storage, and regulatory affairs complicate industrial and clinical translation of natural biological carriers. Furthermore, the negative impact of transfusion-transmitted infectious diseases in the eighties almost decimated RBC drug delivery research. Nevertheless, in the last two decades we are witnessing exponential growth in this arena, and currently, RBCs are being explored for delivery of a variety of therapeutic, imaging, and other useful clinical modalities, including as super-carriers of nanoparticles and other novel nanomaterials. Moreover, several companies are currently pursuing clinical trials [9–12] and many labs are exploring pre-clinical studies of RBC drug delivery. Therefore, it seems timely to address potential unintended consequences and problematic issues of RBC carriage, to establish a rigorous roadmap for addressing safety, industrial translation, and clinical utility of this yet-developing strategy. In this section we focus on the first item, keeping in mind the quintessential guiding medical principle: "First, do no harm".

2. Drug delivery by modified RBCs (mRBC)

Optimization of the pharmacokinetics (PK), e.g., prolongation of drug circulation and boosting bioavailability, is generally viewed as the main pharmacological benefit of RBC carriage. However, RBC carriers may impede circulation of already relatively long-circulating agents such as IgG. For example, RBCs may block immunoglobulin interaction with endothelial FcRn and therefore inhibit the FcRn-mediated IgG recycling mechanism [13]. Further, RBC coated by intact IgG are likely to undergo phagocytosis via multivalent engagement of FcR-gamma, opsonization and other clearance mechanisms that may be activated depending on the nature and degree of RBC modification.

In addition to changing PK, carrier RBCs may otherwise alter the functionality of appended or loaded cargoes. For example, coupling to RBC protects some cargoes from plasma inhibitors, in particular, via masking by RBC glycocalyx [14,15]. RBC carriage also changes drug biodistribution in many ways including: i) redistribution in blood from marginal plasma layer to the mainstream; ii) inhibition of renal glomerular filtration and extravasation via endothelial intracellular and intercellular pathways accessible only to objects smaller than RBC; and, iii) elevated uptake by the spleen [1,16]. Recent studies revealed that in some

cases, agents transfer from the carrier RBCs to endothelial cells [17]. Better understanding of this enigmatic finding may yield a new mechanism for vascular drug delivery.

Many administered drugs are excreted via the urine, while excretion via bile and exhalation plays a relatively secondary role. Loading to RBC carriers shifts the excretion of drug cargo to hepatobiliary and reticuloendothelial uptake. In terms of elimination, RBC are generally biodegradable, unless modified beyond the point of effective biological destruction (for example, fixation), but this degree of modification is usually incompatible with circulation. Yet, even modifications that do not impair RBC degradation do impede RBCs' ability to circulate safely for a prolonged time. In this sense, "biodegradable" and "biocompatible" are different terms.

There are two main approaches for drug loading to carrier RBCs: encapsulation within the inner volume and surface coupling. Both emulate natural transporting functions of RBCs, yet have potentially problematic aspects. These include, but are not limited to destruction and elimination of the modified RBC (mRBC), systemic and local adverse effects caused by mRBC, formation of untoward mRBC/cargo complexes, or unfavorable alteration of the toxicity of the drug cargo itself due to changes in the PK. Some potential risks and challenges of RBC drug delivery are common for encapsulation and surface loading. Some are more typical of a given technique. Some aspects of these rubrics are well recognized, while others have not been substantially addressed in the literature.

3. Risk and challenges of drug loading into carrier RBC

Many within the general public and biomedical science, view RBCs as a *sac de voyage* for hemoglobin, and, therefore, use of their inner space for drug cargoes seems logical. Encapsulation helps isolate drugs from the body, but involves RBC isolation, loading, and infusion. Depending on the loading efficacy and damage to RBCs, the yield of the final formulation may represent a fraction of the initial donor blood, which will place manufacturing demands that may strain the ability to competitively price therapies and even potentially limit drug manufacturing and availability due to socioeconomic factors. Storage and quality control of drug-loaded RBC represent additional regulatory challenges. Given these limitations, the utility of encapsulated RBC-drug delivery in emergency settings, may be more limited than in elective procedures.

Perhaps, more importantly, from the biomedical perspective, encapsulation is also complicated by the very same aspects of RBC that make them an attractive carrier: their relative simplicity (compared to other blood elements). In contrast to other cells, RBCs do not have vesicular pathways for internalization of external compounds. This represents a challenge for encapsulation: with the exception of drugs that freely diffuse into RBCs (which also indicates that they will freely leak from RBCs in the bloodstream), one needs to smuggle drug cargoes across RBC membranes while avoiding damaging this delicate yet incredibly endurable structure.

This multimolecular partition includes the phospholipid bilayer with integral and non-integral glycoproteins, an external layer of negatively charged glycocalyx, and the attached

internal layer of protein cytoskeleton [18–20]. This complex assembly enables RBC circulation for three months, through millions of cycles of squeezing through microvasculature and smashing into the cardiac valves and chambers. To encapsulate drugs in RBCs, one must violate, at least to some extent, this marvelous, elastic, biconcave vehicle, and this comes at the cost of reducing the biocompatibility of RBCs.

In essence, all techniques for drug encapsulation in RBCs that have been advanced to the clinical stage employ osmotic swelling of RBCs. This opens pores in the membrane, allows exchange of hemoglobin or drugs via a concentration gradient, and is followed by resealing of pores ultimately to return to an isotonic solution. Loss of RBCs due to hemolysis during encapsulation is a significant problem, yet impaired biocompatibility of the "surviving" resealed RBCs is, perhaps, an even more serious issue. Osmotic shock affects integrity, plasticity, and mechanical robustness of the RBC membrane and cytoskeleton. Exposure of phosphatidyl serine (PS) increases by orders of magnitude, from normal levels of <0.5% to >5%. These changes lead to fixation of complement and its activation, RBC deformation and increased sensitivity to stress: mechanical, oxidative, immunological. PS exposure on RBCs has also been implicated in activation of coagulation [21]. All these unintended consequences of RBC loading may lead to serious adverse effects.

Some drugs encapsulated in RBCs may diffuse out in plasma and encounter secondary interactions with other blood components, target molecules, and cells. In this scenario, mRBC serve as a systemic depot of therapeutics. This technology is best-developed for the delivery of dexamethasone [9,11,22,23]. Some therapeutics are capable of exerting their effects by acting on target molecules that diffuse into RBCs. For example, many enzymes encapsulated in RBCs can act upon diffusable substrates in blood, thereby providing a long-circulating cellular reactors for detoxifying harmful compounds or eliminating molecules needed for tumor growth [24]. This is also among the most advanced applications of RBC drug delivery, having already entered clinical trials in neurological and oncologic diseases [10,12,25]. Some agents, including anti-inflammatory agents and antigens regulating immune response, are intended to work in phagocytes and other immune cells, which naturally take up mRBCs.

These scenarios provide examples of applications of mRBC that do not require controlled release of the encapsulated cargo. Although controlled drug release from mRBC is a very challenging and fascinating goal, these scenarios are more complicated. The concept was introduced decades ago wherein anchoring of affinity-directed carrier RBCs on the target surface caused deformation of the carrier RBCs and enhanced susceptibility to lysis (and resultant release on mRBC content) via activation of the alternative pathway of complement [26]).

4. New trends in drug encapsulation in RBCs: membrane permeating peptides (MPPs)

Recently, several labs have embarked upon the use of membrane permeating peptides (MPP) to traverse RBC membranes without producing pores or other damage [27]. This is an

intriguing area of research, intertwined with paradigms utilizing MPPs for intracellular delivery of cargoes ranging from small peptides to nanocarriers [28].

In the spirit of the intent of this article, we must note that MPPs destabilize membranes – both artificial, like liposomes, and natural, i.e., cellular. RBCs, in particular, lack cellular organelles and other facilities including enzymatic systems for repairing the membrane. As such, RBCs are vulnerable to lysis: one pore is enough to destroy a RBC. Some MPPs (especially derived from hemolytic venoms) agglomerate in membranes, forming a pore, similar to that formed by complement. The minimal concentration at which an MPP causes hemolysis is a key characteristic of the MPP's activity and variably correlates with transduction activity. The safest MPPs have at least one log of concentration between minimal hemolytic and transduction activities. However, overt hemolysis is somewhat of the tip of a proverbial iceberg. Insertion of MPP into RBC membranes inevitably alters the membrane, making it less resistant to physiological and pathological stresses that every RBC encounters in circulation. Systematic animal studies of the behavior of MPP-modified RBCs have yet to be performed, and it seems advisable to measure expectations (a universal piece of advice, indeed).

Little is known about how conjugated cargoes alter MPP activity and their mechanism of binding to, insertion into, and transport across membranes. Understanding of these processes is limited even for unconjugated MPPs. Further, many MPPs are cationic and their interaction with, and influence on, the RBC glycocalyx may have diverse, and perhaps undesirable, consequences. For example, MPP-treated carrier RBCs may become adhesive or pro-inflammatory to endothelium. Finally, MPPs are promiscuous: they bind to any cell and membrane, and therefore may swap from RBCs to other cells in the body. A glass-half-full perspective may envision an additional drug delivery modality, but viewed from the empty half, one must acknowledge the complexity and lack of control of this approach and recognize the limits this may place on clinical utility at the present time.

5. Surface loading

The RBC surface has important physiological and pathophysiological transport functions. For example, some types of immune complexes are captured and transported to immune cells via binding to circulating RBCs. Taking into account that the collective surface area of RBC membranes represents by far the most extended, dynamic interface in the body, one can safely posit that the transport (and immune regulatory) functions of RBCs have yet to be appreciated.

Several labs are exploring the coupling of drugs, probes, and nanocarriers to RBCs using chemical conjugation, affinity binding, and non-specific absorption of these cargoes to the RBC plasmalemma. Methods have been developed for RBC surface loading both *ex vivo* (e.g., using donor or autologous blood) and *in vivo*, without need for extracorporeal manipulations with RBCs. In comparison with chemical or non-specific mechanisms of coupling to RBCs, using affinity binding (targeting) is advantageous, because it allows either *ex vivo* or *in vivo* loading and effects of specific coupling epitopes may be discerned.

Preclinical animal studies using double-label isotope tracing showed that surface loading of diverse drugs and nanocarriers does not alter RBC circulation, biodistribution, and degradation. Various RBC-coupled drugs and therapeutic fusion proteins, including those loaded to circulating RBCs *in vivo*, have been shown to improve efficacy in a spectrum of conditions, including thrombosis, inflammation, ischemia, and stroke [29–32].

Surface loading is generally less damaging than encapsulation in RBCs as it does not necessitate formation of pores. However, excessive modification of RBCs by conjugation chemistry impedes RBC biocompatibility. Further, even non-covalent attachment of cargoes to RBCs using affinity ligands may have this undesirable effect. For example, RBC-coupled ligands may interfere with functions of a target anchor molecule, block and inhibit CD47 (a glycoprotein expressed on the surface of diverse cells in the body that inhibits their uptake by phagocytes), and impair other protective proteins including inhibitors of complement. Extracellular ligands, even monovalent, can alter deformability of RBC membranes, trigger intracytoplasmic changes in the cytoskeleton, and induce reactive oxygen species generation [33–36]. These changes are likely to be target and epitope dependent.

Effects of RBC modification on the immune system are even more complex, somewhat enigmatic, and, to the present, insufficiently understood. There are reports of reduction of immune reactions, and even induction of tolerance, by loading on or within RBCs [10,37–41]. However, as is evident in allo-immunization after transfusion, the opposite reaction also may occur [42], and is likely to be interdependent on the loading approach and underlying status of the patient.

6. Devising RBC derivatives for drug delivery

Size is an important factor defining the drug delivery properties of RBCs (human RBCs have maximal dimension on ~7 micron). Their large size helps to retain the drugs in bloodstream, yet limits accessibility to extravascular targets, including tumors. In the last two decades, several groups attempted to devise micro- and nanoparticles derived from RBCs. For example, sub-micron vesicles termed "erythrosomes" have been produced from RBC membranes and loaded with cargoes. However, these early *in vitro* studies in vitro have yet to translate into pre-clinical animal studies.

Recently, an intriguing approach has been designed to coat synthetic nanocarriers with fragments of RBC membranes ("RBC-cloaked nanocarriers") [43]. This elegant strategy involves fairly sophisticated methods that help control orientation of the RBC membrane leaflets covering nanoparticles. This precaution is necessary to minimize exposure of constitutively hidden RBC components from blood, and alleviate inevitable impairment of biocompatibility. RBC-cloaked nanoparticles hold promise to evolve into a multifunctional platform for drug delivery and for toxin elimination from the bloodstream, and initial animal studies support for this approach. It is tempting to expect that ongoing studies quantitatively tracing these hybrid vehicles *in vivo* will provide unequivocal proof that the longevity of circulation and safety of RBC-derived particles approaches that of RBCs.

Strategies aimed at re-formulation of RBCs into small membrane vesicles or other semi-natural nanocarriers, may ultimately include membrane vesicles naturally shed, or otherwise formed from cells. In the age of ascendance of cellular therapies employing natural, modified, and reprogrammed cells – lymphocytes and stem cells as exemplary players –it is tempting to posit that "exosomes" and/or "membrane vesicles" might offer new unique delivery options due to their size, tropism, and functionalities. In this context, natural RBC membrane vesicles again represent an attractive, and relatively controllable carrier. Although vesicles derived from pRBC units have not been characterized in depth, they have been used for delivering drugs in cancer patients. In this setting, they serve as immunocompatible, biomimetic nanocarriers that avoid the host immune system while delivering their cargo safely to the site of action and with extended blood circulatory time [44]. However, their behavior and effects in the body must be characterized more systematically.

7. Potentially problematic aspects of RBC-based drug delivery systems

Challenges common to every approach to RBC delivery include loss of plasticity and durability of RBCs, fixation of immunoglobulins and complement, inhibition of the protective functions of CD47, and changes in surface charge (for example, by unintentional removal of sialic acid termini from the glycocalyx). All these alterations lead to elimination of modified RBCs. Such RBCs become adhesive to endothelium and other cell types, get entrapped in the microvasculature (especially in the lungs), and can be lysed by complement and cleared by the RES. Furthermore, unintended or unanticipated generation of erythrocyte microparticles or extracellular vesicles (EVs), may produce adverse inflammatory stimuli, and the important contribution of these EVs to both normal physiology and stored blood components is increasingly recognized [45,46].

These undesirable consequences of RBC loading compromise their transport function, thereby defeating their central purpose, and may precipitate harmful side effects in the patient – local and systemic inflammation, vascular occlusion, endothelial activation, and renal damage by release of free hemoglobin. Furthermore, overload of phagocytes by modified RBCs may impede important host defense functions of these cells including bactericidal and antigen-presenting functions. Even "big eaters" may get indigestion.

In addition to impairment of RBC biocompatibility, drug cargoes loaded to carrier RBCs may cause other problems. Carriage by RBCs produces dramatic changes in drug pharmacokinetics, distribution, and ability to interact with intended and unintended targets. Dosing, timing, and regimens of administration of RBC-loaded or targeted agents need to be evaluated independently and adjusted for these new parameters of their behavior and effects in the body.

RBC drug delivery, as any drug delivery approach, is likely to be intended for use in patients, not healthy individuals. Even prophylactic interventions are often applied to prevent worsening or already underlying pathophysiology. Pathological factors typical of the disease of interest may change both the behavior and effects of RBC-based drug delivery systems. For example, local and systemic levels of cytokines and other mediators of inflammation may result in enhanced opsonization and activation of complement leading to lysis of carrier

RBCs and their uptake by RES, as well as enhanced RBC agglutination and adhesion. Enhanced permeability of barriers – for example, endothelial and blood–brain – can result in extravasation of drug-loaded RBCs into sites of occult diapedesis and/or hemorrhage, resulting in off-target effects in these unintended areas. Thrombi and other vascular occlusions may affect perfusion and RBC access to intended sites of therapeutic interventions.

8. Pre-clinical appraisal of risks of RBC drug delivery

Animal studies are costly and relatively low throughput. To screen formulations of RBC derivatives and carriers across multiple formulations and conditions, one can start with *in vitro* testing of their sensitivity to hemolysis in buffers of varying ionic strength, as osmotic resistance/fragility represents one of the well-known sequelae of altered RBC membrane physiology. If sensitivity of modified RBC formulation to reduced osmolality is similar to that of naïve RBCs, additional challenges can be investigated including, but not limited to, mechanical resistance, deformability, complement resistance, pH, and oxidative stress. These stresses represent some of the several that carrier RBCs are likely to encounter in circulation [47].

RBC formulations or RBC-targeted agents that pass these preliminary exams can then be tested *in vivo* in homologous species. In this vital phase of the testing the methodological aspects of tracing RBCs *in vivo* are paramount. The arsenal of methods includes detection of biotinylated RBCs, RBCs labeled with fluorescent and other optical dyes, detection of biochemical markers of clinical relevance, combined proteomic and mass-spec signatures, or captured immunological detection of RBC antigens [48].

However, these methods yield data convoluted by differences of readouts and their background in various tissues. In most cases, it is difficult, if not impossible, to normalize recovered signal to the injected dose of modified RBC (mRBC). Therefore, these methods do not quantitatively characterize blood clearance, let alone the biodistribution of mRBC.

To circumvent this problem, many labs normalize the signal from mRBC recovered from blood specimen to a reference level that has been detected in the first blood sample taken from an animal, usually 10–30 minutes post injection of mRBC. However, this approach is prone to overestimation of mRBC in the circulation, because damaged, aggregated, or otherwise predisposed mRBC get eliminated before the first blood sample is drawn (alas, this methodological issue is typical for many drug-delivery studies).

This issue can be resolved by injecting of mRBC labeled with appropriate isotopes, e.g., ^{125}I for membrane components or RBC-loaded cargo and ^{51}Cr for intracellular content. This method provides direct, accurate, and quantitative measurement of mRBC in blood and tissues.

Of note, studies in naive animals represent just the first step; they must be reproduced in appropriate models of the human pathology of interest. As discussed above, pathological factors may alter behavior and effects of carrier mRBCs and their cargoes in unpredictable ways, and this parameter of safety should be carefully characterized. Having this

information in place, one can characterize effects of RBC-delivered agents in animal models at safe, and therapeutically relevant, doses.

9. Conclusion & future perspective

The authors of this admittedly opinionated article, representing three generations of biomedical researchers, are unquestionable enthusiasts of RBC drug delivery. We do believe that RBC drug delivery systems represent viable (and, in some cases, preferable) alternatives to synthetic counterparts. For this reason, we also believe that this exciting area of biomedical research is coming to the point when it is necessary to apply the highest standards of scientific rigor and scrutiny to the characterization, mechanistic understanding, and appraisal of benefit/risk balance of RBC drug delivery. In particular, a brutally sober prospective analysis of potentially problematic issues of this drug delivery approach is vital.

Impairment of biocompatibility of the carrier RBCs and other adverse and unintended effects should become the top priority for each translational RBC drug delivery approach. Raising these difficult questions is not intended to disrupt the momentum of the field, dissuade young talents, sour the translational outlook for developers and investors, or put off medical practitioners who will eventually apply RBC drug delivery. Rather, we feel that focusing research efforts on approaches that are unlikely to produce adverse effects in patients will help to bring this day sooner, and ultimately allow RBC drug delivery to truly flourish.

Acknowledgments

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