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Red blood cells: Supercarriers for drugs, biologicals, and nanoparticles and inspiration for advanced delivery systems☆,,☆☆

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Abstract

Red blood cells (RBCs) constitute a unique drug delivery system as a biologic or hybrid carrier capable of greatly enhancing pharmacokinetics, altering pharmacodynamics (for example, by changing margination within the intravascular space), and modulating immune responses to appended cargoes. Strategies for RBC drug delivery systems include internal and surface loading, and the latter can be performed both *ex vivo* and *in vivo*. A relatively new avenue for RBC drug delivery is their application as a carrier for nanoparticles. Efforts are also being made to incorporate features of RBCs in nanocarriers to mimic their most useful aspects, such as long circulation and stealth features. RBCs have also recently been explored as carriers for the delivery of antigens for modulation of immune response. Therefore, RBC-based drug delivery systems represent supercarriers for a diverse array of biomedical interventions, and this is reflected by several industrial and academic efforts that are poised to enter the clinical realm.

Keywords

Red blood cells; Erythrocytes; Drug delivery; Diagnostics; Nanoparticles

1. Introduction: artificial, natural, and hybrid drug delivery systems

Drug delivery systems (DDSs) can be divided into three types: artificial, natural (biological) and hybrids. Artificial DDSs, including nanocarriers made of synthetic or natural components, offer advantages of rational design, diversity, and control of materials. Biological DDSs including cells and their fragments offer biocompatibility and utilization of natural mechanisms for transport, localization, and responsiveness to factors of

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microenvironment in the body. Hybrid systems offer the theoretical possibility of combining these advantages while mitigating shortcomings.

This article reviews a class of DDSs that largely falls within the second and third categories, namely, erythrocytes or red blood cells (RBCs). RBCs represent natural transport agents that can be used to improve vascular and systemic delivery of drugs and probes, either encapsulated in the cell's inner volume, or coupled to the surface of RBCs. This review focuses on the second approach for loading drugs to RBCs and explores the role of RBCs in other drug delivery strategies, including: (i) their effect on other DDSs; (ii) DDSs using RBC as a "supercarrier"; (iii) artificial DDSs imitating features of RBCs; and (iv) hybrid DDSs that combine artificial elements and RBC components.

2. RBC as carriers in drug delivery: brief overview

RBCs naturally deliver "cargoes" including oxygen throughout the body via prolonged circulation in the vascular system. RBCs circulate in humans for about 3 months and in mice for about 40 days. RBCs lack organelles and have a biconcave shape; hence their entire inner volume and extended surface can be used for carriage of diverse compounds. From a practical standpoint, RBCs represent the most abundant, biocompatible, affordable, and easy to handle biological carrier for DDSs [1–5].

These natural carriers can be employed for drug delivery, for example, via loading drugs into donor or autologous RBCs prior to transfusion to a patient. For more than forty years, RBCs have been explored for delivery of many drugs in models ranging from *in vitro*, to small animal models and primates, as well as in clinical studies in human patients. This section provides a background pertaining to key aspects of drug delivery by RBCs. Detailed reviews of this subject can be found in this volume (see Magnani) and elsewhere [2,4–7].

2.1. Pharmacokinetics, biodistribution, and targets of RBC-loaded drugs

RBC carriage offers unique features advantageous for delivery of many agents for improved management of some pathological conditions. First, carriage by RBCs cardinally changes the pharmacokinetics (PK) of drugs by prolonging their circulation time in the bloodstream, facilitating redistribution in blood from plasma to cellular elements and redistribution in tissues. This change in PK is especially desirable for drugs that: (i) require a long-lasting depot in blood; (ii) have intra-vascular therapeutic targets accessible to RBCs; or (iii) are supposed to act in the extravascular compartments accessible to RBCs. In many cases, these changes to PK effectively enhance drug bioavailability, allowing reduced dosages and therefore alleviating adverse effects.

Carriage by RBCs shifts the pathway for drug clearance from renal filtration to bile excretion, typical of products of hemoglobin degradation. This switch in clearance mechanism results from: (i) RBC size, which, by many orders of magnitude, exceeds the limit of glomerular filtration; and (ii) macrophages in the reticuloendothelial system (RES), which have direct access to blood cells (particularly, in the hepatic sinuses and splenic follicles) and normally eliminate senescent and damaged RBCs. As such, these host defense cells represent natural targets for drug delivery using RBC carriage.

With the exception of these compartments of the RES, “normal areas” for RBCs in the body are limited to areas connected by the vascular system. However, RBCs often exit this space in pathological sites including: (i) the CNS (for example, in stroke or traumatic brain injury); (ii) penumbra of the myocardial infarction zone in immediate post-ischemic period; (iii) alveolar and airway compartments in acute lung injury, pulmonary hypertension, and other conditions which lead to damaged endothelium; and (iv) other numerous cases of pathological vascular permeability and vessel damage. In theory, RBCs may provide “passive” drug delivery to these sites.

In the absence of these pathological conditions, the list of main targets accessible to RBCs includes components of blood, endothelium, and a few constituents of the RES. RBC-coupled drugs do not act upon targets inaccessible to their carrier, which limits non-specific, systemic, and other undesirable off-target effects. Furthermore, coupling to RBCs impedes a drug’s freedom to interact with some targets even within the vascular compartment. For example, fibrinolytic plasminogen activators bound to RBC surfaces can act upon soluble therapeutic targets in blood, but not with endothelial receptors, which circumvents a main side effect of free plasminogen activators [8–10].

RBCs are attractive carriers for drugs that: (i) metabolize or capture agents circulating in blood; (ii) regulate blood clotting and thrombi transformations; (iii) require delivery to the endothelium; (iv) replace deficient lysosomal enzymes; and (v) regulate immune system (antigens, anti-inflammatory agents, stimulators, and inhibitors of phagocytes).

2.2. Loading drugs in RBC inner volume and to RBC surface

Drugs and probes can either be encapsulated into the RBC inner volume or coupled to the RBC surface (Fig. 1). Drugs encapsulated in RBC may interact with molecules diffusing from blood through the RBC membrane, but not with molecules outside the RBC membrane including components of blood and vascular walls. Thus, encapsulation in RBCs may reduce immune reaction to biotherapeutic agents or protect from pathways of inactivation.

In most protocols, loading drugs into the carrier RBC inner volume necessitates *ex vivo* manipulation. Hypotonic loading is achieved by incubating washed RBCs or blood with the cargo at hypotonic solutions which induce the formation of transient pores in the plasma membrane of swelling cells. This approach is currently used for several RBC carriers in clinical trials (Table 1) for the treatment of oncologic, inflammatory, and neurological diseases and commercial development is underway (Table 2). Newer developments include attempts to use cell-penetrating peptides to import therapeutic proteins in the carrier RBCs [11] and fusion of RBCs with drug-loaded liposomes [12].

Surface loading to RBCs can be achieved *ex vivo* by incubating either intact or modified RBCs with drugs or drug carriers. In such an approach, the drug of interest may react chemically with the RBC surface to form a covalent linkage or be non-specifically adsorbed. Most recently, biotechnological methods to culture genetically engineered RBCs *ex vivo* that bear recognition sequences have allowed for site-specific, covalent coupling of therapeutic proteins by way of transpeptidases [13]. Alternatively, drugs and carriers can be conjugated or fused with antibodies, antibody fragments, peptides, or other ligands that bind to RBC’s

surface. In the latter approach, RBC-targeted drugs can be used in two main protocols. First, this can be employed for *ex vivo* surface loading on donor or autologous RBCs, to avoid multi-step and inevitable damaging loading by encapsulation. Second, a simple intravascular injection of these RBC-targeted agents leads to rapid binding to RBC circulating in the bloodstream [14].

2.3. RBC surface loading: effects on cargo

RBC's surface can be used for the coupling of drugs, their carriers, and targeting agents. The prototype for the latter approach was designed in the early 1980s for targeting RBC-encapsulated drugs to the sites of vascular injury [15–18]. Antibody-coated RBCs have been studied *in vitro* and *in vivo* for targeting drugs to diverse cells—endothelium, smooth muscle cells, leukocytes, etc. [19–21]. More recently, RBCs painted with affinity ligands have been tested for targeting to circulating leukocytes [22,23].

Anchoring of drugs to RBC surface changes not only their PK and distribution but in some cases also modulates their functional features. For example, as discussed above, coupling of plasminogen activators including tPA and urokinase to RBC's surface restricts their ability to interact with receptors on other cells [9]. This mitigates adverse effects mediated by such interaction of soluble plasminogen activators, which is especially dangerous in the CNS [24]. As a result, RBC-bound tPA demonstrates an unparalleled safety profile in very challenging animal models including stroke [25], TBI [26], hypoxia [27], and thrombosis [28] in the cerebral vasculature [29].

On the other hand, coupling of drugs and carriers to RBC's surface, as well as the features of RBC-associated agents is influenced by the glycocalyx [30]. For example, RBC-coupled tPA is protected against plasma inhibitors by RBC glycocalyx [31]. Furthermore, coupling to RBC carrier creates high local concentrations of tPA, allowing expedient focal dissolution of blood clot matter in the close vicinity of RBC–tPA complexes, which in models of thrombotic occlusion of blood vessels results in the formation of patent channels spanning the plug and permitting reperfusion prior to dissolution of the thrombus [32,33]. Therefore, drug loading to carrier RBCs profoundly alters their pharmacological profile, in some cases, enabling effects simply unavailable to free drugs. It is tempting to postulate that RBCs may also significantly and favorably modulate delivery and therapeutic effects of nanocarriers, among other artificial drug delivery systems, and these interactions with nanocarriers are detailed below.

2.4. Immunological and biotechnological aspects of surface-loading of RBCs

When considering surface-coupling as a strategy for RBC-based DDSs, one must also consider the potential for immunomodulatory effects, which may be favorable or unfavorable depending on the desired application. In transfusion medicine, immune responses to allogeneic RBCs represent a significant obstacle to multiple transfusions in clinical practice. Despite decades of experience in the transfusion of RBCs and the observation of a multitude of allo-antibodies thereafter, the precise mechanisms by which transfused RBCs induce alloantibody formation in certain individuals and in certain clinical

scenarios remain to be fully understood and they are the subject of extensive investigation and are reviewed elsewhere [34,35].

A key observation is that despite the high degree of polymorphism of RBC antigens, and the fact that most transfusions are not matched for antigens other than that of ABO and RhD systems, only a subset of RBC recipients ultimately develop allo-antibodies [36]. Contributory factors likely include recipient immune status and degree of mismatch between donor and recipient populations [37]. Recently developed models of RBC alloimmunization to the human Kell antigen have revealed several interesting features of immunologic responses to red blood cells [38,39], including the observation that C3 is involved in "antigen modulation" on the RBC surface [40], and that a subset of allo-geneic RBCs may be resistant to clearance by antibodies in mouse models [41].

Of particular interest are recent studies that coupling of antigens to RBCs may induce tolerance. Hubbell demonstrated that injection of albumin conjugated with peptides binding to RBCs leads to T-cell deletion and immune tolerance to the antigen [42]. The authors also demonstrated efficacy in a model of autoimmune type 1 diabetes. It was speculated that such a strategy exploits natural regulatory mechanisms of eryptosis (programmed RBC death) whereby the body's own pathways are designed to clear RBCs via mechanisms that avoid auto-reactivity and provide tolerogenic signals. This same group recently furthered this approach by coupling of *E. coli* L-asparaginase (ASNase) [43], a biomolecular therapeutic used for treatment of acute lymphoblastic leukemia and non-Hodgkin lymphoma that was previously explored in RBC DDSs using internal loading approaches [44–46]. This therapeutic is limited by the high frequency of development of anti-ASNase antibodies, many of which are drug-neutralizing [47]. The authors found that *in vivo* loading of ASNase onto RBCs resulted in >1000-fold reduction of anti-ASNase antibodies, >10-fold improvement in pharmacodynamic effect, and tolerized mice to subsequent challenges with the wild-type enzyme.

These findings carry major implications for RBCs as DDS. Immunogenicity of protein therapeutics continues to complicate their repeated administration in clinical settings, including drug-neutralizing antibodies and infusion reactions, which can be severe [48]. Although strategies for immune tolerance induction have been developed [49], many include potent immunosuppressive agents such as rituximab and corticosteroids. RBC and platelet internal loading [44,50] and particle-based delivery systems [51] have demonstrated promise in overcoming these limitations, but the efficacy of the RBC surface loading for induction of tolerance is remarkable given its relative simplicity, and opens the possibility of RBCs as carriers of virtually all protein therapeutics, including enzyme replacement therapies (for example, FVIII replacement in hemophilia) where development of inhibitors remains a major clinical hurdle [52]. Overall, these important studies, together with decades of experience in transfusion medicine, have revealed that the immune responses to red blood cells may represent a complex balance between immunogenicity and tolerogenicity, dependent on a multitude of factors, including the inflammatory or disease state of the host.

Further expanding the RBC DDS platform, the recent progress in *ex vivo* differentiation of stem cells [53] (including peripheral blood CD34+ stem cells, umbilical cord stem cells,

human embryonic stem cells, and inducible pluripotent stem cells (iPSC)) into mature, enucleated RBCs has opened the possibility of *ex vivo* production of molecularly engineered RBCs as drug carriers. The early focus of *ex vivo* production of RBCs has largely been placed on the production and feasibility of transfusable packed red cell units, and has been reviewed elsewhere [54]. Such an approach offers the promise of overcoming limitations in donor supply, alloimmunization, and infectious disease safety in human blood products. However, production of multiple units of transfusable red blood cells faces major hurdles with respect to the ability to produce sufficient transfusable RBCs and the scalability of these approaches, although significant advances have been made [55]. In 2011, the group of Giarratana et al. demonstrated successful *ex vivo* GMP-grade production and transfusion of 10^{10} RBCs into a healthy donor [56]. Using ^{51}Cr labeling, they demonstrated survival of the cultured RBCs in circulation was comparable to what has been reported for native RBCs. In drug delivery applications, far fewer numbers of carrier RBCs might be expected to be required, which lessens concerns over scalability.

Indeed, building upon these successes in *ex vivo* RBC production, the group of Lodish and Ploegh demonstrated the successful production of engineered RBCs expressing modified proteins on the surface of *in vitro*-differentiated reticulocytes [13]. The modified RBCs were labeled with various probes, demonstrated long-circulation, and were able to be targeted by attachment of single domain antibodies. Modified RBCs produced with these technologies have enormous potential as drug carriers, potentially with cytoplasmic as well as surface cargoes, and have already prompted industrial development (see Table 2). Further complementing this approach is the significant recent advancement in genome editing strategies, such as CRISPR-Cas9 and zinc finger nucleases [57–59]. The optimal source of stem cells, and whether an autologous or allogeneic source should be used, remains an open question. The enucleated nature of red cell carriers, also lessens concerns over undesired effects of genetic manipulation such as the potential for neoplastic transformation.

2.5. Surface loading of RBC using targeting ligands

The surface of RBCs offers myriad membrane proteins representing potential sites for carriage of therapeutics. The diversity of RBC membrane proteins has been largely informed by the decades of experience in transfusion medicine, where phenotypic diversity across individuals [60], attributed in part to evolutionary pressure for protection against infections, can result in allo-antibody formation after transfusion. Although, in transfusion medicine, the focus is largely on epitopes present only in fractions of the population (and its subsequent potential for alloimmunization), drug delivery using RBCs is likely to focus on epitopes with very high frequency across populations. Several such targets have been described and are reviewed elsewhere [1,61] and a summary is provided in Table 3.

Despite the multitude of potential sites for drug coupling, few of these epitopes have been assessed for their suitability in drug targeting. Clinical experience in RBC membrane disorders, such as hereditary spherocytosis, demonstrate the critical importance of preserving (or allowing control of) RBC integrity, deformability, membrane-fluidity, shape, rheology, and resistance to phagocytosis and clearance [62–64]. Indeed, it has been demonstrated that extracellular ligands, including monovalent Fabs, can significantly alter

membrane rigidity, with studies suggesting that extracellular ligands can promote strong or transient interactions between membrane proteins, thereby impeding local distortion [65]. Such alteration of surface proteins can significantly alter RBC physiology, for example, by impeding malarial parasite entry of RBCs [66], altering oxidant susceptibility [67], RBC margination and hemodynamics [68], and interaction with platelets and vessel walls [69]. Considering these potential effects, the surface ligand chosen for RBC coupling should be chosen as to maximize biocompatibility, and will likely also depend on the number of ligands that may need to be coupled for therapeutic effect.

Among the earliest targets for surface coupling of therapeutics to RBCS was complement receptor type 1 (CR1). In normal RBC physiology, CR1 serves as a ligand for complement C3b, wherein immune complexes containing the activated component of complement C3b bind to CR1 on RBC, resulting in transfer to macrophages and clearance without untoward damage to other tissues [70]. In primates and humans, the large majority of CR1 (>90%) is found on RBCs (although at relatively low numbers per cell, ~500–1500 copies per cell, relative to other RBC proteins) [71,72]. Several decades ago, the group of Ron Taylor proposed emulating this natural mechanism using bispecific antibodies to clear pathogens or toxins by coupling them to RBC CR1 [73]. Importantly, the binding of anti-CR1 conjugates to RBCs apparently facilitates pathogen clearance without overt RBC phagocytosis [74] or damage to cells [75,76]. This approach has demonstrated efficacy for clearance of bacterial and viral pathogens and pathogenic antibodies, including in primate models [77–82], as well as coupling of therapeutics [83]. CR1 constitutes the Knops blood group, of which many high-frequency epitopes have been described, although their levels of expression are variable [84]. Interestingly, alloantibodies to the Knops antigens are not associated with hemolysis of transfused RBCs, suggesting that this target can tolerate drug loading with little adverse effects.

Among the most abundant proteins on the RBC surface are the band 3 and glycophorin A proteins. Band 3, an anion exchange protein, represents approximately 20% of the protein mass on the RBC surface, and exists both in isolation and in multiprotein complexes that are critical for maintaining linkages to the spectrin skeletal network below [85,86]. Band 3 carries a large portion of the ABO blood group sugars and is central to the maintenance of RBC membrane integrity and structure, as evidenced by the various membrane disorders associated with band 3 mutations. It may represent an attractive target for surface coupling due to the very large number of available sites such that only a relatively small fraction would need to be occupied. Band 3 is classified as the Diego blood group and high-frequency epitopes (such as Wr(b)) have been described [87]. Glycophorin A (GPA) is a type I membrane-bound sialoglycoprotein that functions as a receptor for complement, a chaperone for band 3 transport, and contributes to RBC structural integrity and the negative charge of the RBC glycocalyx [88]. It is similarly present in very high numbers and, like band 3, is also expressed on portions of the kidney. This protein has been successfully used to couple therapeutics to RBCs in animal models [14,89,90], and is among the most commonly used markers for RBC lineage. Glycophorin A is classified as the MNS blood group and high-frequency epitopes, such as En(a) have been described. Additional glycophorins on the red cell surface, although fewer in number, include glycophorins B, C,

and D which are part of the MNS (glycophorin B) and Gerbich (glycophorins C and D) blood groups.

Other potential targets for surface coupling include the Kell, ART4, ICAM-4, CD59, DAF, GLUT1, and Rh family proteins, among many others. These proteins have a range of expressions from low to high copy numbers, facilitating design of therapeutics according to the number of moieties that should be coupled to RBCs. The Kell family, although relatively low in expression (1e4/cell) [91], has been successfully modified in genetic engineering of *ex vivo* derived RBCs [13], although when considering *in vivo* loading, it should be noted that Kell is also expressed on many other tissues including parts of the brain, and lack of Kell is associated with a severe clinical phenotype [92]. ART4 constitutes the Dombrock blood group, and although it is known to encode an ADP-ribosyl-transferase, the enzymatic function in RBC physiology is unknown [93,94]. ICAM-4 constitutes the LW (Landsteiner Weiner) blood group and is involved in RBC adhesion and erythropoiesis [95, 96]. CD59 and DAF are involved in complement regulation, and although CD59 has yet to be designated as a specific blood group [97], DAF constitutes the Cromer system [98]. GLUT1 is a highly expressed, minimally polymorphic RBC antigen involved in glucose transport that is also expressed in many other tissues [99]. The Rh family is among the most well-described in transfusion medicine, is thought to be involved in ammonia transport, has moderate expression levels, and is relatively erythroid-specific [100–102]. High-frequency epitopes for all the above blood groups have been characterized.

Binding of antibodies and other multivalent ligands to some high-density determinants such as GPA (e.g., TER119 antibody) causes RBC aggregation, and therefore monovalent scFv fragments are likely to be preferable and, in addition, support modular recombinant engineering of diverse fusion proteins. Indeed, TER119 antibody-derived scFv-fusions have been used by John Atkinson's and other labs for coupling to RBC of therapeutic fusions including those controlling RBC damage by complement [14,90,103]. Interestingly, Rh-antigen D targeted ligands using remodeled monovalent scFv in multivalent format renders agglutinating ability without activation of complement [104], and multivalent anti-Rh(D) scFv/CR1 fusion restores the function of this natural RBCs glycoprotein involved in host defense [105]. Selection of ligands using phage display libraries provides a means to discover novel peptide and antibody ligands [106] that facilitate binding of diverse cargoes and improve their pharmacokinetics [107].

3. RBC and nanocarriers

Artificial carriers—liposomes, polymeric carriers, dendrimers, filomicelles, and other nanoparticles—are capable performing diversified biomedical applications [108]. Their features can be controlled via addition of surface modifications (inclusion of hydrophilic polymers, self-peptides, targeting moieties, etc.) [109], inclusion of stimuli-responsive functions (e.g. enzyme, pH, near-infrared light, or magnetic responsiveness) [110], or modifications to their physical parameters (e.g. size, shape, elasticity) [111]. It is tempting to speculate that hybridization of artificial DDS with RBC may help overcome some of the problematic issues that limit the utility of nanocarriers, such as inadequate behavior in the

bloodstream. On the other hand, RBCs represent an important and influential, yet underappreciated in the past, counterpart of DDSs.

Indeed, RBCs normally occupy ~50% of blood volume and, due to such high concentration and peculiar hemodynamics, greatly influence circulation of other particles and their interaction with vascular walls [112]. In arteries, where blood perfusion is associated with high levels of fluid shear stress, RBCs tend to occupy the mainstream and avoid the micron-scale marginal layer of blood that directly contacts endothelial surface, thereby pushing other circulating particles to the cell-free layer (Fig. 2a) [113]. This hydrodynamic phenomenon intensifying interactions of particles with endothelial cells, known as “margination” [114], is of extreme importance in designing vascular targeted nanocarriers [115]. Margination, or margination potential, can also be described as a particle’s ability to either travel from bulk flow to vessel walls or, more generally, the ability to travel close to vessel walls. In general, this has been studied using perfused model vessels [116] and computations simulating interactions between nanocarriers and endothelium in differently sized vessels, both with and without RBC presence [113,117].

The amplitude of margination effect depends on a variety of factors including hydrodynamic conditions, vessel diameter and shape, and geometry of particles [112,118–120]. Here, we will focus on the role that circulating RBCs, either alone or interplayed with the above factors, have on influencing particle margination. In one of the earliest reports, a particle-cell computational model was leveraged to show how nanocarriers more effectively marginate to vessel walls in the presence of RBCs (Fig. 2b), as compared to a RBC-free vessel (Fig. 2c) [113]. These effects were attributed as the cell-free layer effect (Fig. 2a), which captures interactions between particles and RBCs. RBC-mediated margination effects are also dependent on particle shape, as shown by a recent dissipative dynamics model where both spherical and ellipsoidal particles increase as hematocrit levels increase, with spheres marginating more efficiently [120]. Other computational studies have reported mixed results regarding margination advantages of higher aspect ratio particles in the presence of RBCs, where in some cases higher aspect ratio particles marginate better [121], and in other cases worse [120], than spherical particles. However, it should be noted that a recent *in vitro* microfluidic study, in contrast to the above computational studies, has reported enhanced margination of high aspect ratios particles in the presence of RBCs [122]. Other shapes, notably discoidal particles, have exhibited enhanced margination in the presence of RBCs, both *in vitro* [123] and *in vivo* [123,124].

Particle size has also been shown to greatly impact margination in the presence of RBCs. Overall, the majority of computational [120,125] (exception here: [113]), *in vitro* [126,127], and *in vivo* reports [123, 125] have reported enhanced margination of larger particles, independent of shape, as compared to their smaller counterparts in the presence of RBCs. A recent *in vivo* study highlighted how small 200 nm particles (Fig. 2d) are distributed randomly and non-specifically throughout a vessel section, whereas larger 1000 nm particles (Fig. 2e) show enhanced accumulation at the vessel’s outer layers or the vessel walls [125]. Indeed, quantitative analysis revealed that the 1000 nm particles were 3× more concentrated at the vessel walls than their smaller 200 nm counterparts, highlighting their enhanced margination abilities [125].

More advanced *in vitro* models are combining RBCs, flow, and vessel geometry effects in devices containing inflamed endothelial cells, so as to better simulate binding to pathological tissues [128]. While it is widely known that functionalization of nanocarriers occurs with affinity ligands that bind to endothelial surface determinants such as intracellular cell adhesion molecule 1 (ICAM-1, which is used in several examples given below in this article) [129,130], it has only recently been shown that affinity-mediated targeting is further enhanced due to the cell-free layer and RBC-mediated nanocarrier-margination effects [126].

The interaction of nanocarriers with endothelial luminal surface is greatly influenced by the cellular glycocalyx [131]. One of the postulated functions of this brush-like structure formed on the surface of endothelial and other cells by anionic carbohydrate chains of glycoproteins is to control vascular adhesion of blood cells [132]. The interplay between cellular interactions of the glycocalyx and circulating nanocarriers awaits more definitive characterization. For example, the glycocalyx spatial barrier may inhibit binding of neutral and anionic particles, facilitating the binding of cationic counterparts; however, the effects of circulating RBCs on glycocalyx–nanocarrier interactions are not well known. Treatment with glycocalyx-degrading enzymes in animals markedly augments endothelial binding of polymeric spheres (diameter ~100 nm) targeted to ICAM-1 [133], so it seems logical that blood cells and other circulatory molecules can further influence interactions between nanocarriers and the glycocalyx. Additionally, the endothelial glycocalyx modulates endocytic uptake and thus is involved in the intracellular delivery of nanocarriers [134]. The glycocalyx also modulates the interaction of endogenous regulatory entities (e.g. plasma cofactors and inhibitors) with biotherapeutic agents bound to endothelium, similarly as RBC's glycocalyx protects bound serine proteases from plasma inhibitors [31,135].

4. Particles/carriers designed for targeting to or carriage by RBC

In the 1980s, Daan Crommelin and coworkers showed that injection of liposomes carrying targeting Fab fragments to RBCs results in the prolongation of the attached liposomes at the cost of enhanced clearance and hepatic uptake of RBC themselves [136]. In other words, RBC-adhered particles circulate for longer times, but the modified cells exhibit more rapid clearance than unmodified cells. In this study, RBCs were just a model target cell to test an approach for the elimination of tumor cells from the circulation. In a modern application, RBC-targeted liposomes are being studied for delivery of anti-malarial agents. In these studies, antibodies and semi-specific agents such as heparin have been used to target liposomes to *Plasmodium*-infected RBCs [137,138].

An important corollary of targeting DDSs to RBCs is that, theoretically, similar to RBCs being utilized as a carrier for drugs, RBCs can also be used for carriage of nanocarriers. Encapsulation of drug-loaded hydrogel nanoparticles in RBCs and other nano-scale drug formulations has been studied *in vitro* [139,140]. Loading in RBCs and laser-triggered release of gold nanoparticles has also been reported [141]. Encapsulation of iron oxide nanoparticles into RBCs renders them responsive the magnetic field *in vitro* [142]. These ideas might evolve in mechanisms for controlled release and magnetic guiding of RBC-carried nanocarriers for therapeutic and imaging purposes [143]. The main challenges of

these approaches are the loss of RBC's compatibility and the need for extracorporeal loading. As such, surface loading of nanocarriers to RBCs seems preferable [144].

Recent iterations of binding carriers to RBC supercarrier, called "cellular hitchhiking" may combine the advantages of synthetic nano-particles and the natural delivery abilities of circulatory cells [145, 146]. RBCs represent arguably the simplest biological carrier for cellular hitchhiking.

4.1. Loading nanoparticles on/in RBCs

There are a variety of methods to attach nanoparticles of various compositions, surface chemistries, sizes, and shapes to the RBC surface (Fig. 3a, b and c), or encapsulate nanoparticles in RBCs (Fig. 3d). In either case, it is essential to consider both the properties of the particles and the end application of the RBC delivery system so that the chosen method offers both optimal attachment/inclusion while limiting damage or impaired function of the RBC. Here, we will review the three main strategies used to combine nanoparticles and RBCs: (i) non-specific adsorption of nanoparticles to RBCs, (ii) specific binding, either ligand-receptor mediated or through chemical conjugation, and (iii) encapsulation of nanoparticles within RBCs.

The simplest method involves non-specific physical adsorption of the particle to the RBC surface. Similarly to other cells, RBC's plasma membrane contains relatively hydrophobic domains, which present an area for binding hydrophobic particles. These interactions have been shown to be independent of charge, as both positive and negatively charged hydrophobic particles bind to RBC surfaces (Fig. 3a) [147]. In other examples, cationic particles bind to the anionic RBC's glycocalyx [148], while mesoporous silica nanoparticles with silanol functional groups have been shown to adsorb to RBCs via interacting with the phosphatidyl choline present on RBC membranes (Fig. 3b) [149].

Covalent and non-covalent loading protocols with variable degrees of molecular specificity have also been reported. Proteins and carbohydrates on RBC's surface contain an abundance of amine and thiol groups [150] available for conjugation via a variety of chemistries involving modification of the RBC surface. For example, chemical conjugation of biotin to RBC surface allows avidin functionalized particles to readily bind and attach to the biotinylated RBC [151–154]. Coating particles with antibodies or other ligands of RBC proteins offers most specific loading [1]. Recently, phage display has been exploited to discover novel peptides that bind to RBCs [42,107] where some of these peptides have been shown to facilitate nanoparticle binding to RBCs [106].

Generally, particle encapsulation in RBCs is based on the same principle of hypotonic swelling that opens up pores in the membrane, allowing nanoparticles to diffuse into the cell before returning the cell to isotonic conditions to seal the pores (Fig. 3d) [142]. This method requires that the nanoparticles be small enough to enter the pores (<100 nm [141]) during hypotonic treatment. Typically, inorganic nanoparticles (e.g. iron oxide or gold) have been utilized for encapsulation inside RBCs for imaging applications [155,156].

4.2. RBC-bound carriers

Coupling to RBC supercarrier prolongs blood circulation and alters tissue distribution of nanoparticles in animals. Hydrophobic polystyrene particles bound to RBC circulated in animals for markedly longer time than free counterparts with particle sizes ranging from 100 to 1100 nm, with 220 nm and 450 nm nanoparticles exhibiting the longest circulation times when hitchhiked onto RBCs [157]. This outcome was verified using adsorption onto RBCs of anionic and other surface chemistries nanoparticles, showing prolongation of circulation of hitchhiked particles of 220 nm for all surface chemistries examined [147].

Hitchhiked nanoparticles detach from circulating carrier RBCs in a time-dependent manner likely via shear stress-based forces and physical contact with vascular walls [147]. This manifests as transfer from RBC and pulmonary accumulation of RBC-hitchhiked nanoparticles, while simultaneously lowering uptake in the liver and spleen (i.e. clearance organs) [158]. Lung accumulation of RBC-hitchhiked nanoparticles was as high as 7-fold as compared to free nanoparticles. Transfer of nanoparticles from RBC surfaces to lung endothelium was confirmed via independent tracking of RBCs and nanoparticles.

Pulmonary accumulation of RBC-carried nanocarriers was further enhanced via attachment of lung-specific antibodies to the surface of RBC-adsorbed nanoparticles (Fig. 3c) [158]. In a follow-up study [159], lung accumulation and clearance organ avoidance of RBC-hitchhiked nanoparticles were improved further by leveraging the use of rod-shaped particles, which have recently been used for lung targeting [160]. Spherical and rod-shaped particles, RBC-hitchhiked and non-hitchhiked, were functionalized either with clearance organ targeting moiety, IgG, or with ICAM antibodies providing affinity to endothelium and directly compared for their liver, spleen, and lung uptake (Fig. 4a). The formulation which provided the best lung targeting, while decreasing uptake in the liver and spleen, leveraged synergistic contributions from RBC-hitchhiking, lung-targeting antibodies, and rod-shaped nanoparticles [159].

In other RBC-hitchhiking work, focus has been placed on transferring the long-circulation abilities of RBCs to hitchhiked inorganic nano-particles for diagnostic and imaging purposes. For example, 35 nm iron oxide nanoparticles with a silica shell were non-specifically adsorbed to the surface of human RBCs, and hitchhiked RBCs were shown to respond to a constant magnetic field (Fig. 4b) [161]. In this study, human RBCs that were injected into mice showed negative contrast enhancement in the liver and spleen, which increased over a period of 24 h (Fig. 4c), likely due to an increased clearance of RBC-hitchhiked iron oxide particles [161].

Alternative approaches used RBC-encapsulated nanoparticles as a means to increase their circulation time [162]. These studies have also shown that iron oxide-loaded RBC can be propelled and controlled using both magnetization and ultrasound stimulation, while preserving the integrity of the cell during stimulation [142]. In one of the first examples of using RBC-hitchhiking for a therapeutic benefit, RBCs were loaded with both iron oxide nanoparticles and aspirin for the magnetic delivery and treatment of arterial thrombosis in dogs [163]. Given the large number of clinically approved iron oxide-based imaging agents [164], RBC-hitchhiking could potentially be integrated with a number of these clinically

relevant imaging agents given non-traumatic conditions for particle loading into RBCs [155]. RBC encapsulation of gold nanoparticles can potentially be used in tandem with X-ray absorption to track RBC flow in vessels [156].

The RBC-hitchhiking approach offers then combination of drug delivery and imaging. For example, coupling to biotinylated RBCs of avidin-conjugated iron oxide nanoparticles (14–18 nm) prolonged their blood circulation and provided magnetic targeting in a tumor model in mice, which improved tumor visualization using fluorescent and MR imaging (Fig. 4d) and tumor shrinkage by doxorubicin encapsulated into the RBC interior by diffusion and a photodynamic agent loaded onto the RBC-bound nanoparticles [151].

These examples illustrate diverse and powerful potential biomedical utility of RBC-coupled nanocarriers and provide a strong impetus to both extend the versatility of this DDS platform and rigorous analysis of its translational and clinical aspects, including adverse effects (see below in Section 5).

5. RBC-inspired and RBC-hybrid nanocarriers

The aim of RBC mimicry is to yield carriers capable of navigating the vasculature and remaining in circulation similarly to RBCs. To date, no synthetic carrier has proven capable of circulating for up to a month, and in fact, most are cleared from the circulation in minutes [165]. Carriers capable of long-circulation will shift the paradigm of nanoparticle drug delivery and diagnostics, and as such, synthetic versions of RBCs are of high interest for applications in drug delivery and imaging. There are two main modern approaches to combining the advantages of synthetic materials and RBCs [166]. The first is to design carriers mimicking desirable physical features of RBCs—size, shape, elasticity, and lack of adhesion [167,168]. The second is to design synthetic carriers that contain proteins [169] or cell membranes [170] or other components of natural RBCs.

5.1. Synthetic RBCs

Synthetic RBCs (sRBCs) have long been the Holy Grail of bioengineering [171], given the demand for blood substitutes in transfusion medicine [172,173]. New technological developments offer hope that this task will be ultimately achieved.

A technique of particle replication in non-wetting templates (PRINT®) has been devised (Fig. 5a) for the high-throughput automated synthesis of hydrogel sRBCs. Injection in mice of iterations of sRBCs produced using this technique showed that highly elastic PEG-hydrogel sRBCs (Fig. 5b) with diameters similar to RBC (6μm) were capable of circulating for longer times than rigid sRBCs, which were unable to deform through identical channels (Fig. 5c) [168]. Further, sRBCs with a diameter most closely matching that of RBCs circulate for the longest times [174]. Moreover, PRINT® sRBCs were designed to harbor hemoglobin and were shown to possess oxygen binding capacity (Fig. 5d) [175]. PRINT® sRBCs were also engineered to possess functions that natural RBCs do not have, namely, pH-modulated swelling (Fig. 5e–i and e–ii)) and the ability to control the spatial conjugation (e.g. exterior versus interior) of small molecules (Fig. 5f–i and f–ii) [176]. These studies highlight the role of size, shape, and elasticity in designing drug carriers, whereas the

versatility of the PRINT® approach gives hope that it may yield medically useful sRBCs with the potential of functionalities that RBCs themselves do not possess.

Two other recently devised approaches, layer-by-layer (LbL) particle templating of polyelectrolytes, and electrohydrodynamic jetting were used independently and in combination to create a variety of sRBC formulations (Fig. 6a), including sRBCs made of: (i) natural RBC proteins and synthetic polyelectrolytes created via LbL on hollow polystyrene spheres subjected to core dissolution (Fig. 6b), (ii) PLGA prepared from electrohydrodynamic jetting (Fig. 6c), and (iii) a combination of protein/polyelectrolyte LbL templating on sRBCs prepared from electrohydrodynamic jetting with core dissolution (Fig. 6d). These particles were shown to possess RBC-like features, including geometry (Fig. 6e), elasticity allowing transport through capillaries smaller in diameter than the sRBC (Fig. 6f), and oxygen binding capabilities. Further, sRBCs have been tested for loading and release of heparin, and loading of iron oxide nanoparticles (Fig. 6g) [167]. Other methods for synthesis of LbL [177] and hydrogel [178] sRBCs are also being devised and may evolve to yield DDSs formulations that might find medical utility.

5.2. Hybrid nanoparticles containing RBC components

An alternative approach is to employ RBC components as elements of synthetic DDSs to improve biocompatibility. Historically, exosome-like fragments of RBC, i.e. vesicles called “nanoerythrosomes”, which could theoretically reach target sites including tumors that are typically inaccessible to RBCs, were proposed two decades ago [179,180]. Initial *in vitro* studies showed some promise of anti-tumor effects of prototype derivatives of RBC membrane ghosts loaded with cytotoxic agents. Notwithstanding, this DDS apparently had not been pursued to the level of systematic studies *in vivo*, perhaps due to very rapid clearance from blood, as this formulation lost biocompatibility [181]. In one recent study, nanoerythrosomes filled with anti-malarial agents were cleared in rats within a few hours (although slightly slower than free drug) [182].

Recently, alternative strategies emerged. In one strategy, RBC membranes were used to form a shell that shields the synthetic nanoparticles from direct contact with the immune system (so-called “RBC-cloaked” nanoparticles). In this approach, RBC membranes isolated after hypotonic lysis (RBC ghosts) are extruded alongside carboxylated PLGA nanoparticles (~70 nm) to form the RBC-cloaked particles (Fig. 7a and b) [170]. These particles have prolonged circulation versus PLGA-based and, more impressively, PEGylated particles (Fig. 7c) [170]. These hybrid formulations, which can also be synthesized with an inorganic core [183], offer additional functions and possibilities to conjugate targeting moieties [183], controlled release of chemotherapeutics (e.g. doxorubicin) [184], acoustical propulsion [185] and photothermal effect [186]. In a therapeutic application of this, RBC-cloaked gold nanocages were shown to provide enhanced photothermal tumor ablation versus non-cloaked counterparts [186].

RBC-cloaked carriers, in theory, can provide a platform for targeted drug delivery [187], vaccination [188], and toxin absorption [189], among other applications. For example, toxoid (a pore-forming staphylococcal α-hemolysin, Hla) damages cell membranes and can lead to skin damage if used subcutaneously. Mice immunized with toxoid incorporated in

RBC-cloaked versus control particles showed dramatic resistance to Hla subcutaneous infection as compared to unvaccinated and heat-treated Hla vaccinated groups.

In another strategy, RBC components such as RBC proteins that inhibit RBC's clearance by phagocytes, or peptides derived from these proteins, are conjugated to the surface of particles to provide more specific mechanisms to avoid immune system clearance as compared to widely used "stealth" methods of PEG conjugation, which persists with unresolved problematic issues [190]. For example, a glycoprotein CD47, which regulates the RBCs ability to be recognized as "self" and thus avoid clearance by macrophages (Fig. 8a), has recently been exploited as a functional molecule that can be incorporated in nanocarriers [191]. In prototype studies, coating of polystyrene particles by human CD47 inhibited their uptake by human phagocytes even when particles were opsonized with IgG [191]. In the follow-up study [169], small "self" peptides derived computationally from human CD47 were shown to increase the circulation time of 160 nm polystyrene nanoparticles (Fig. 7b) by inhibiting uptake by macrophages. As a result of improved circulation, CD47 and CD47 "self" peptide particles loaded with paclitaxel showed increased tumor accumulation (Fig. 7c) and tumor shrinkage, compared to "non-self" control formulations [169]. CD47 and "self" peptides present one of the only examples of a molecule that give off an active "self" signals, which, unlike non-active hydrophilic coatings that delay opsonization and clearance, addresses the clearance issue directly to succeed even in cases where opsonization has occurred.

6. Current challenges and opportunities

In this decade, a few RBC-based DDSs have entered (Table 1), are entering, or are approaching the clinic.

For RBC-based DDSs, the dependence on blood typing and storage conditions on their circulation and therapeutic effect should be defined. Concerning RBC-specific cellular therapies, questions concerning the source (e.g. autologous versus allogeneic), storage, and extent of chemical modification of RBCs need to be addressed. Drug loading might affect RBC's biocompatibility, and strict control of this aspect is prescribed to ensure the safety of the medical use of RBC drug carriers. The key issue in this approach is to avoid RBC damage by cross-linking cell membrane or other mechanisms associated with an excessive coupling of therapeutic molecules to RBC surface. For example, drugs endowed with an affinity to RBC surface determinants may inhibit molecules in RBC membrane that normally protect against phagocytosis or damage by complement system, alter plasticity of RBC membrane, or interact in an undesirable or unintended fashion with components of blood and other accessible vascular targets. The therapeutically effective and potentially toxic levels of drugs and nanocarriers remain to be determined for each formulation [158]. Loading schemas that affect RBC biocompatibility, for example, by reducing their elasticity and deformability [149], are likely to be problematic [192]. While many studies have focused on the positive aspects of RBC drug delivery, few have investigated the potential negative effects.

Non-specific physical adsorption of nanoparticles onto RBCs has been shown to have minimal hemolysis effect on the modified RBCs [149,158] and also have minimal effects on RBC structure following desorption from the RBC surface [158]. Specific methods for RBC loading by nanoparticles have not been widely explored for particle binding, and as such, it is not clear if specific binding or chemical modifications will damage or impair the hitchhiked RBCs; however, it has been shown that some methods conjugating drugs directly to RBCs can lead to damaged membranes and subsequently rapid clearance *in vivo* [5]. *In vitro* studies characterized how particle size, genesis, and surface chemistry of super-paramagnetic iron oxide nanoparticles (SPION) influence the cell integrity of the RBCs following encapsulation and implicated agents used for particle dispersion [155], particle loads, and duration of the loading [156]. Loading conditions producing the least RBC damage *in vitro* must be recapitulated *in vivo* [162].

In general, other limitations and advantages for RBC delivery systems designed to deliver drugs or nanoparticles exist. For example, RBCs are typically restricted to the vascular space, and as such are naturally limited in their abilities to penetrate and cross tissue barriers. This can affect the delivery of both drugs and nanoparticles for RBC delivery systems, and consideration of the best application for RBC delivery systems should be taken. Alongside this, tissue penetration issues for RBC delivery systems through microscopic barriers (e.g. the blood–brain barrier, endothelial barriers) also translate down to nanoscale barriers (e.g. cellular internalization). For these barriers, contact and interactions between nanoparticles/drugs loaded onto/into RBCs may be difficult depending on the location of the target cell.

Although RBC-hitchhiking brings together cellular therapies and nanotechnology, it may compete with both approaches. Future key areas of interest include: (i) investigating highly perfused tissues other than the lungs as potential targets for RBC-hitchhiking therapies, (ii) developing methods that can allow for on-demand detachment of the particle from the RBC so as to facilitate nanoparticle accumulation at any site in the body, and (iii) examining whether or not covalent attachment of particles to RBCs could improve nanoparticle circulation times even further. Hybrid RBC–nanocarrier approaches are intriguing and offer a chance to avoid immunological issues of PEG coating [190]. Exciting studies of RBC-cloaking and “self-signaling” DDSs warrant rigorous analysis of biocompatibility and toxicity.

Overall, RBC drug carriers present complex regulatory challenges. The regulatory challenges will be dictated by whether the materials are entirely synthetic, hybrid biologic/synthetic, or entirely cell-based. For entirely synthetic nanomaterials, these challenges have been reviewed elsewhere [164,193] and although they remain difficult and often costly, successful examples of clinically approved nanomaterials include liposomal formulations, protein-based nanoparticles, imaging agents, and device components [194]. For methods incorporating RBC elements into hybrid materials, the same standards can be expected to apply, with the additional consideration that they now contain biopharmaceuticals and, potentially, blood-derived elements that are subject to additional regulatory procedures. As part of the FDA, the Center for Biologics Evaluation and Research (CBER) regulates blood

products and biological therapies [195] and provides guidance to investigators and manufacturers regarding these additional regulatory procedures.

Although the Center for Devices and Radiological Health (CDRH) regulates most medical devices, CBER continues to regulate those used with blood and cellular products, for example, the equipment expected to be used for the collection and processing of RBC products and other cellular therapies. Alteration of donor red blood cells (for example, leukoreduction, irradiation, and washing) is already practiced in the clinical setting through hospital blood banks. Such modifications lead to the designation of blood banks as "manufacturers" of drugs, and makes them subject to FDA licensure, registration, and regulation [196], including FDA inspection every two years. If *ex vivo* loading of red cells is to become widely used in the clinic, the resulting complex regulatory and compliance issues should be addressed early in the translational process for successful RBC-based carriers. Important practical considerations include the logistics, technique, and timing of cell collection, processing, and reinfusion. For example, peripheral blood stem cells for hematopoietic transplantation are usually harvested by apheresis and subsequently frozen before later reinfusion, while other cellular therapies such as chimeric antigen receptor T cells are generated on demand and reinfused upon successful manufacture. The techniques by which RBC-based delivery systems would be collected and manufactured will likely depend on their application and source. As one example, the EryDex system for RBCs loaded with dexamethasone utilizes a simple 50 mL peripheral blood draw that is then loaded using a proprietary RBC loading device [202,203].

Although red cell transfusion has long been successfully practiced clinically, cellular therapy using engineered cells, both autologous and allogeneic, represents a field still in its earliest clinical stages and with regulatory issues that remain to be fully defined and addressed [197–199]. Nonetheless, successful examples of cellular therapies that involve autologous cells manipulated *ex vivo* are growing in number [200,201]. As a potential alternative, methods permitting the *in vivo* loading of RBCs [14,42] are advantageous over those requiring *ex vivo* cellular loading and reinfusion in this respect, as the manufactured product (such as a recombinant protein) can be separately subjected to well-established quality control and manufacturing practices and likely faces better-defined regulatory hurdles.

7. Conclusion

Since the first exploratory studies four decades ago, red blood cell-based drug delivery systems have evolved into a diverse series of DDS platforms for drugs and nanocarriers loaded either inside or on the surface of this supercarrier, which profoundly changes the PK, biodistribution, clearance, and metabolism of the cargoes. In many (if not all) instances, RBC carriage also changes the activity, regulation, and pharmacodynamic characteristics of the cargoes, including their immunological and therapeutic features. Furthermore, naive RBCs circulating in the bloodstream influence behavior *in vivo* and interaction with target and non-target cells of carriers that do not (or at least are not supposed to) interact with RBCs. Using RBCs as "mother ship supercarriers" enables previously unrecognized options for transfer of nanocarriers to the vascular cells, in some cases, augmenting the efficacy of targeting of nanocarriers directed to cells of interest by affinity ligands. Both coupling to

RBC and hybridization of RBC with synthetic components are being explored to combine affinity, geometry, plasticity, and features of nanocarriers with unique biocompatibility and transporting features of RBCs. New biotechnological techniques to produce modified RBCs, RBC carrier hybrids, and RBC carriers are currently undergoing transition from academic to industrial and clinical domains.

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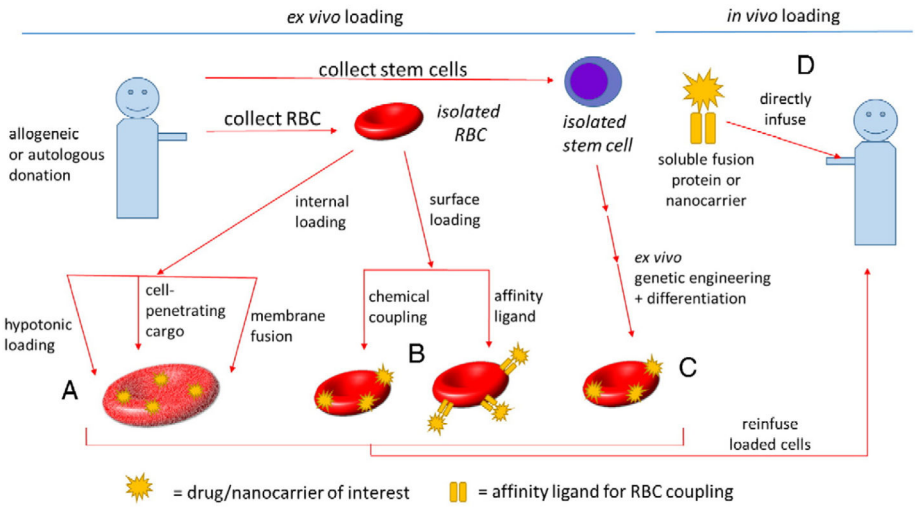

Fig. 1.

Strategies for RBC-based DDSs. Approaches can be broadly categorized as *ex vivo* versus *in vivo* RBC loading. Among *ex vivo* approaches are included: (A) internal loading of drugs/nanocarriers by penetration through the RBC membrane, for example, by hypotonic loading, cell-penetrating cargo, or fusion with liposomes; (B) surface loading by chemical coupling and binding of RBC-targeted affinity ligands; (C) differentiation of engineered, enucleated RBCs derived from stem cells (peripheral blood CD34+ cells, iPSCs, etc.). All *ex vivo* approaches would ultimately require reinfusion of the manipulated cellular RBC product after quality control testing. *In vivo* loading would be comprised of (D) drugs or nanocarriers fused or coupled to RBC-targeted affinity ligands such that they would bind rapidly to RBC in circulation.

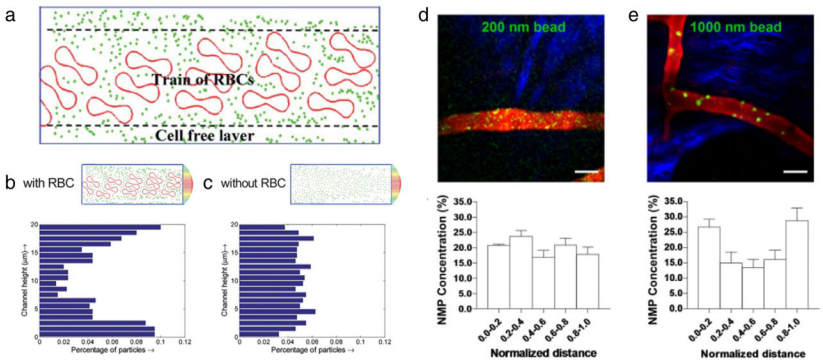

Fig. 2.

Interactions between red blood cells and nanocarriers in microcirculation. (a) Schematic of the cell-free layer with flowing nanocarriers and RBCs. Distribution of particles flowing in a straight channel in a particle cell computational model (b) with and (c) without RBCs. Intravital microscopy and quantitative analysis of the radial distribution of nanocarriers of (d) 200 nm and (e) 1000 nm diameter. (a, b, and c) Adapted from [113] with permission from The Royal Society of Chemistry. (d and e) Adapted by permission from Macmillan Publishers Ltd.: Scientific Reports [125], copyright (2013).

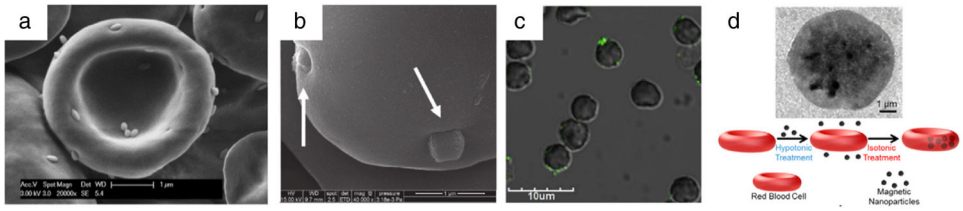

Fig. 3.

Attachment/inclusion of nanocarriers to/in red blood cells. (a) Polystyrene rod-shaped, (b) mesoporous silica, and (c) antibody-functionalized polystyrene spheres adsorbed to the surface of RBCs. (d) Hypotonic swelling and subsequent sealing of RBCs to include nanocarriers inside RBCs. (a) Reprinted from [159], Copyright (2015), with permission from Elsevier. (b) Adapted with permission from [149]. Copyright (2011) American Chemical Society. (c) Adapted with permission from [158]. Copyright (2013) American Chemical Society. (d) Adapted with permission from [142]. Copyright (2014) American Chemical Society.

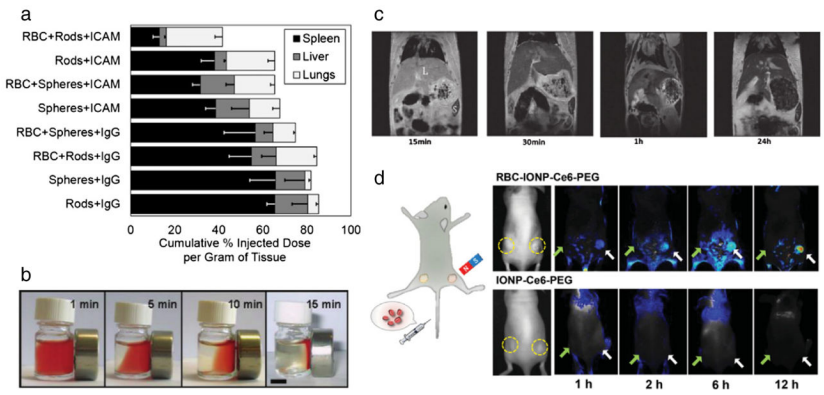

Fig. 4.

Applications of RBC-bound or loaded nanocarriers. (a) The adsorption of spherical or rod-shaped polystyrene particles functionalized with endothelium targeting antibody or immune system targeting antibody exhibit distinct distribution in liver, spleen, and lungs. (b) Magnetic responsiveness of RBC-adsorbed iron oxide nanoparticles, used for (c) magnetic resonance imaging contrast. (d) Biotinylated RBCs with avidin-conjugated iron oxide nanoparticles used for enhanced magnetic targeting in a tumor model in mice. (a) Reprinted from [159], Copyright (2015), with permission from Elsevier. (b and c). Reproduced from [161]. (d) Reproduced from [151].

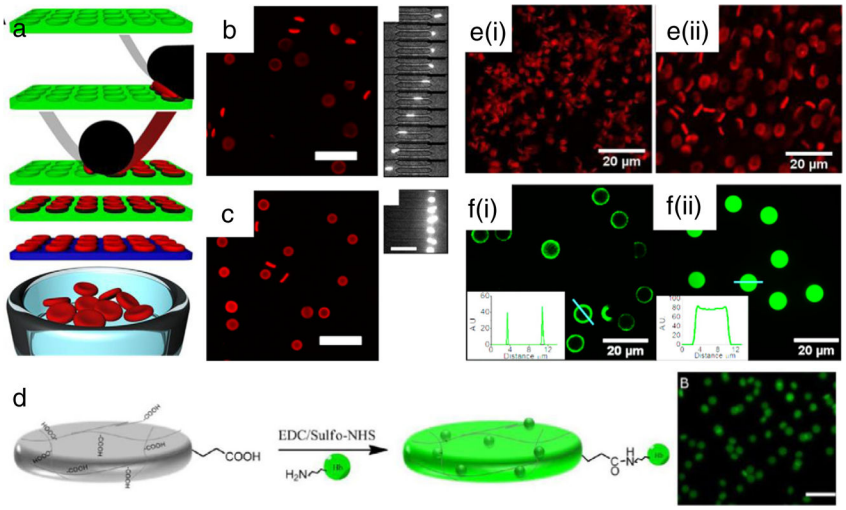

Fig. 5.

PRINT® for synthesis of sRBCs. (a) Schematic of the PRINT® process. (b) Deformable PEG-hydrogel sRBCs squeeze through small diameter channels, whereas (c) stiffer counterparts cannot. (d) PRINT® sRBCs functionalized with hemoglobin exhibit oxygen binding capacity. Additional functions of PRINT® sRBCs include (e) pH modulated swelling and (f) control over spatial (e.g. exterior versus interior) conjugation of small molecules. (a–c) Reprinted from [168]. (d) Adapted with permission from [175]. Copyright (2012) American Chemical Society. (e and f) Adapted with permission from [176]. Copyright (2014) American Chemical Society.

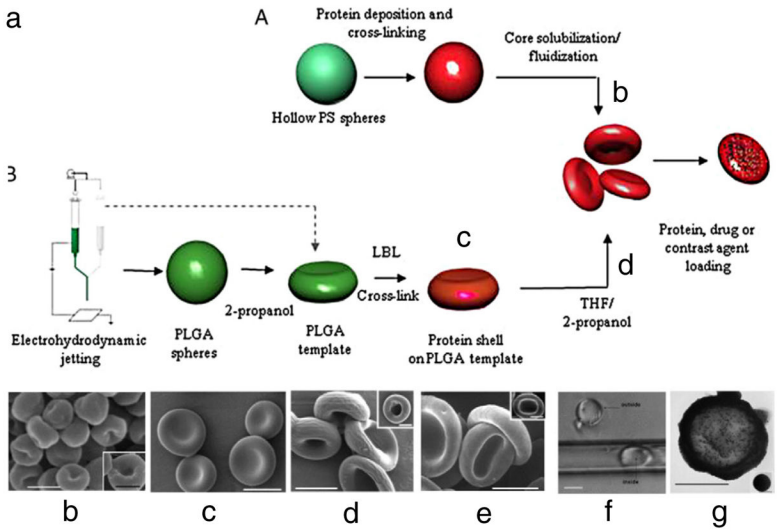

Fig. 6.

Layer-by-layer (LbL) and electrohydrodynamic jetting for sRBC synthesis. (a) Schematic illustrating the synthesis mechanism for both sRBCs consisting of (b) natural RBC proteins and synthetic polyelectrolytes made via LbL, (c) PLGA made via electrohydrodynamic jetting, and (d) a protein/polyelectrolyte mixture leveraging both methods. (e) Reference image of mouse RBCs. (f) sRBCs were able to travel though capillaries smaller in diameter than the sRBCs themselves. (g) sRBCs loaded with iron oxide. (a–g) Reprinted from [167].

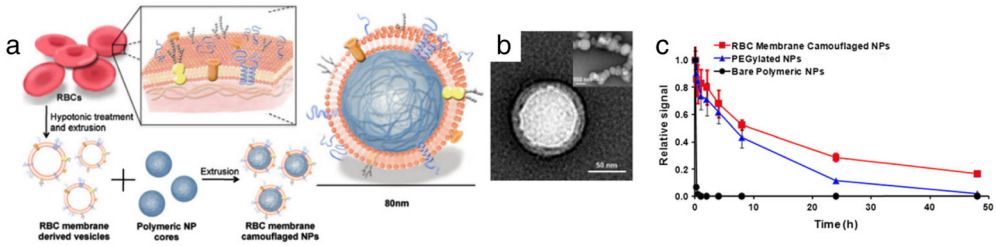

Fig. 7.

RBC-cloaked nanoparticles. (a) Schematic highlighting the extrusion approach for synthesis of RBC-cloaked particles. (b) TEM-image of final RBC-cloaked PLGA particle. (c) RBC-cloaked particles exhibit enhanced circulation as compared to both the PLGA template particle and its PEGylated counterpart. Reprinted from [170].

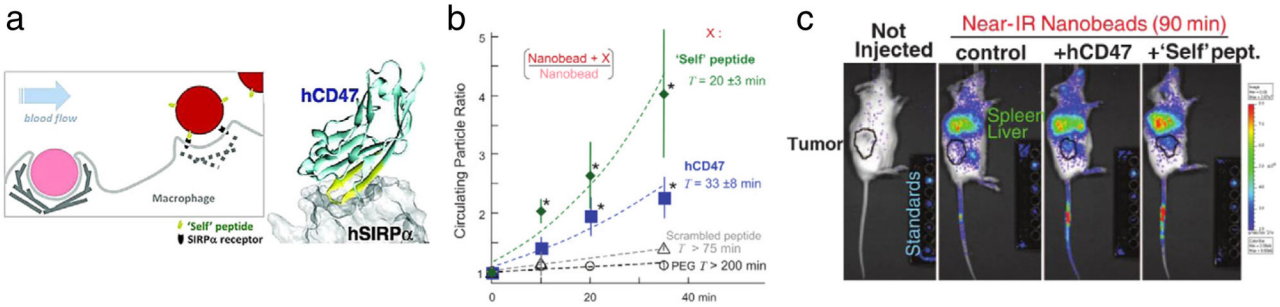

Fig. 8.

"Self" peptide particles inspired by RBC stealth protein, CD47. (a) Mechanism by which phagocytes actively avoid CD47-based particles. (b) Polystyrene particles functionalized with either a CD47-derived "self" peptide or human CD47 exhibit enhanced circulation as compared to scrambled peptide or PEG-coated particles. (c) By virtue of CD47-mediated extended circulation, stealth particles exhibit enhanced tumor accumulation. (a–c) From [169]. Reprinted with permission from AAAS.

Table 1

List of clinical trials for RBC DDS which are currently active or recruiting (retrieved from www.clinicaltrials.gov).

Sponsor (product)	Principal investigator	Drug and loading method	Application/indication	ClinicalTrials.gov Identifier
Erydel: EryDex	Larry J. Dumont	Dexamethasone sodium phosphate loaded into RBCs (A)	Determining *in vivo* recovery and survival of dexamethasone sodium phosphate loaded into RBCs	NCT02380924
ERYtech Pharma:	• Richard A. Larson	L-asparaginase loaded in RBCs	• Acute lymphoblastic leukemia (H)	• NCT01910428
• ERY-ASP	• Xavier Thomas		• Acute myeloblastic leukemia	• NCT01810705
• GRASPA	• Yves Bertand		• Relapse of acute lymphoblastic leukemia	• NCT01518517
• GRASPA	• Pascal		• Progressive metastatic pancreatic carcinoma (in combination with gemcitabine or FOLFOX)	• NCT02195180
• ERY001	• Hammel		• Acute lymphoblastic leukemia (combination with polychemotherapy)	• NCT02197650
• GRASPA	• Yves Bertand			
• University of Iowa	• John A. Widnes)	Biotin labeled RBCs (A)	• Investigating biotin labeled RBC survival in patients that previously developed antibody response against biotin labeled RBCs	• NCT02077751
• VA Office of Research and Development	• Robert Cohen		• Investigating RBC survival in diabetics and pre-diabetics	• NCT01204216
North Shore Long Island Jewish Health System	Lawrence A. Yannuzzi	Indocyanine green loaded RBCs (A)	Diagnosing retinal pathologies via quantification of capillary blood flow	NCT02445001

Table 2

Commercialization of RBC DDS.

Company	Technology	Target disease/application	Current status and results	Refs
Erydel	Hypotonic swelling and resealing for loading of dexamethasone sodium phosphate	Ataxia telangiectasia	Beginning phase III studies Received ODD in both US and Europe	[202,203]
Erytech	RBC encapsulated: **1** L-asparaginase **2** Methionine-y-lyase, Arginine deiminase **3** Therapeutic enzymes **4** Tumor antigens	**1** Various leukemias including acute lymphoblastic leukemia and acute myeloblastic leukemia and solid tumors including pancreatic cancer **2** TBD cancer target **3** Enzyme replacement **4** Tumor vaccination	1. Acute lymphoblastic leukemia: Phase I/II and Phase II trials completed Currently being investigated for Phase IbODD in Europe and USA Acute myeloblastic leukemia: Phase IIb initiated ODD in Europe Pancreatic cancer: Phase I study completed Phase II study started	[204,205]
Orphan Technologies	Encapsulation of thymidine phosphorylase	Mitochondrial neurogastrointestinal encephalomyopathy	ODD by both FDA and EMA If approved, this will be the only treatment available for any mitochondrial disease	[206]
Rubius	Genetically modified hematopoietic stem cells are turned into RBCs in culture that eventually express therapeutic proteins	Treat metabolic diseases, autoimmune diseases, and cancer	Company recently launched (December 2015)	[13]
Anokion	Engineered proteins are attached to circulating RBCs following IV injection, and via natural cell death the RBCs and the attached engineering proteins are processed by immune cells for enhanced antigen processing	Inducing protein specific immune tolerance without immunosuppression	Expanding preclinical applications	[42,43,207]
NanoBlood	A 7 nm particle containing a hemoglobin core, a hydrated PEG outer shell, and an inner superoxide dismutase mimetic gold shell	Alternative to RBC transfusions with applications in stroke treatment, trauma situations, traumatic brain injuries, and sickle cell disease	Patent granted	[208,209]
Arytha Biosciences	Developing nanoparticles that mimic key properties of RBCs by using natural RBC membranes to	Improving nanoparticle therapies (e.g. extended circulation, vaccine applications, toxin removal applications)	Expanding preclinical applications	[170,184,188,210]
RxMP Therapeutics	RBC derived microparticles synthesized from packed RBCs	Hemostatic agents to prevent bleeding in trauma situations	Currently in preclinical development	[211,212]

The technologies farthest along the commercialization path are focused on the encapsulation of therapeutics into RBCs, as these RBC delivery strategies were developed earlier than the other RBC delivery systems highlighted here (e.g. cellular hitchhiking, RBC-inspired carriers, or genetically engineered RBCs). For example, both Erytech and Erydel have entered clinical trials (see Table 1). The technologies that have received Orphan Drug Designation from either the FDA or EMA are also RBC-encapsulation based. Other RBC-based delivery strategies are being commercialized that make use of either engineered RBCs (Rubius) or engineered proteins (Anokion) attached to the surface of RBCs. These specific technologies will require a commercialization strategy that deviates from RBC encapsulation technologies, since they rely on genetic modifications or protein engineering, which will likely necessitate additional considerations during the approval process. While more recently described in preclinical settings, a few particle-based technologies are being commercialized to enhance current approaches toward blood transfusions, hemostatic agents, and nanoparticle biotechnologies (e.g. drug delivery, vaccination, and toxin removal). The majority of RBC delivery systems being commercialized have academic roots, highlighting the central role of academia in the development of RBC DDS.

Table 3

Summary of RBC surface targets and estimated copy numbers.

RBC membrane protein	Blood group designation	Approximate copy number per RBC
Band 3 (anion exchanger 1, AE1)	Diego	1,000,000
Glycophorin A (GPA)	MNS	1,000,000
GLUT1	None identified	500,000
Glycophorin B (GPB)	MNS	250,000
RHCE and RHD	Rhesus	100,000–200,000
CD59	Undefined	10,000–20,000
Kell	Kell	10,000
Decay accelerating factor (DAF, CD55)	Cromer	3000–5000
ICAM-4	LW	3000–5000
Complement receptor 1 (CR1)	Knops	1000
ADP-ribosyltransferase 4 (ART4)	Dombrock	Uncertain

For more detailed review of red cell antigens, see Reid et al. [61]or Klein and Anstee [213].