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The Effective Use of Red Blood Cells as Vehicles for Chemical and Biological Therapeutic Agents (L-Asparaginase, Anti-Infective Drug Loaded Red Cells and Anti-Thrombotic- Loaded Red Cells)
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Summary

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 nano-carriers to RBCs, and to optimize their pharmacokinetics, distribution, and effects in the body. Newer approaches include design of drugs with an affinity to circulating RBCs, encapsulation into RBCs using membrane permeating compounds. For several decades, researchers have used erythrocytes for drug delivery of a wide variety of therapeutics in order to improve their pharmacokinetics, bio-distribution, controlled release and pharmacodynamics. Approaches include encapsulation of drugs within erythrocytes, as well as coupling of drugs onto the red cell surface. This review focuses on the latter approach and examines the delivery of red blood cell (RBC)-surface-bound anti-inflammatory, anti-thrombotic, as well as RBC carriage of anti-tumor cells agents. Herein, we discuss the progress that has been made in surface loading approaches, and address in depth the issues relevant to surface loading of RBC, including intrinsic features of erythrocyte membranes, immune considerations, potential surface targets and techniques for the production of drug loaded red blood cells. 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. Based on the variety of approaches, the future of drug delivery vehicles is very promising.

Key words: *Effective use, red blood cells, biological therapeutic agents, drug loaded red cells, anti-thrombotic- loaded red cells.*

Introduction

How to design a drug carrier that can specifically deliver drug to the site of disease is always the main focus of current pharmaceutical research (Chertok *et al.*, 2013; Hubbell *et al.*, 2013). Thanks to recent advances in material science and nanotechnology, many nano-formulations, such as liposomes, micelles and nanoparticles (NPs). For artificial DDS, its “non-self” property may lead to some adverse effects. In contrast, the autologous cells-based carriers with the similar membrane structure of body cells are considered as the “self” and thus demonstrate much better biocompatibility and lower toxicity. This cell or cell membrane-based DDS can be produced in a simple way with minimum membrane protein loss. The retained membrane structure consequently endows the carriers various bio-functions and/or targeting specificities as their parent cells without further modifications. For example, carrier erythrocytes (red blood cells, RBCs) were widely used to encapsulate or bind small-molecule agents, nucleic acids, proteins, and NPs to treat systemic disease owing to their long lifespan and high biocompatibility (Hamidi *et al.*, 2007; Millan, *et al.*, 2004). Stem cells (SCs) could transport therapeutic cargoes to tumor microenvironment via their intrinsic tumor-tropic properties. Drug delivery has the potential to tremendously impact the treatment of certain diseases large number of drugs are produced every day that can effectively treat these diseases but are limited by delivery issues such as crossing blood barriers (blood- brain and blood-eye barriers) or the need to mitigate side effects as they come in contact with organs or tissues not specific for them.

The use of red blood cells has been recently developed for the effective vascular delivery of several classes of therapeutic agents. This has also helped in the localization of drugs in the target sites.

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 (Hu *et al.*, 2012).

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. The aim of this review is to demonstrate the effectiveness of the use of red blood cells as a new technology to deliver drugs to target sites and also to highlight the role of Medical Laboratory Scientists in the development, progress and effectiveness of this technology.

Drug Delivery Systems; History and Types

Drug delivery system is the method or process of administering a pharmaceutical compound to achieve a therapeutic effect in humans or animals. It controls the rate at which a drug is released and the location in the body where it is released. A targeted drug delivery system can allow doctors to transport medicine to an exact location in the body. Drug Delivery System is the controlled release of therapeutic agents. Drug Delivery System monitors the drug released and the location in the body where it is released. The upcoming drug delivery systems include the researches in the areas of crossing the Blood-Brain Barrier (BBB) in the brain, diseases and disorders, enhancing targeted intracellular delivery, combining diagnosis and treatment at exact location in the body.

Too often, we take for granted how easy it is to get and use medicine that treats a variety of ailments, both minor and major. Get a headache, develop arthritis, catch a cold or suffer an allergy attack, and you can swallow a pill and feel better. Topical medicines can take care of itching, rashes and pain. Meanwhile, a strategically placed shot can ease joint pain, and vaccinations can help ensure we don't get sick at all with a particular illness.

It's actually pretty amazing that we can introduce a foreign substance into our bodies in a variety of ways — oral, topical, injection — and create a desired

effect. Drug delivery methods, however, can have drawbacks, too. Some medicines may not move easily throughout the circulatory system or through tissues and cells. Others may lose efficacy as they pass through the digestive system. Or medicines may cause harmful side effects to healthy organs and tissues.

Drug delivery systems combine one or more traditional drug delivery methods with engineered technology. These systems create the ability to specifically target where a drug is released in the body and/or the rate at which it gets released. This ability benefits patients in multiple ways.

A targeted drug delivery system can allow doctors to transport medicine to an exact location in the body — a cancerous tumor, for example — while minimizing or even eliminating systemic side effects and/or damage to tissues surrounding the treatment site. Targeted delivery can also help ensure the medicine reaches the area where it's needed without any degradation that might occur if it has to pass through bodily systems like the digestive tract or circulatory system.

This delivery method can also help bypass the body's natural defenses that may block foreign substances — even needed medicine — from entering individual cells. The medication reaches the diseased or damaged location quickly and at maximum efficacy. Controlled release drug delivery systems are a natural evolution of the concept that's made timed-release oral medications successful. These systems time the release of medication that may be administered in multiple ways, such as orally, by injection or implantation. This can allow physicians to maintain a specific level of medication within the patient's body, reduce the need for repeated administrations of a medicine, optimize the efficacy of a drug, and even bypass the pitfalls of patients failing to take medicine as prescribed.

Biomedical engineering has contributed substantially in making us understand the physiological barriers to efficient drug delivery such as transport in the circulatory system and movement through cells and tissues. Drug delivery provides the means to improve patient's compliance, increase efficacy and reduce the side effects.

Erythrocytes and its basic structure

Red blood cells, also called erythrocytes, are the most abundant cell type in the blood. The primary function of red cells is to serve as an oxygen cargo to body cells

and carbon dioxide to the lungs. A red blood cell has what is known as a bi-concave shape. Both sides are curved inwards like the interior of a sphere. The shape aids its ability to maneuver through tiny blood vessels to deliver oxygen to organs or tissues (Bailey, 2017).

Red blood cells have a unique structure. Their flexible disc shape helps increase the surface area-to-volume ratio of these extremely small cells (Bailey, 2017)

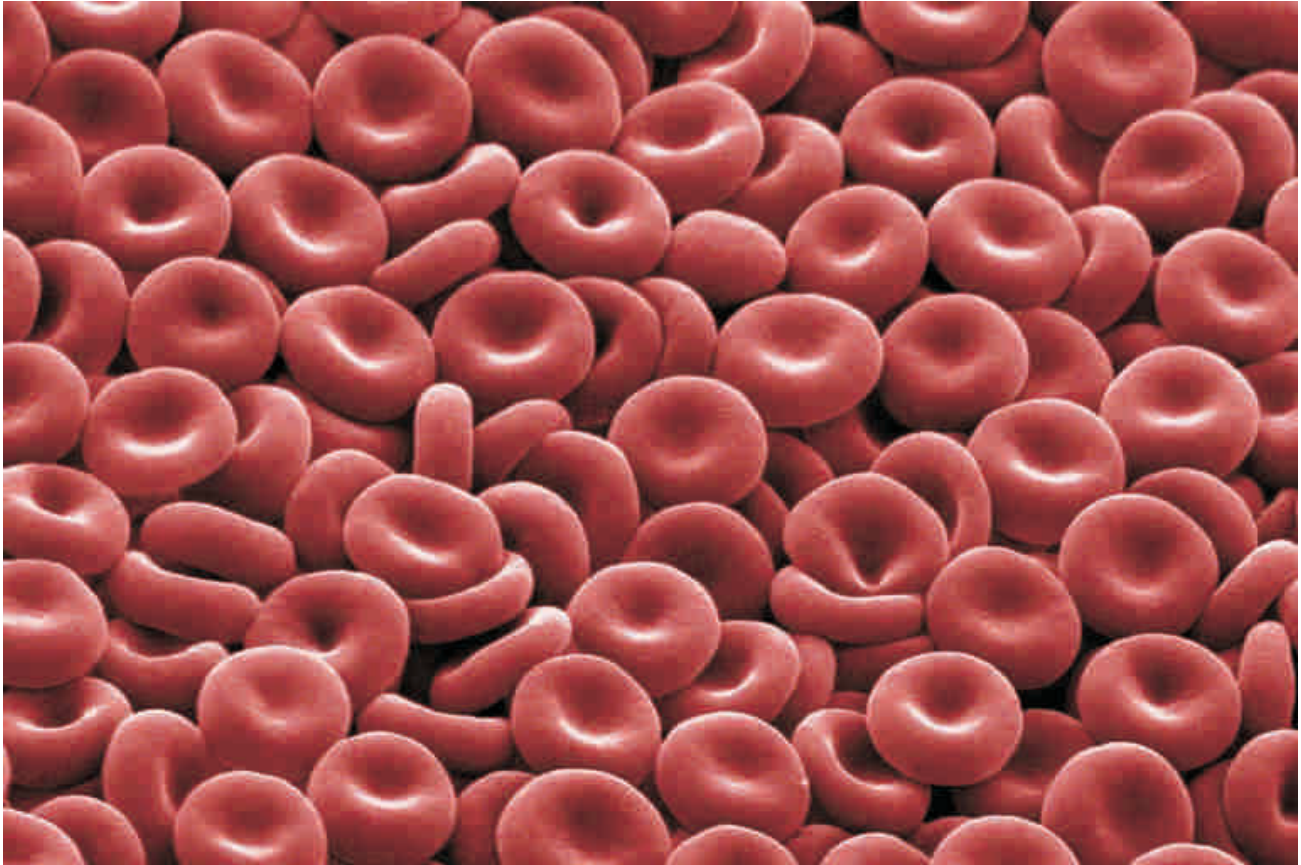


Figure 1: Red blood cells showing their biconcave structure, giving them a large surface area for gas exchange and highly elastic nature which helps them pass through narrow capillary vessels (Bailey, 2017)

THE USE OF RED BLOOD CELLS AS A DRUG DELIVERY SYSTEM

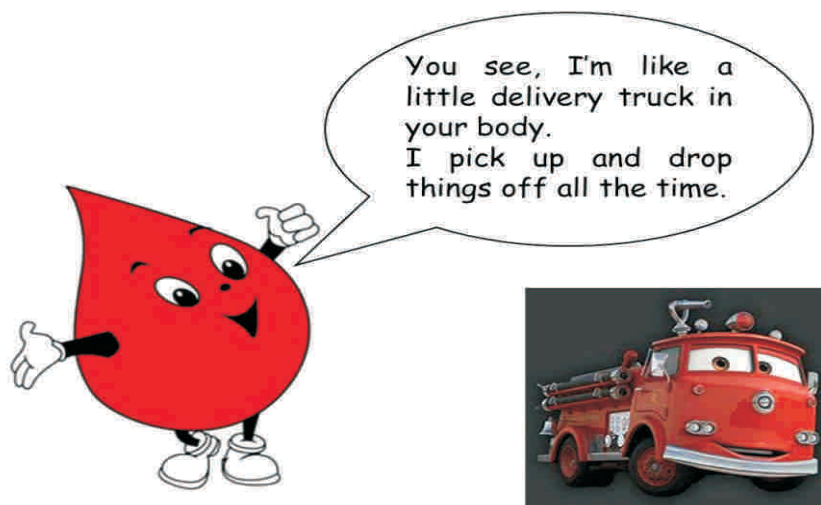


Figure 2: Showing the red blood cell as a moving truck that picks and delivers substances (Hopkins, 2015).

The optimization of vascular delivery of drugs is an important biomedical problem. This problem is especially acute in case of delivery of potent and specific, yet labile and complex bio-therapeutic agents including enzymes, which in most cases require precise localization in the target site. One means to achieve this goal is coupling drugs to carriers, such as synthetic or natural polymer structures of diverse geometries, phospholipid liposomes, albumin, antibodies or other biological molecules (Simone *et al.*, 2008).

Use of drug carriers promises to enhance the specificity, effectiveness and safety of therapeutic, diagnostic or prophylactic interventions. Functions of drug carriers include:

- I. Providing the optimal drug half-life in circulation and clearance mechanism
- II. Restriction of unintended drug uptake by and effects in non-target tissues
- III. Targeting to the intended therapeutic site
- IV. Providing optimal timing of the action, i.e., its timely initiation and termination.

Among other carriers, erythrocytes (red blood cells, RBC, non-nuclear biconcave discs with a diameter of $\sim 7 \mu\text{m}$, thickness $\sim 2 \mu\text{m}$ and plasma membrane surface area $\sim 160 \mu\text{m}^2$) represent a potentially attractive and, in some aspects, unique carrier for drug delivery. One microliter of human blood contains about 5 million RBC and total number of RBC, the most abundant cellular constituent of the blood (>99%), in the human body approaches 30 trillion. Human erythrocytes normally have a life span of 100–120 days (of note, mouse RBC life-time is about a third of human's counterpart), travel ~ 250 km through the cardiovascular system and function as natural carriers for oxygen. RBC life-span and size markedly exceed those of other drug delivery systems (e.g., <10 h and <100 nm for PEG-modified “stealth” liposomes). Thus, RBC are attractive carriers for intravascular delivery, e.g., for prolonging drug circulation and restricting unintended extravasation (Coller, *et al.*, 1992; Muzykantov *et al.*, 1986; Dale *et al.*, 1979).

Injected substances interact with RBC, which partition and metabolize more than 50 known drugs (e.g., captopril, sulfanilamide, testosterone, insulin, catecholamines, tacrolimus, antibiotics and other agents) (Hinderling, 1997). Therefore, RBC may unintentionally serve as a natural blood compartment participating in bio-distribution, pharmacokinetics, slow release, metabolism and action of diverse drugs including anti-tumor agents (Dumez *et al.*, 2001) and

genetic materials (Sakurai *et al.*, 2001). However, the intentional use of RBC as drug carriers is arguably of even higher interest.

However, the intentional use of RBC as drug carriers is arguably of even higher interest. This area of research has been incepted in early seventies, when it has been proposed that effects of certain drugs might benefit from encapsulation into autologous or immunologically compatible donor RBC that can be safely injected in the host, thereby improving circulation of a protected drug and its delivery to certain targets in the body (Ihler *et al.*, 1973). A prolonged life-time in the circulation, availability, considerable surface and volume (mean corpuscular volume of human and mouse RBC is ~ 90 and $\sim 50 \mu\text{m}^3$, respectively), high biocompatibility and natural mechanisms for safe elimination of RBC represent attractive features of this tentative drug carrier. Erythrocytes are champions among other drug delivery systems from the standpoint of longevity of circulation, biocompatibility and restriction of unintended permeation of drugs into extravascular compartments. Hydrodynamic forces driving RBC to the blood mainstream in the circulation and endothelial glycocalyx minimize RBC interaction with vascular walls (Vink *et al.*, 1996). Normally, RBC does not undergo extravasation from the circulation into tissues except hepatic sinuses and interstitium in the splenic follicles, i.e., open to circulation sites of RBC genesis and elimination, part of reticulo-endothelial system (RES). RES macrophages in the spleen and liver rapidly and effectively take up senescent, damaged and modified RBC via phagocytosis leading to their lysosomal degradation.

Drug delivery is frequently hindered by inadequate pharmacokinetics (PK) of the therapeutic agent. Rapid elimination impedes drug delivery to the intended targets, limits efficacy and may dictate the need for high doses and multiple administrations. These issues are especially pertinent to the use of expensive and labile bio-therapeutics. Several approaches have been devised to enhance drug bioavailability within the bloodstream, including chemical modification, artificial carriers (such as polymers) and natural carriers (such as blood proteins and cells).

As a successful example of the first approach, chemical conjugation with polyethylene glycol (PEG), which prolongs circulation and reduces uptake by the reticulo-endothelial system (RES), is widely used in research and clinically (Yang *et al.*,

2011; Wang *et al.*, 2005; Dumez *et al.*, 2004). Loading drugs within PEG-coated carriers, especially durable polymeric carriers that adapt to flow, can further extend circulation times. Still, no existing synthetic carrier can begin to match the intravascular longevity of erythrocytes (Magnani, 2012; Muzykantov 2010).

Erythrocytes (red blood cells [RBCs]) are natural carriers for drugs that require sustained intravascular delivery (Coller *et al.*, 1992) as they have evolved to deliver what might be considered the most vital biologic cargo, oxygen.

Starting in the 1970s, several laboratories attempted to improve drug delivery by loading drugs within autologous or donor RBCs prior to transfusion (Ihler *et al.*, 1973). Initial studies provided rather mixed results, likely due to damage to RBCs inflicted during drug loading (Alvarez *et al.*, 1996; Perez *et al.*, 1996). During the 1980s, prospects of using carrier RBCs were impeded by the outbreak of HIV and other blood-transmitted infections. Meanwhile, use of erythrocytes was overshadowed by novel carriers, particularly liposomal systems. However, a few laboratories persisted in efforts to improve RBC delivery systems, devised less traumatic methods for loading and tested RBC carriers (Rossi *et al.*, 2001) in animal models and in pilot trials, in human patients

(Bax *et al.*, 2000; Hambye *et al.*, 1997). At the present time, several RBC drug delivery strategies are in industrial development and clinical testing.

Three main strategies for RBC drug delivery are generally employed (Figure 3). Two approaches involve infusion of RBCs preloaded with a drug *ex vivo*, using either encapsulation within RBCs, or conjugation to the RBC surface. These strategies involve *ex vivo* manipulations with RBCs and create a situation where in a relatively minor fraction of circulating RBCs carries the drug (unless exchange hemo-transfusion is employed to load a greater fraction of the patient's RBCs). Hundreds of publications using this approach have been reported in the last four decades and have been the subject of comprehensive reviews, including important clinical studies showing the feasibility and medical promise of infusion of drug-loaded RBCs, such as RBCs loaded with encapsulated dexamethasone and therapeutic enzymes (Bax *et al.*, 2013; Patel *et al.*, 2008; Hamidi *et al.*, 2007). The third strategy involves targeting drugs directly to the RBC surface. Our article focuses on this novel approach. We will review: (i) pertinent features of the RBC as a carrier for surface-bound drugs; (ii) evolution, status and perspectives involving this strategy; and, (iii) medical and translational aspects of the envisioned clinical use.

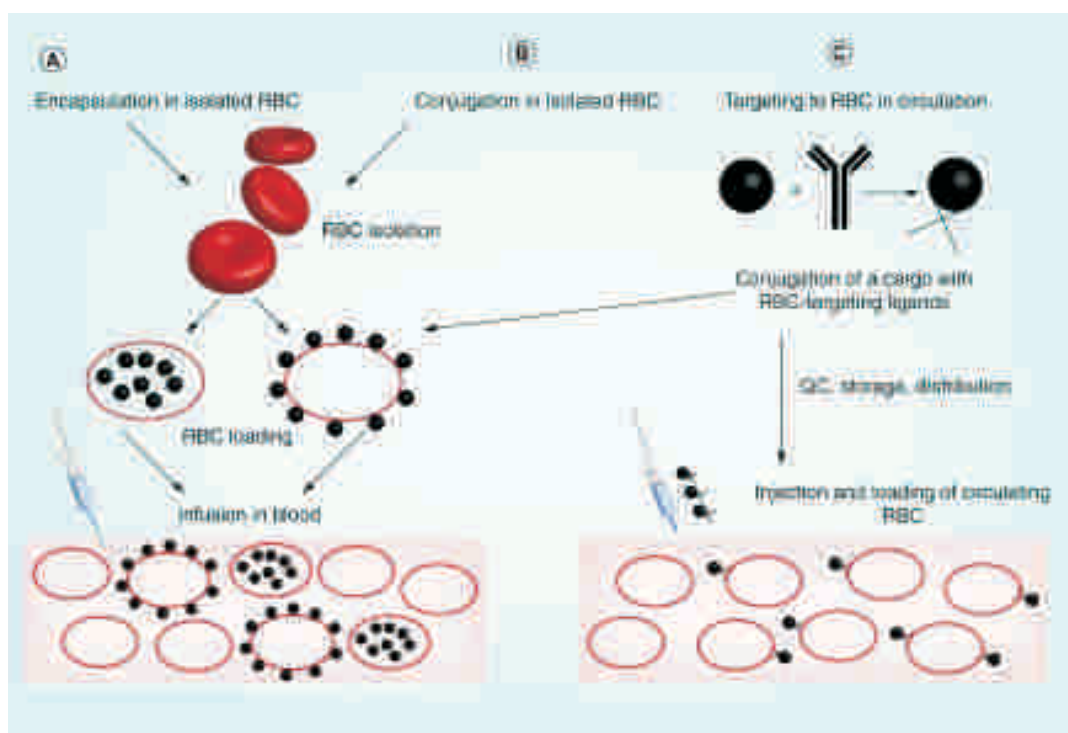


Figure 3: Strategies for drug delivery by red blood cells (Hamidi, 2007).

Relationship Between the Structure of Red Blood Cells and Its Ability to Serve as Vehicles for Therapeutic Agents

1. Red blood cell membrane and plasticity:

In order to examine RBCs as intravascular drug carriers, one should consider their innate properties that contribute to their utility as delivery vehicles. Chief among the structural features that RBCs possess to execute their function as oxygen carriers is their complex and unique membrane (Mohandas, *et al.*, 2008). The RBC membrane is in a perpetual balance between tensile strength and deformability, which is required for RBC to repeatedly pass through capillaries with cross-sections as narrow as one-third of the cell diameter (Sosa *et al.*, 2013). RBC must simultaneously maintain membrane integrity to avoid intravascular haemolysis with release of free haemoglobin, which may cause renal (Tracz *et al.*, 2007) and endothelial injury (Schaer *et al.*, 2013). When extensive, haemolysis can be fatal, as evidenced by the significant mortality associated with acute haemolytic **transfusion reactions** (Janatpour *et al.*, 2008).

The RBC membrane is supported from within by its cytoskeleton, in particular, the hexagonal actin-spectrin lattice that underlies the plasmalemma. This lattice interconnects with anchoring and membrane proteins, including glycophorin A and band 3 proteins, two of the most prevalent integral glycoproteins among the more than 300 proteins found in the RBC plasma membrane (Pasini *et al.*, 2006). Dynamic rearrangements of the cytoskeleton and plasma membrane plasticity enable the cells to undergo millions of reversible cycles of deformability through capillary networks (Kalfa *et al.*, 2006). Hydrodynamic forces drive RBCs to the center of the vascular lumen and the endothelial glycocalyx helps to minimize interactions with the vascular walls.

The organization of the RBC membrane that allows for deformation while maintaining integrity is based on a series of 'vertical' and 'horizontal' linkages between membrane protein complexes within the fluid phospholipid membrane bilayer. This structural organization allows the sub-membrane skeleton to undergo reversible changes in conformation from tight to expanded networks (Nans *et al.*, 2011) that confer resistance to shear stress, while concurrently permitting deformation and maintenance of cell volume and surface area. As in other eukaryotic membranes, the phospholipid bilayer of RBCs also includes distinctly organized populations of cholesterol, lipid rafts and phospholipids including

phosphatidyl serine (PS), a phospholipid that, although normally restricted to the inner leaflet of plasma membranes, can be exposed to the extracellular surface as cells senesce or incurs damage (Sathi *et al.*, 2014; Lubin *et al.*, 1981).

2. Elimination of red blood cells; complement and Reticuloendothelial system

Having noted the important features of the RBC membrane, one must also consider the pathways by which aged and damaged RBCs are cleared, as they provide insight into potential adverse effects on cell lifespan and function when RBCs are employed as drug carriers. Erythrocytes do not leave the bloodstream except transiently during passage through hepatic sinuses and the interstitium in the splenic follicles. Tissue macrophages in the spleen and liver are the principal sites where 'compromised' RBCs are eliminated. Senescence, pathological changes (e.g., in malaria or sickle-cell disease) and, potentially, drug loading, all reduce RBC biocompatibility, enhancing their clearance and increasing the risk for harmful haemolysis.

Some surface RBC glycoproteins that are expressed at low and variable levels protect RBCs from damage and elimination. These include **complement** inhibitors such as DAF and CD59 (Zaltzman, *et al.*, 1995) and signaling molecules such as CD47 (Oldenborg *et al.*, 2000) and SHPS-II (Ishikawa *et al.*, 2006). CD47, a membrane protein, inhibits phagocytosis of RBCs by macrophages through binding to SIRP α , which transduces an inhibitory 'do not eat me' signal (Burger *et al.*, 2012). Loss or inhibition of CD47 may be a key step in the pathologic process known as haemophagocytic lymphohistiocytosis, a syndrome characterized by excessive immune activation that leads to engulfment of erythrocytes and other haematopoietic cells by macrophages in the bone marrow and elsewhere (Kuriyama *et al.*, 2012).

L-ASPARAGINASE LOADED RED BLOOD CELLS

L-Asparaginase is an enzyme that is used as a medication and in food manufacturing. As a medication it is used to treat acute lymphoblastic leukemia (ALL), acute myeloid leukemia (AML), and non-Hodgkin's lymphoma. It is given by injection into a vein, muscle, or under the skin. A pegylated version is also available. In food manufacturing it is used to decrease the acrylamide (Gokmen, 2015).

Common side effects when used by injection include

allergic reactions, pancreatitis, blood clotting problems, high blood sugar, kidney problems, and liver dysfunction. Use in pregnancy may harm the baby. As a food it is generally recognized as safe (Gokmen, 2015). Asparaginase works by breaking down the amino acid known as asparagine without which the cancer cells cannot make DNA. Asparaginase was approved for medical use in the United States in 1978 (Kim et al., 2016).

E. coli strains are the main source of medical asparaginase (Muller, 1998). Branded formulations (with different chemical and pharmacological properties) available in 1998 include Asparaginase Medac, Ciderolase, and Oncaspar (Muller, 1998).

Asparaginase can be produced by two organisms. The one produced by *Erwinia chrysanthemi* instead is known as crisantaspase (BAN) and is available in the United Kingdom under the trade name Erwinase.

One of the *E. coli* asparaginases marketed under the brand name Elspar for the treatment of acute lymphoblastic leukemia (ALL) is also used in some mast cell tumor protocols (Apple et al., 2007). Unlike most of other chemotherapy agents, asparaginase can be given as an intramuscular, subcutaneous, or intravenous injection without fear of tissue irritation.

The use of L-Asparaginase in the treatment of acute lymphoblastic leukemia

The discovery and development of asparaginase as an anti-cancer drug began in 1953, when scientists first observed that lymphomas in rat and mice regressed after treatment with guinea pig serum (Kidd, 1953). Later it was found out that it is not the serum itself which provoke the tumour regression, but rather the enzyme asparaginase (Broome, 1963) After researchers comparing different kinds of asparaginases, the one derived from *Escherichia coli* and *Erwinia chrysanthemi* turned out to have the best anti-cancer ability. *E. coli* has thereby become the main source of asparaginase due to the factor that it is also easy to produce in large amount.

Asparaginases are a cornerstone of treatment protocols for acute lymphoblastic leukemia (ALL) and are used for remission induction and intensification treatment in all pediatric regimens and in the majority of adult protocols. Extensive clinical data have shown that intensive asparaginase treatment improves clinical outcomes in childhood ALL. Three asparaginase preparations are available; the native asparaginase derived from *Escherichia coli* (*E. coli*-asparaginase), a pegylated form of this

enzyme (PEG-asparaginase) and a product isolated from *Erwinia chrysanthemi*, i.e. *Erwinia* asparaginase. Clinical hypersensitivity reactions and silent inactivation due to antibodies against *E. coli*-asparaginase, lead to inactivation of *E. coli* asparaginase in up to 60% of cases. Current treatment protocols include *E. coli*-asparaginase or PEG-asparaginase for first-line treatment of ALL. Typically, patients exhibiting sensitivity to one formulation of asparaginase are switched to another product to ensure they receive the most efficacious treatment regimen possible. *Erwinia* asparaginase is used as a second- or third-line treatment in European and US protocols. Despite the universal inclusion of asparaginase in such treatment protocols, there is much debate regarding the optimal formulation and dosage of these agents.

Despite being an essential drug used in all treatment protocols for ALL, there is much debate regarding the optimal formulation and dosage of asparaginase. Asparaginases are associated with a unique set of side effects. Hypersensitivity reactions, due to anti-asparaginase antibody production, have been observed in up to 60% of patients at some time during *E. coli* asparaginase therapy (Panosyan et al., 2004). The development of these antibodies appears to be more commonly observed with native *E. coli*-asparaginase (Larson et al., 1998; Avramis, et al., 1994; Woo, et al., 1961) compared with the pegylated enzyme (Hawkins et al., 2004). Symptoms of clinical hypersensitivity include anaphylaxis, pain, edema, Quincke's edema, urticaria, erythema, rash and pruritis (Woo et al., 2000). The route of administration determines the clinical symptoms with a greater incidence of major skin reactions observed with intramuscular (IM) administration compared with intravenous (IV) administration. An asparaginase encapsulated into homologous red blood cells has recently been proposed as a new approach to maintain enzyme activity, while reducing formation of anti-asparaginase antibodies (Bertrand et al., 2008) bringing about L-asparaginase-loaded red blood cells.

Mechanism of action

The rationale behind asparaginase is that it takes advantage of the fact that acute lymphoblastic leukemia cells and some other suspected tumor cells are unable to synthesize the non-essential amino acid asparagine, whereas normal cells are able to make their own asparagine; thus leukemic cells require high amount of asparagine (Fernandes et al., 2017). These leukemic cells depend on circulating asparagine. Asparaginase, however, catalyzes the

conversion of L-asparagine to aspartic acid and ammonia. This deprives the leukemic cell of circulating asparagine, which leads to cell death (Broome, 1981).

Tumor cells deficient in asparagine synthetase (ASNS) are unable to synthesize enough L-asparagine (Asn) to meet metabolic demands and, therefore, depend upon circulating Asn in the plasma for survival. L-asparaginase, which depletes Asn, has been used in the treatment of acute lymphoblastic leukemia (ALL) for over 30 years, since the majority of such tumors are deficient in ASNS. Recent evidence suggests that a subset of pancreatic tumors is deficient in ASNS, providing rationale for testing L-asparaginase against such tumors. However, L-asparaginase is associated with high immunogenicity and severe side effects. Therefore, it has been shown in patients with ALL, that L-asparaginase loaded into red blood cells (GRASPA) allows to strongly reduce side effects and to improve L-asparaginase pharmacokinetics.

L-asparaginase loaded in homologous red blood cells (GRASPA) is a new pharmaceutical formulation of the enzyme. This cell-based medicinal product allows a better safety profile and an improvement of the pharmacokinetics and pharmacodynamics of the enzyme.

L-asparaginase remains active entrapped inside the red blood cell (RBC), while asparagine is constantly and actively “pumped” through the membrane of the red cells thanks to N⁺ channel system. Thus, L-asparaginase loaded RBCs act as “cellular bioreactors”. Indeed, plasmatic asparagine diffuses through the RBC membrane to the intra cellular compartment where it is cleaved by the entrapped L-asparaginase. Thanks to the RBC membrane, the enzyme is protected from body reaction thus reducing the side effects.

An L-asparaginase loaded red blood cell is a cell-based medicinal product for personalized medicine. The physician prescribes the drug, then the hospital orders the product to the company. The patient weight, the ABO blood type and a valid irregular antibody screening (IAS) have to be joined with this order. The qualified person on the manufacturing site, orders immediately to a blood bank a leukocyte reduced packed RBC unit compatible with the patient.

Production of L-asparaginase loaded red blood cells.

The product is manufactured under cGMP using a 3-

hours automated process:

- (1) A washing step removes the preservative solution from the packed RBC,
- (2) L-asparaginase is mixed to the RBC washed suspension,
- (3) The mixture is dialyzed against a hypotonic solution and resealed
- (4) A final washing step allows to purify the product, finally
- (5) The preservative solution is added. According to the prescribed dosage in (IU/Kg)

The volume of GRASPA® is adjusted in the final product PVC bag. Indeed, the product release specifications are constant and reproducible from batch to batch such as the corpuscular concentration of L-asparaginase (117±19, IU/ml), extracellular hemoglobin (0.11±0.03 g/dL), osmotic fragility (<3.5 g/L of NaCl), extracellular L-asparaginase (0.4±0.2 IU/ml ie <1% of the total activity). Based on these specifications, the qualified person releases the product and ships it (kept at 2–8°C) to the prescriber, meaning the delay between order and delivery is less than 2 days. Currently a 72h shelf-life for the final product is considered (Bailly, 2011).

Anti-Infective Drug Loaded Red Blood Cells. Azidothymidine loaded red blood cells for HIV treatment

Phagocytosis of RBC intentionally modified in the drug loading process to be taken up by macrophages in the RES serves as a natural delivery path for agents that help to eliminate invaders residing in these cells (Magnani et al., 1992). For example, anti-viral drug azidothymidine (AZT) was loaded in carrier RBC (Benatti et al., 1996) that delivered cargo to macrophages and provided enhanced anti-viral potency vs free drug in cell culture (Rossi et al., 1998) and in a mouse model of HIV infection (Fraternali et al., 2002). Encouraging results have been obtained in cells with RBC-mediated delivery of other anti-viral agents (Magnani et al., 1996) including antisense agents inhibiting HIV-1 replication in infected cells. These studies provide a basis for a wide-range antiviral strategy posed for systematic testing in animal models and human studies. It has to be noted, however, that loading of some anti-microbial drugs into RBC (i.e., anti-malarial agent clotrimazole) predisposes RBC to oxidative damage and oxidized RBC are rapidly taken up by hepatic RES macrophages (i.e., Kupffer cells) via scavenger receptor-mediated phagocytosis (Terpstra et al., 2000).

Antigen-presenting function of macrophages and

other immune cells eliminating altered RBC also offers an attractive natural mechanism for delivery of antigens to boost immune response in addition to optimized delivery of antigens, carrier RBC plays a role of the adjuvant, by presenting multiple copies of antigens and stimulating non-specific immune response. Further, encapsulation of cytokines including interleukins and interferon into RBC facilitates delivery of these agents to macrophages, resulting in stimulation of immune response in animal models.

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

IFN- α and RIBA-loaded red blood cells for treatment of hepatitis C

IFN- α and ribavirin (RIBA) are established treatments for hepatitis C. Therapeutic success is moderate but can be significantly improved by raising IFN- α dosage and associating antivirals such as RIBA. High toxicity limits increase of dosage of both drugs (Sulkowski et al, 2000). The aim was to find innovative ways of enhancing delivery of IFN- α and RIBA to the liver. Homologous or donor erythrocytes loaded with both drugs (IFN- α -ribavirin-loaded red blood cells; RBC-IFN- α -RIBA) and made susceptible to the action of phagocytes by opsonization induce their selective removal by targeting them to the liver macrophages. Encapsulation of IFN- α and RIBA into RBC and targeting to the liver allows:

- i) Organ-specific liberation of high amounts of IFN- α and attainment of higher therapeutically efficient concentrations in the liver
- ii) Autocrine stimulation of liver (and spleen) macrophages by IFN- α to enhance cell-mediated antiviral defense
- iii) Control of viral proliferation within the macrophage.

Antithrombotic Agent Loaded Red Blood Cells

When we are wounded, either externally (for instance, when we cut ourselves) or internally (for instance, due to gastric ulcer or brain hemorrhage), blood clots which are sponge-like plugs that are rapidly formed in response to the injury by activated blood platelets and fibrin in a process called

coagulation — prevent profound bleeding. Thus, good or haemostatic clots save our lives.

However, under pathological conditions blood clots can also form inside vessels. Such bad or thrombotic clots occlude blood vessels and cause oxygen starvation of vital organs including the brain (stroke), heart (acute myocardial infarction) or lungs (pulmonary embolism). Thrombosis is one of the leading causes of morbidity and mortality from cardiovascular and other disease conditions.

Diverse anti-thrombotic means are being developed. For instance, anticoagulants (such as heparin) and platelet inhibitors (such as aspirin) help to prevent formation of clots (blood thinners). Fibrinolytics, known as plasminogen activators (such as tissue-type plasminogen activator, or tPA) dissolve formed clots by degrading the fibrin meshwork. Both types of therapeutics are widely used in medical practice, e.g., for treatment of two forms of ischemic heart disease caused by thrombi in coronary vessels — acute myocardial infarction and unstable angina.

Unfortunately, these anti-thrombotic therapies are not free of serious (sometimes fatal) side effects, the most prominent being bleeding. Thus, fibrinolytics do not discriminate between good, haemostatic clots that should be spared, and bad, thrombotic clots that must be dissolved. In addition, plasminogen activators are eliminated from the bloodstream within a few minutes (which makes them impractical for prophylactic use) in part due to filtration into the extravascular tissues, where they might cause side effects by degrading normal tissue components.

Arguably, the situation is worst in surgical patients. On the one hand, the wounds must be protected against bleeding by good clots. On the other hand, due to the instability of the balance for blood coagulation and the patient's immobilization, clots tend to form in the post-operative period despite anticoagulant means. These bad nascent clots formed during the recovery after surgery (that takes from days to several weeks), embolize vital organs and must be dissolved as soon as possible.

A new paradigm, utilizing erythrocytes or red blood cells (RBC) as a drug delivery carrier, promises to solve at least some of these problems and permit effective and safer use of these drugs for thromboprophylaxis, including surgical patients. By many parameters, RBC could become ideal carriers for the intravascular delivery of drugs, whose prolonged activity in the bloodstream is desirable

(Magnani et al., 2002). RBC circulates normally for several weeks and do not leave the blood vessels (their diameter is 1,000 times bigger than that of plasminogen activators). For the same reason, RBC does not permeate already formed blood clots.

A new thromboprophylaxis concept, published recently in *Nature Biotechnology* (Murciano et al., 2003) is based on the coupling of tissue plasminogen activators (e.g., tPA) to RBC, in order to optimize their delivery in the vasculature. The idea is that coupling tPA to RBC would prevent its diffusion into good haemostatic clots (sparing them from unwanted dissolution) and extravascular tissues (minimizing side effects). On the other hand, RBC carriage would prolong tPA life-time in the bloodstream, permitting its incorporation into the bad nascent clots, which then would be dissolved from within (a Trojan Horse for fibrinolysis).

This animal study tested whether coupling tPA to the surface of RBC generates a novel fibrinolytic agent (RBC/tPA) capable of dissolving nascent clots from within, while only minimally affecting pre-existing haemostatic clots. After intravenous injection in mice and rats alike, the fibrinolytic activity of RBC/tPA persisted in the bloodstream at least 10-fold longer than did free tPA. Experiments were limited to short-term duration, 3 hours, but the shape of the kinetic curve of RBC/tPA blood clearance strongly suggested that duration of its fibrinolytic activity would persist beyond this time frame. In a model of venous thrombosis induced by aggregation in the pulmonary vasculature of intravenously injected fibrin microemboli (Murciano et al., 2002), soluble tPA lysed pulmonary clots lodged before, but not after the tPA injection. In contrast, RBC/tPA lysed pulmonary clots lodged after, but not before the RBC/tPA injection. Free tPA injected either before or after occlusive thrombosis of carotid artery failed to dissolve clots, whereas RBC/tPA injected before, but not after thrombosis, restored blood flow.

Therefore, RBC-based drug delivery strategy changes the fibrinolytic profile of tPA, providing the basis for prophylactic fibrinolysis. In this particular study, tPA was coupled to isolated RBC chemically, using a biocompatible conjugation technique developed in the prior studies (Muzykantov et al., 1996). In principle, this technique is applicable in the haemotransfusion settings associated with thrombotic disorders (for example, sickle cell anemia) or in elective surgeries where autologous transfusion is a standard practice.

Furthermore, it is possible to couple therapeutic proteins to RBC directly in the bloodstream. Human red blood cells possess the complement receptor 1 (CR1) that binds immune complexes and transfers them to macrophages without opsonization of RBC themselves (Taylor et al., 1991). CR1 monoclonal antibodies and anti-CR1 immunoconjugates injected into animals, bind to RBC and circulate for a prolonged time without RBC damage. Preliminary studies show that, in principle, an anti-CR1/tPA conjugate can be used for thromboprophylaxis without the need for RBC extraction, modification or transfusion.

The proposed strategy may also enhance the safety of cerebral fibrinolysis. Free tPA improves reperfusion in ischemic stroke but may enhance cerebral bleeding in haemorrhagic stroke. Besides unintended dissolution of haemostatic clots in the cerebral vasculature, diffusion of plasminogen activators into the brain aggravates cerebral edema and matrix remodeling, and causes collateral damage including cytotoxicity to neurons. Therefore, RBC-carried tPA might be safer due to better retention within the vascular compartment.

Limitations and Risk Factors of Red Cells as Drug Delivery Cargoes

Many within the general public and biomedical science view RBCs as a sac de voyage for haemoglobin, 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 durable structure.

This multi-molecular 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 (Burton, et al., 2011; Gallagher, et al., 2008). 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 haemoglobin 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 haemolysis 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 (Whelihan et al., 2012) 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 (Chessa et al., 2014). 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 diffusible substrates in blood, thereby providing a long-circulating cellular reactors for detoxifying harmful compounds or eliminating molecules needed for tumor growth (Magnani et al.,

1993). This is also among the most advanced applications of RBC drug delivery, having already entered clinical trials in neurological and oncologic diseases (Hunault et al., 2015). 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 (Muzykantov et al., 1996).

Advantages and Disadvantages of The Use of Red Cells as Drug Delivery Cargoes

The advantages of Drug Delivery Systems: -

- Ease administration of drugs
- Quick absorption and onset of action
- Availability of Simple formulations
- Favorable environment
- Bio-availability satisfactory
- Reduced hospital outpatient care
- Accurate consistent dosing
- Lower manufacturing costs

The disadvantages of Drug Delivery Systems:

- Untoward immunogenic reactions may occur
- Insufficient availability of data for penetration enhancers
- Pathology may adversely affect (Madhavi et al. 2013)

The Role of Medical Laboratory Science in The Use of Red Cells as Vehicle for Drugs

The basis of red cell drug delivery is transfusion medicine whereby autologous or homologous red cells coupled with drugs are transfused into a patient for treatment. The Medical Laboratory (Blood Transfusion Laboratory) is where the blood components for transfusion are prepared. For a successful drug delivery, the basic rules of transfusion science have to be followed and this is ensured by a well-trained Medical Laboratory Scientist in the field of haematology. The Medical Scientist oversees most of the packaging process and makes sure that the red blood cells to be transfused are;

- (a) Blood grouped and immunogenically compatible,
- (b) That they are stored at appropriate conditions and temperature
- (c) They are to ensure proper preparation of packed red cells
- (d) Ensure proper transfusion technique to limit damage to cells.

In general, the Medical Laboratory Scientist (Haematologist/Blood Transfusion Scientist), Pharmacist and the Physician all have basic roles to play in the production of drug loaded red cells.

The Application of Red Cells as Drug Delivery Cargoes

1. To improve the efficacy and pharmacodynamics of poorly stable, fragile or potentially immunogenic drugs.
2. To transport antibiotics to their target areas in macrophages.
3. It is used in the fibrinolysis of post-operative clots and also as a thromboprophylaxis before operative procedures.
4. It is used to transport anti-tumor agents directly to target tumors thereby reducing the side effects of chemotherapeutic drugs.

The Development of This Technology in Nigeria

As Nigeria is known to be a developing country, the use of this technology has not been implemented in its hospitals, but research is being carried out on drug delivery systems in general by some universities in Nigeria. Among them is the University of Nigeria, Nsukka in the Department of Pharmaceutical Technology and Industrial Pharmacy, by a research group - Drug Delivery and Nanotechnology Research Unit (RUNDD) led by Prof. S. I. Ofoefule – the Initiator and group coordinator. This research group focuses on the uses of one of the drug delivery systems that could be used (use of Nanotechnology).

Nanotechnology is the understanding and control of matter at the nano-scale, at dimensions between approximately 1-100 nanometers. It involves the use of very minute matter that cannot even be seen by light microscope at 1-100nm to delivery drugs. As time goes on and more research group are found, the use of red cell as a drug delivery mechanism will be more grounded in Nigeria.

Conclusion

The work described here illustrates the therapeutic potential for erythrocytes as drug delivery vehicles, which will enable the targeted and less toxic delivery

of agents for a variety of disease states. Red cells may be loaded with anti-cancer agents to protect against severe side effects, diminished toxicity and reduced immunogenicity. The targeted removal of RBCs in the RES may be utilized to enhance the delivery of molecules for modulation of the immune system. Anti-infective agents may also be delivered to the RES system in this manner. The membrane of RBCs may be modified to more effectively target different organs of the RES. In the case of anthracycline-based cytotoxic agents, the cardio-toxic effects may be attenuated with RBC encapsulation. Although this work focused mainly on encapsulated drugs alternative strategies such as anchoring therapeutic proteins and fusion to the surface of RBC offer the potential to address additional pathologies, for example, thromboprophylaxis. Based on the variety of approaches, the future of RBCs as drug delivery vehicles is very promising and is highly advocated.

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