Expert Opinion





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# Erythrocyte-based drug delivery

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The use of a physiological carrier to deliver therapeutics throughout the body to both improve their efficacy while minimising inevitable adverse side effects, is an extremely fascinating perspective. The behaviour of erythrocytes as a delivery system for several classes of molecules (i.e., proteins, including enzymes and peptides, therapeutic agents in the form of nucleotide analogues, glucocorticoid analogues) has been studied extensively as they possess several properties, which make them unique and useful carriers. Furthermore, the possibility of using carrier erythrocytes for selective drug targeting to differentiated macrophages increases the opportunities to treat intracellular pathogens and to develop new drugs. Finally, the availability of an apparatus that permits the encapsulation of drugs into autologous erythrocytes has made this technology available in many clinical settings and competitive with other drug delivery systems.

**Keywords:** cell-based drug delivery, drug targeting, erythrocytes as carriers, nucleoside analogues, protein delivery

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## 1. Introduction

The main problems associated with systemic drug administration are essentially related to the biodistribution of pharmaceuticals throughout the body. The indiscriminate distribution of a drug in the body means that, to achieve a required therapeutic concentration, the drug has to be administered in large quantities, the major part of which is just wasted in normal tissues. A perfect drug should ideally exert its pharmacological activity only at the pathological site, at the lowest concentration and without negative effects on nontarget compartments. The conjugation of a drug molecule with an appropriate carrier may solve many of these problems. The coupling or the entrapping of a selected drug to or into a carrier system should be able, on one hand, to prevent its premature degradation, inactivation or elimination from the organism and, on the other hand, to reduce the manifestation of undesirable immune response and toxic side effects, as the pharmaceutical is confined into a limited compartment such as the carrier. A selective targeting of the drug of interest could be achieved using vector molecules or carriers possessing high affinity toward the affected zone.

The delivery systems currently available enlist carriers that are either simple, soluble macromolecules (such as monoclonal antibodies, soluble synthetic polymers, polysaccharides and biodegradable polymers) or more complex particulate multicomponent structures (microcapsules, microparticles, cells, cell ghosts, lipoproteins, liposomes, erythrocytes). Among these, erythrocytes (red blood cells [RBCs]) constitute potential biocompatible carriers for different bioactive substances, including protein drugs, as they feature some unique advantages [1-3]. For example:

- They are completely biodegradable without generation of toxic products and show high biocompatibility especially when autologous erythrocytes are employed.
- They can be easily handled by means of several techniques for the encapsulation

of different molecules, after which one can obtain loaded erythrocytes with morphological, immunological and biochemical properties similar to those of native cells.
- As they lack a nucleus and other organelles, leaving most of their volume available, they have a large capacity in which to encapsulate drugs and, therefore, a high degree of encapsulation can be achieved.
- They protect the encapsulated substance from premature inactivation and degradation by endogenous factors and, at the same time, the organism against the toxic effects of the drugs, thus avoiding immunological reactions.
- A wide variety of chemicals can potentially be entrapped, even peptides of high molecular weight presenting significant biotechnological applications.
- They have a longer lifespan in circulation as compared with other synthetic carriers.
- They act as bioreactors due to the presence of several enzymatic activities that can directly affect the loaded molecules and, in the case of loaded prodrugs, give rise to the active drug itself.

Furthermore, exploiting another main feature of the RBC, a selective targeting of drugs directly to macrophages without affecting the nontargeted compartments, could be achieved. After their natural lifespan (~ 120 days) in systemic circulation, the senescent RBCs are recognised by the cells of the phagocytic system (the reticuloendothelial system [RES] otherwise known as the monocyte–macrophage system) and removed from circulation to be destroyed. It is possible to artificially induce these senescent signals on the RBC membrane, in order to specifically target the drug-containing erythrocytes to the phagocytic cells, in particular to the monocyte-derived macrophages [4].

The main physiological mechanism used to remove RBCs from circulation is immune-mediated. The senescent cells expose some new antigenic sites on the membrane that are recognised by autologous immunoglobulins (IgG) and complement and then opsonised [5]; the opsonised cells are then recognised by Fc and C3b macrophage receptors and phagocytosed. As this cellular type represents an important *in vivo* reservoir for several kinds of viruses and microrganisms (such as *Herpes simplex*, HIV-1, *Leishmania* spp., *Mycobacteria* spp.), a drug-targeting system that selectively delivers drugs to macrophages should prove beneficial.

Keeping in mind all these physiological features in addition to therapeutic perspectives, erythrocytes as drug carriers could be employed for various primary purposes (**Figure 1**):

- They could retain an active payload and thus be used as circulating bioreactors to remove undesired molecules from the bloodstream.
- They could be used as a drug delivery system providing a sustained release of the drug into the body, allowing therapeutic levels to be maintained in the blood for long periods of time.
- They could be used as drug-targeting systems for the selective delivery of pharmacological substances to cells responsible for or capable of erythrophagocytosis (monocyte–macrophage system), which are often the sites of various clinical and pathological conditions.
- They could be loaded with magnetic-responsive particles and targeted to a specific site of the body using external magnetic fields.

Thus, erythrocyte-based drug delivery represents an attractive and versatile carrier system suitable for several clinical purposes, depending on the drug to be transported or on the cells to be targeted. The range of the substances that have been loaded into or associated with erythrocytes is wide and includes agents such as therapeutic proteins (TPs; enzymes and vaccines), nucleic acids, oligosaccharides, cancer chemotherapeutics, chemical markers and other active agents such as antiviral drugs and metabolic modulators. The therapeutic applications of engineered erythrocytes are obviously related to the agent incorporated and to the ways by which this active agent becomes available to perform its intended function. Based on the range of active agents that have been loaded into or associated with RBCs, therapeutic applications have been suggested in treating cancer [6], circulatory disease [7], metabolic and immunological disorders [8], and in detoxification treatment modalities [9]. Moreover, recent studies report the use of erythrocyte ghosts (EGs) as a biocompatible nonviral delivery system for extended circulation and prolonged expression of plasmid DNA in the blood, indicating the potential of EG as a safe, prolonged and blood-targeted delivery system of therapeutic genes [10].

In this review, some examples of erythrocyte-based drug delivery are summarised, mainly according to experience that has led to the application of this procedure to the development of a new apparatus for the encapsulation of nondiffusible drugs in RBCs to be used for clinical application.

## 2. Erythrocytes as carriers of encapsulated drugs

### 2.1 How to prepare carrier red blood cells

Different methods have been proposed to encapsulate substances into RBCs in order to obtain an appropriate delivery of drugs, enzymes or peptides [11-14]. These methods are performed to reach a dual objective: to seek an enhanced performance of the encapsulated substance, while ensuring that the RBCs undergo the fewest possible alterations. Thus, it is important to obtain erythrocytes as similar as possible to normal ones in order to ensure their proper survival and circulation in functional terms. The most widely used methods are commonly based on the remarkable property of the RBC to increase in volume when placed under conditions of reduced osmotic pressure, such as in the presence of a hypotonic solution. Three variations of the hypotonic haemolysis procedures are available: the dilutional, preswell dilutional and dialysis methods. The hypotonic dialysis method (**Figure 2**) is the best

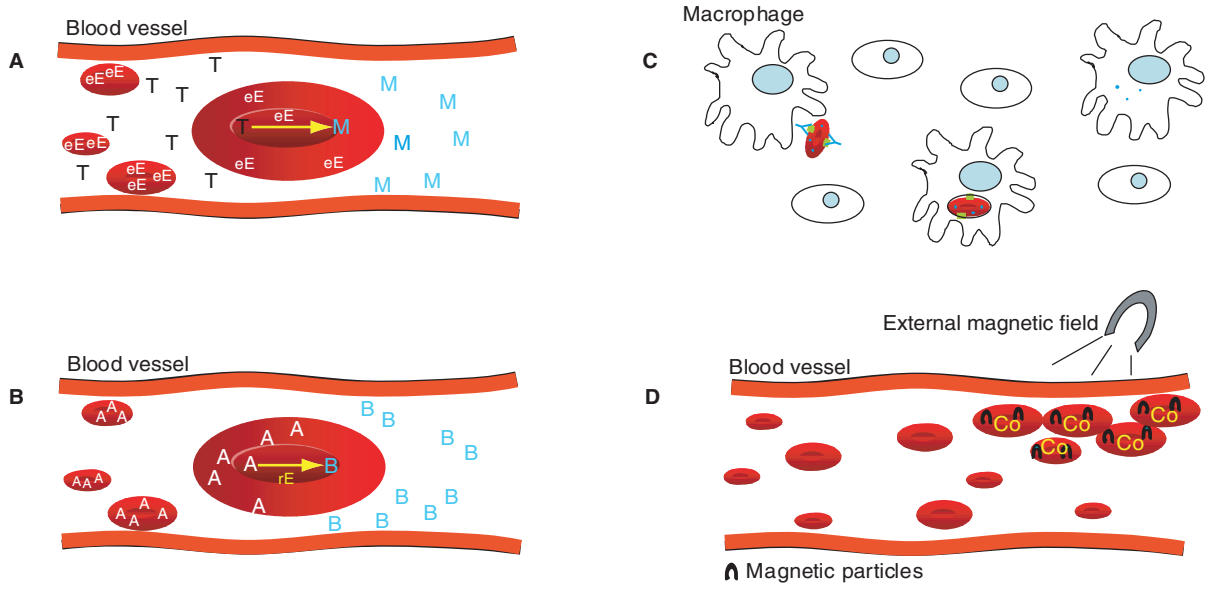


**Figure 1. Schematic representation of various modes by which erythrocytes can be used as drug carriers. A.** Circulating bioreactors to remove undesired molecules; **B.** circulating bioreactors to release diffusible drugs in blood vessel; **C.** drug-targeting system for the selective delivery of drugs to macrophages; and **D.** drug-carrier systems to release therapeutics by using external magnetic fields.
A: Nondiffusible prodrug; B: Diffusible active prodrug; Co: Coentrapped drug; eE: Encapsulated enzyme; M: Diffusible nontoxic metabolite; rE: Red blood cell resident enzyme; T: Diffusible toxic molecule.

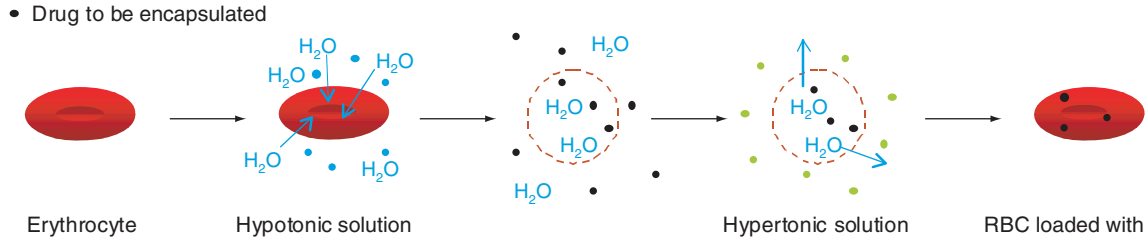


**Figure 2. Schematic representation of drug encapsulation in erythrocytes by hypotonic dialysis method.** Hypotonic solution causes membrane swelling and opening of pores. The added drug crosses pores. Cells are resealed by using hypertonic solution. Drug has been encapsulated.
RBC: Red blood cell.

of these because it preserves the biochemical and physiological characteristics of the erythrocytes resulting from the process, and furthermore, it results in the highest percentage of encapsulation. More specifically, erythrocytes at a haematocrit of 70% are placed in a dialysis tube and then immersed in a hypotonic solution. During the incubation, the membrane pores appear and open. At this point, a drug added externally can be incorporated inside the erythrocyte by a passive mechanism. A drug can be added to the erythrocytes before dialysis only if the molecular weight of the substance to be encapsulated is greater than the cutoff of the dialysis tube. On the contrary, if the substance is rapidly dialysable, it should be added to the external dialysing buffer if it is available in large amounts or, after the dialysis step, incubating the dialysed RBCs with the substance directly, when it is available in limited quantities. In the latter situation, the maximum concentration to be loaded may be limited by the need to avoid high osmolalities, which interfere with the lysis procedure. Finally, by raising the salt concentration to its original level, the pores close, the RBCs reassume their normal biconcave shape and the substance remains encapsulated inside the cells at a suitable concentration. Successively, the nonentrapped substance is washed out and the loaded RBCs are ready to be used as carriers for the delivery of the encapsulated drugs. Furthermore, by inducing the formation of an antigenic site on the RBC membrane, it is possible to specifically target the drug-containing erythrocytes to macrophages (**Figure 1C** and **Figure 3** ). In particular, when $ZnCl_2$ is added to loaded RBCs externally, it is able to induce the reversible clusterisation of the band 3 protein (an anion transporter on the RBC surface) that, once stabilised by the addition of the crosslinker agent bis(sulfosuccinimidyl)suberate ($BS^3$), becomes an antigenic site readily

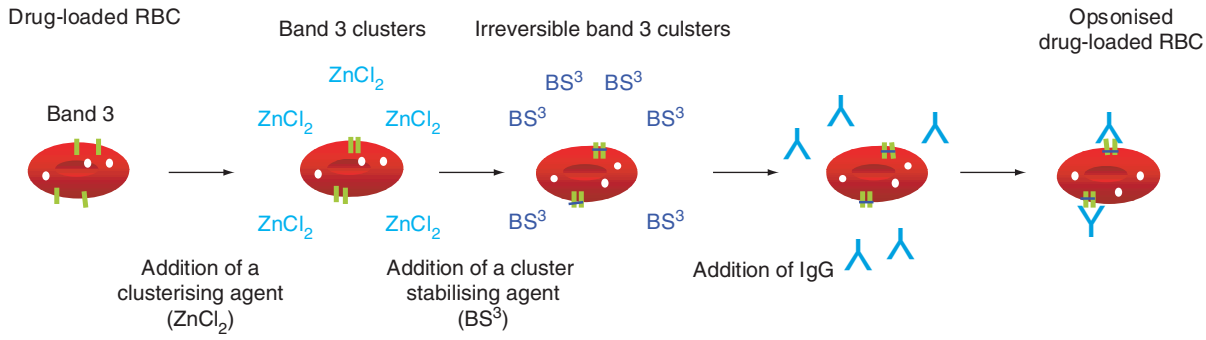


**Figure 3. Schematic representation of erythrocytes opsonisation.** The drug-loaded cells are treated with $ZnCl_2$ to induce band 3 clustering and $BS^3$ to make these clusters irreversible. Serum autologous IgG molecules recognise band 3 clusters and opsonise the RBC.
$BS^3$: *bis*(sulfosuccinimidyl)suberate; RBC: Red blood cell.

recognised by autologous IgG and complement. All this mimics the physiological senescent process and promotes phagocytosis of the RBC by macrophages [15]. Phagocytosed RBCs enter the endocytic pathway that leads to the degradation of the carriers in the lysosomal compartment and the release of their content within the macrophages [4]. Moreover, it is possible to modulate the *in vivo* survival of the treated cells by controlling the extension of band 3 clustering by varying the amount of $Zn^{2+}$ used [16]. This method allows an estimation of the amount of drug to be delivered to phagocytic cells, thus controlling the rate of RBC removal from circulation.

### 2.2 Red blood cells as nucleotide analogue carriers

Nucleoside analogues are currently used in a variety of HIV-1-infected patients [17]. However, due to their short plasma half-life, frequent administrations of drugs are necessary to maintain therapeutically useful drug levels. Moreover, to achieve adequate concentrations of drugs in the cerebrospinal fluid, high doses of analogues must be administered. As a consequence, severe side effects arise. In addition, several of these drugs are DNA-chain terminators; thus, to be incorporated into the growing DNA chain, they must be phosphorylated in the target cells in order to yield nucleotide analogue 5′-triphosphates, whereas some cell types (quiescent cells, such as macrophages) possess low levels of the specific kinases for drug activation. To bypass these limitations, erythrocytes as carriers of nucleotide analogues could be used. In fact, as erythrocytes are naturally endowed with a series of enzymes involved in bioconversion reactions, it is possible to design and synthesise a metabolically more remote precursor of the desired drug (in this case nucleotide analogues) to obtain a membrane-diffusible active drug. At the same time, active phosphorylated forms of nucleoside analogues (or their prodrugs) can be encapsulated into erythrocytes and selectively targeted to macrophages, the role of which in the pathogenesis of AIDS is well known [18]. As the first phosphorylating step is critical, the direct administration of the partially phosphorylated form would overcome these limitations. Knowledge of the biochemistry of RBCs is a key factor in designing the most appropriate prodrug presenting charged chemicals groups. Once the chemical groups have been hydrolysed by resident RBC enzymes, the prodrug is converted into an active drug that can diffuse through the erythrocyte membrane and thus be released into the circulation or at specific sites where RBC targeting is achieved (macrophages).

To give examples of the first case (drug release in circulation), the following compounds, 3′-azido-3′-deoxythymidine-monophosphate (AZT-MP) [19], di-(thymidine-3′-azido-2′,3′-dideoxy-D-riboside)-5′-5′-$p^1$-$p^2$-pyrophosphate ($AZTp_2AZT$) [20], 2-fluoro-ara-AMP [21] and $p^1$-thymine-3′-azido-2′,3′-dideoxy-D-riboside-5′-$p^2$(+)2,2′-(ethylendiimino)di-1-butanol pyrophosphate ($AZTp_2EMB$) [22], have been successfully encapsulated into erythrocytes, and *in vitro* experiments have been performed. The phosphate bridge, when present, was first hydrolysed by a dinucleotide pyrophosphatase, followed by the removal of the phosphate group by a 5′-nucleotidase to generate the corresponding active drug, thus ensuring long-lasting plasma concentrations while reducing toxic side effects. As examples of the second case (nucleotide analogues released into macrophages), the following homo- and hetero-dinucleotides, $AZTp_2AZT$ [23], $p^1$-(thymine-3′-azido-2′,3′-dideoxy-β-D-riboside-5′-$p^2$-guanine-9-(2-hydroxyethoxymethyl) pyrophosphate ($AZTp_2ACV$) [24], $p^1$,$p^2$-*bis*[2-(adenin-9H-yl)ethoxymethyl]phosphonate (*Bis*-PMEA) [25], 9-[(*R*)-2-(phosphonomethoxy)propyl]adenine (AZTpPMPA) [26], ACVpPMPA [27], have been used *in vitro* to treat virus-infected macrophages. These compounds were shown to be efficiently encapsulated in RBCs and stable enough to be hydrolysed when they are phagocytosed by the macrophages together with their carriers. In fact, the dinucleotide pyrophosphatase promotes the activation of the drugs directly in the macrophages with the release of acyclovir monophosphate (AZT-MP) (or ACV-MP or PMEA or PMPA) and they are now able to display their pharmacological action on HIV-1 or herpes simplex virus infection, thus bypassing the first critical phosphorylating step. The role of RBCs as nucleotide analogue carriers has recently been reviewed [28,29].

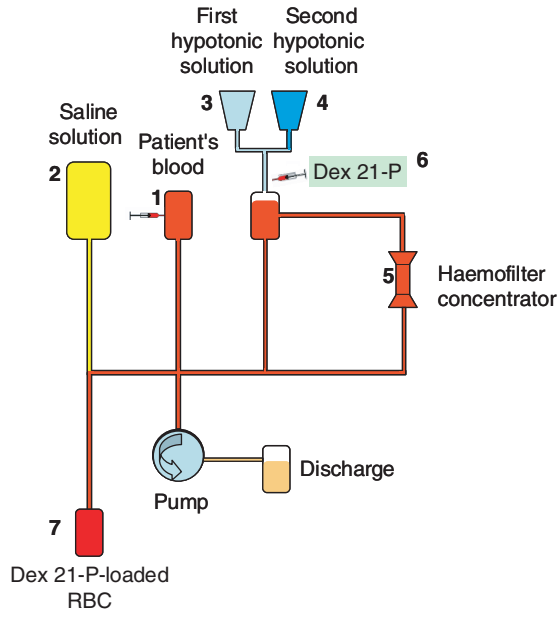


**Figure 4. Diagram of the apparatus used for the encapsulation of drugs in human erythrocytes**. **1.** Blood drawn from a patient is transferred by the pump into a rotation bowl. **2.** The erythrocytes are washed with a saline solution, and plasma, platelets and white cell buffy coat are removed. **3 – 4.** Two different hypotonic solutions (the first to swell erythrocytes, the second to lyse them) are added in sequence. **5.** The lysed erythrocytes are concentrated in a haemofilter. **6.** Dex 21-P is introduced in the bag in which the lysed and concentrated erythrocytes have been collected. A resealing solution is then added and the resealed erythrocytes are finally washed and collected in a disposable plastic bag ready to be infused (**7**).
Dex 21-P: Dexamethasone 21-P; RBC: Red blood cell.

### 2.3 Red blood cells as glucocorticoid analogue carriers

The use of RBCs as a delivery system to target nondiffusible glucocorticoid analogues, such as dexamethasone 21-phosphate (Dex 21-P), to activated monocytes/macrophages (inducing band 3 clusterisation on the RBC membrane), or to use the same system for the slow release of dexamethasone in circulation has been investigated. In macrophages, the expression of several inflammatory mediators (cytokines, chemokines, cell adhesion molecules, enzymes) is regulated by the NF-κB/inhibitor κB (IκB) pathway. The involvement of nuclear transcription factor NF-κB in immune and inflammatory responses makes it a promising target for therapeutic intervention. In unstimulated cells, NF-κB is retained in the cytoplasm in a latent form through an interaction with a member of the -kB group of protein inhibitors (IκB), so that the NF-κB nuclear localisation signal (NLS) is blocked. In response to stimulatory agents such as pathogens and proinflammatory cytokines, IκB protein is phosphorylated and subsequently degradated by the ATP-dependent 26S proteosome complex, thereby promoting the release and nuclear translocation of DNA-binding NF-κB from the cytoplasm to the nucleus. Glucocorticoids that bind to a cytoplasmic glucocorticoid receptor (GR) translocate to the nucleus, and express part of their anti-inflammmatory action by interfering with NF-κB activity and particularly by increasing the transcription of the IκB gene. Consequently the new synthesis of IκB compensates for the rapid degradation of the inhibitor on cell stimulation, thus allowing the maintainance of NF-κB in its inactive state. The targeting of RBCs loaded with Dex 21-P to *in vitro* stimulated macrophages was shown to downregulate TNF-α secretion [30] and, more recently, the efficiency of this system in interfering with the NF-κB activation pathway was documented in *in vitro* studies [31].

The nondiffusible prodrug Dex 21-P has also been encapsulated into human erythrocytes with the goal of obtaining a slow but sustained release of the corresponding diffusible drug dexamethasone (Dexa) into the circulation [30]. A single administration of Dex 21-P-loaded RBCs provides concentrations of the diffusible drug within therapeutic values for several days, whereas to maintain similar Dexa concentration, the free drug must be repeatedly administered at intervals of a few hours. This is of great clinical interest, as the administration of the drug in such a way that its presence in low but sustained concentrations in the bloodstream is assured, could avoid the undesirable, but inevitable, toxic adverse effects due to a prolonged exposure to high plasma concentrations. In the mean time, if the release of the diffusible drug from the carrier is slow enough, a constant and therapeutically useful concentration is obtained.

Furthermore, the use of erythrocytes as a glucocorticoid analogue delivery system has recently found a clinical application in the treatment of inflammatory disease [32,33], as the loading procedure, based on the hypotonic haemolysis method, has been opportunely modified to be performed in a specifically designed apparatus under blood-banking conditions [34]. A standard blood-processing apparatus (Dideco Compact Advance, Dideco SpA, Modena, Italy) has been adapted to perform the encapsulation of drugs into human erythrocytes by means of equipment specifically designed for this purpose (**Figure 4**). The procedure consists of two sequential hypotonic dilutions of washed RBCs, followed by a concentration step carried out with a haemofilter and ultimately the resealing of the loaded RBCs. The preparation of Dex 21-P-loaded RBCs guarantees a high percentage of drug encapsulation (30 ± 3%) and a good cell recovery (30 – 50%). It is easy to perform, reproducible and can be completed within 2 h. The procedure is safe, this being confirmed by > 700 infusions of drug-loaded erythrocytes in patients with inflammatory diseases in which no clinically significant adverse effects have been observed. Furthermore, the administration of Dex 21-P-loaded RBCs *in vivo* provides a low and sustained Dexa plasma concentration (0.02 – 0.05 μM), which is still detectable 1 month after a single infusion. In effect, the low doses of Dexa released from the autologous drug-loaded RBC received monthly by patients give the proper clinical benefits of corticosteroid treatment without the toxic side effects.

## 2.4 Red blood cells as protein carriers

### 2.4.1 Delivery of enzymes

One of the applications of carrier RBCs is the encapsulation and the consequent transport of enzymes that metabolise cell membrane permeating substrates to correct congenital metabolic disorders or to clear undesired molecules from the bloodstream [35-37]. In the treatment of metabolic disorders, deficient or missing enzymes could be replaced by administering them through intravenous injection. Unfortunately, this is not without serious consequences as the therapeutic use of enzymes includes some problems such as the shorter circulation half-life of the enzymes, the appearance of immunological disorders and/or allergies and, in some cases, tissue toxicity. According to the different enzymes loaded and the related disorders, these enzyme-carrier cells could: act as bioreactors by which substrates can enter into the cell, interact with enzymes and generate products; target the enzymes to the cells of the RES, where their action is required; or release the enzyme in a particular site on haemolysis for future catalysis (fibrinolytic agents).

One of the most important applications of enzyme-loaded erythrocytes as therapeutic tools is the encapsulation of L-asparaginase for the treatment of paediatric neoplasma (lymphosarcoma and acute lymphoblastic leukaemia) [6]. Furthermore, erythrocytes loaded with aldehyde dehydrogenase (AIDH) were shown to favour the depletion of acetaldehyde and ethanol in normal or alcoholic mice [38]; whereas alcohol oxidase-loaded RBCs were efficiently used to deplete methanol and formaldehyde during methanol mice poisoning [9]. Moreover, the coentrapment of glucose oxidase and hexokinase was found to be effective in regulating blood glucose at approximately physiological concentration in hyperglicemic mice [39]. Furthermore, the administration of glucocerebrosidase-loaded RBCs could be beneficial in patients with the rare hereditary disorder, known as Gaucer's disease, which results in accumulation of the lipid glucocerebroside within macrophages due to a deficiency in lysosomal β-glucocerebrosidase. Thus, through the selective targeting of glucocerebrosidase-loaded RBCs directly to macrophages [40], the missing enzyme could be present in the site where its action is requested in significant amounts.

A new conceptual approach is the encapsulation of therapeutic agents, which act on coagulation mechanisms to prevent thrombosis in individuals having high risk factors. Previously, several *in vitro* and *in vivo* experiments were carried out by loading RBCs with factors IX and X [41], heparin [42], aspirin [43] and brinase [44]. Among the fibrinolytic agents, urokinase and streptokinase have been encapsulated in RBCs and administered in this form to prevent thrombosis [45].

### 2.4.2 Delivery of toxins

Erythrocytes loaded with the haemolytic toxin listeriolysin O (LLO) were administered *in vitro* to human macrophages infected with *Mycobacterium avium* [46]. This was performed with the goal of reaching the pathogen directly in the site of its replication and to perturb its environment. In fact, many pathogens, such as mycobacteria, have evolved several strategies to elude host defence mechanisms and to survive within the intracellular environment undisturbed. In particular, *M. avium* has a peculiarity; once it is phagocytosed by macrophages, it survives and multiplies in a specialised vacuole, which does not fuse with lysosomes but, retaining immature features, maintains the ability to fuse with early endosomes [47]. Keeping this in mind, to reach the pathogen directly inside its vacuole, the administration of a drug in a way that it is phagocytosed by macrophages (i.e., as in drug-loaded RBCs) and retained inside a phagosome, could represent an effective strategy. LLO has attracted interest because its activation (the activation of the ability to form pores in membranes) occurs at acidic pH, after phagosomal acidification once LLO-loaded RBC are phagocytosed by macrophages, and at no other time. This activation could lead to lyses of the erythrocyte membrane, release of the toxin and perturbation of the mycobacterial phagosome. The administration of LLO-loaded RBC to *M. avium*-infected macrophages resulted in a 50% inhibition of the replication of the pathogen without affecting the host cells. The strategy described could also be useful against other pathogens and constitute a basis for future studies on the use of LLO-loaded RBCs in combination with traditional antimycobacterial drugs, thus providing a feasible approach in delivering new biologicals to the mycobacterial vacuole. Furthermore, in general, LLO-loaded erythrocytes could be used to improve the intracellular trafficking of coencapsulated therapeutic molecules.

### 2.4.3 Delivery of peptides

The targeting of peptides or proteins to cells is an important task in the biomedical field. It has been demonstrated that the RBC-mediated delivery of proteins is feasible, and interesting results have been obtained for both the delivery of an ubiquitin analogue [48] and for the *in vivo* delivery of glutathione [49]. The RBC delivery of a ubiquitin mutant has been performed as a fascinating alternative to glucocorticoid administration to modulate NF-κB activation via targeting of IκB-α ubiquitylation and/or degradation. It is known that IκB-α degradation occurs through a highly selective degradation pathway that involves the covalent conjugation of multiple ubiquitin molecules to target proteins leading to the formation of a multi-ubiquitin chain. This chain presents a polymeric structure in which the C-terminus of each ubiquitin is linked to Lys48 of the preceding ubiquitin. It is shown that the ubiquitin mutant in which Lys48 has been replaced by arginine can block polyubiquitin chain elongation. Consequentially, the authors reasoned that administration of K48R ubiquitin to macrophages using an erythrocyte-based delivery may be efficient in the control of NF-κB activation. It has been reported that this system permits the internalisation of the ubiquitin analogue into macrophages and that it is able to compete with the endogenous ubiquitin of cells and consequently to modulate IκBα degradation and NF-κB gene transcription on stimulation with lipolysaccharide (LPS).

Regarding glutathione (GSH)-loaded RBC, the fact that erythrocytes are able to serve as potential GSH carriers avoiding the oxidation that rapidly occurs in biological fluids when it is administered in its free form has been demonstrated. In particular, the use of RBCs as GSH carriers in the treatment of retroviral infections was evaluated. It is known that macrophages constitute important targets for HIV-1, that they serve as virus reservoirs and represent the most important targets in the CNS. Current antiretroviral therapies are of limited efficacy in this cellular compartment; thus every new approach may be of great clinical relevance. As previous findings [50] have demonstrated that the administration of GSH at elevated doses to mice infected with the retroviral complex LP-BM5 (murine model of AIDS) is effective in reducing the disease, RBCs were used to deliver GSH selectively to macrophages, thus protecting the CNS, in combination with the approved antiretroviral drugs (known to be quite ineffective in this compartment). A substantial protection of the brain was obtained, suggesting that GSH has an antiviral activity and that redox control is an important strategy for oxidative stress-associated disorders, including HIV infection, and that RBCs can be used to enhance the delivery of GSH to the CNS, thus improving therapy for AIDS dementia and related encephalopathies. The *in vitro* and *in vivo* results concerning GSH-loaded RBCs are reviewed in Fraternale *et al.* [51].

### 2.5 Red blood cells as antisense peptide nucleic acid carriers

Peptide nucleic acids (PNAs) are synthetic oligonucleotide analogues. They form very stable complexes with complementary DNA or RNA strands for which they show very high affinity while possessing a strong resistance to enzymatic degradation in cells, fluids and tissues exhibiting an exceptional stability in biological environments [52]. PNAs are principally studied for their potential antisense and antigene activities; however, their permeability across the cell membrane is, by nature, low [53] and, therefore, limits their application. Thus, the delivery of PNA to tissues and cell targeting tend to be more difficult and methods need to be improved. The use of loaded opsonised autologous erythrocytes to selectively deliver PNAs to macrophages has been proposed; in particular, the capacity of PNA-loaded RBCs to control the production of a mediator of inflammation, nitric oxide (NO), was evaluated. This signal metabolite is synthesised by three distinct isoforms of NO synthase (NOS): the brain and endothelial enzymes are constitutively expressed and their enzymatic activity is calcium-dependent; in contrast, the inducible NOS (iNOS) is regulated at the transcriptional level by endotoxins and cytokines. The ability of a homopyrimidine PNA probe to specifically bind murine iNOS mRNA and consequently to inhibit its translation has been explored *in vitro* [54]. In particular, the inhibition of iNOS expression and NO production following the selective targeting of loaded RBCs to murine macrophages after stimulation with LPS has been studied. PNA-loaded RBCs administered to LPS-activated macrophages were able to inhibit iNOS levels and reduce nitrite production. These results are particularly encouraging considering that, in order to obtain a similar inhibition with free antisense oligonucleotide in a similar cellular model [55], a much higher concentration is necessary. The study was extended to an *in vivo* murine model confirming the results reported above. This system overcomes the obstacle represented by the crossing of intact macrophage membranes by PNAs. Moreover, it represents a valid alternative in the use of drugs, which are already available, but are often toxic and lacking in specificity, thus suggesting its possible useful therapeutic applications.

### 2.6 Coupling proteins to the surface of red blood cells

As an alternative to drug loading inside the RBC, TPs, such as antibodies or enzymes, could be coupled to the surface of the plasma membrane of the carrier RBC. This coupling can elude some problems related to loaded drugs based, not on the amount of the drug encapsulated, but rather on steric restrictions. Concerning enzyme-loaded RBCs, these can effectively interact only with membrane-permeable substrates such as methanol or glucose. Furthermore, even enzymes that react with small, diffusible substrates are more active when bound to the RBC surface than when incorporated within cells if the substrate concentration is higher in plasma than in the RBC. As a second instance, coupling antibodies or enzymes to the RBC surface could be used to facilitate their immunotargeting to intravascular targets and to regulate pathological situations that often occur intravascularly, such as abnormalities in coagulation and fibrinolysis. The biocompatible coupling of drugs or target moieties to a RBC carrier must satisfy some requirements: the complex must be stable and retain the activity of the coupled protein; the activation of the complement-mediated phagocytoses and the activation of the immune system must be avoided (biocompatibility); and lifetime in circulation and biodistribution in tissues should not be compromised (bioavailability). A specific biocompatible and effective approach for the coupling of proteins to RBCs is represented by the use of a streptavidin (SA)–biotin crosslinker. Briefly, the coupling of TPs to carrier RBCs using this system requires the separate biotinylation (b) of RBCs and TPs by a biotin succinimide ester, to form RBC-b and TP-b. Successively, SA binds to RBC-b and provides sites for the stable attachment of TP-b, thus permitting the coupling of TP-b to RBCs via SA (**Figure 5A**). The degree of RBC biotinylation is an important parameter of this crosslinking system. It should be stressed that only monovalent coupling via SA to RBCs allows prolongation of the functional half-life of the TP as this does not compromise RBC biocompatibility [56], whereas polyvalent SA coupling to RBC-b inactivates complement regulating proteins decay accelerating factor (DAF) and CD59 [57], which leads to RBC haemolysis [58]. One example of therapeutic proteins amenable to RBC carriage would be antithrombotic enzymes (**Figure 5B**). The coupling of fibrinolytic agents, such as clinically applicable tissue-type plasminogen

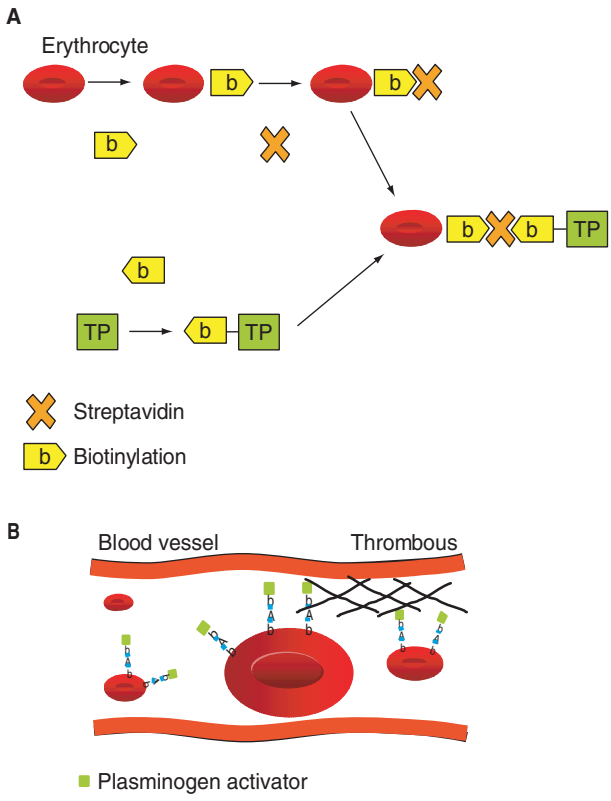


**Figure 5. Coupling of therapeutic proteins to carrier RBCs throughout SA–biotin crosslinker. A.** Schematic representation of the coupling procedure. RBC and TP are biotinylated separately by a biotin succinimide ester to form RBC-b and TP-b. Successively, SA binds to RBC-b, providing sites for stable attachment of TP-b. **B.** Schematic representation of action of antithrombotic agents coupled to RBC surface by SA–biotin crosslinker.
A: Avidin; b: Biotin; RBC: Red blood cell; SA: Streptavidin; TP: Therapeutic protein.

activator (tPA) and urokinase (uPA), could provide, *in vitro*, a longer lifetime in circulation, maintaining high fibrinolytic activity without compromising the biocompatibility of the carrier. These were also tested *in vivo* in an animal model in which it was demonstrated that blood level and tissue distribution of RBCs carrying tPA or uPA were similar to those of control RBCs. Furthermore, when compared with plasminogen activators, RBC-coupled enzymes showed a dramatically prolonged lifetime and a higher bioavailability in the bloodstream of intact animals [59,60]. As further examples of plasminogen activators coupled to RBCs, streptokinase retains its ability to convert its substrate, plasminogen, into plasmin *in vitro*, whereas RBCs carrying both collagen antibody and streptokinase were even able to bind immobilised collagen and degrade fibrin clots formed over the collagen target [61].

In some cases, coupling an enzyme on the surface of RBCs could be more useful than loading it inside the RBC. This is true of uricase, the enzyme responsible for uric acid degradation. Different approaches have been used to reduce serum urate levels, such as the promotion of uric acid excretion and the administration of free uricase, whereas others have proposed uricase-loaded RBCs to degrade uric acid *in vitro* [62] and *in vivo* [63]. Unfortunately, the RBC membrane is less permeable to uric acid; thus, despite the amount of uricase loaded, the degradation capacity of enzyme-loaded RBC is very low. Furthermore, as a product of the uricase reaction, hydrogen peroxide is generated. To overcome these two limitations, the possibility of coupling uricase to the extracellular erythrocyte membrane was evaluated [63]. The ability of coupled uricase to degrade uric acid in the bloodstream was maintained and, furthermore, its level was maintained at low concentration for several days in animals receiving the enzyme bound to RBCs. This strategy could be used to optimise the therapeutic profile of a wide spectrum of drugs such as antithrombotic agents, antigens, cytokine antagonists and other enzymes. Finally, the biocompatible coupling of proteins could represent an attractive approach to the intravascular administration of therapeutic proteins for which prolonged action, restricted to the blood, is required.

## 3. Conclusion

Data summarised in this review show some of the numerous potential biomedical applications of erythrocytes as drug delivery systems. RBCs feature some unique advantages compared with other delivery systems making them not only natural, safe and abundant carriers, but, being endowed with enzymes involved in bioconversion reactions, also active bioreactors. Recently, the role of erythrocytes as drug carriers has been documented as shown by several and extensive reviews [64-68]. Herein we report, initially, some information regarding the preparation of drug-loaded RBCs and successively, numerous examples of RBCs as drug carriers. In particular, RBCs as carriers of nucleotide analogues, proteins, glucocorticoid analogues and antisense peptide nucleic acids are reviewed. Altogether, from the analysis of the data, the following conclusions can be drawn:

- Phosphorylated nucleoside analogues (e.g., AZT-MP, $AZTp_2AZT$) can be encapsulated into RBCs, which act as bioreactors for the slow delivery in circulation of diffusible nucleoside analogues (i.e., AZT), thus ensuring their long-lasting plasma concentrations while reducing their toxic side effects.
- Phosphorylated or phosphonate nucleoside analogues (e.g., $AZTp_2ACV$, Bis-PMEA, AZTpPMPA) can be encapsulated into RBCs and selectively targeted to macrophages (known reservoirs of numerous pathogens) where they are hydrolysed to compounds (i.e., AZT-MP, ACV-MP, PMEA, PMPA) able to efficiently display their antiviral activity in those cells where nucleoside analogues are usually ineffective.
- Proteins can be efficiently encapsulated in or coupled to RBCs, thus preventing their premature degradation and

reducing undesirable immune response and toxic side effects. Results confirm the ability of enzyme-loaded RBCs to remove undesired molecules from the bloodstream (such as acetaldehyde and ethanol in alcoholism, methanol and formaldehyde in methanol poisoning, and glucose in diabetes). Moreover, a selective protein targeting to macrophages can be achieved by using opportunely modified protein-loaded erythrocytes. This is the case of the toxin LLO (delivered to macrophages infected with *M. avium* to inhibit pathogen replication), of the K48R ubiquitin analogue (delivered to macrophages to modulate NF-κB activation in inflammatory diseases) and of GSH (delivered to macrophages to avoid oxidation in oxidative stress-associated disorders).

- The anti-inflammatory Dex 21-P can be encapsulated into RBCs to obtain a slow but sustained release of dexamethasone in the bloodstream without the appearance of toxic side effects. This strategy has been successfully used in some inflammatory diseases (chronic obstructive pulmonary disease, cystic fibrosis and inflammatory bowel disease). Moreover, Dex 21-P-loaded RBCs can be selectively targeted to macrophages to inhibit the expression of inflammatory mediators by interfering with NF-κB activity.
- Modified oligonucleotides (such as PNA) can be encapsulated into RBCs and selectively targeted to macrophages to exploit their antisense and antigen activities, while bypassing their low cellular permeability. It was demonstrated that by using RBCs, a PNA probe that binds inducible NO synthase mRNA works better than its free form in inhibiting NO production in a cell inflammation model.

From the results obtained from our and other laboratories [69,70], we are confident that erythrocytes have great potentialities in the field of drug delivery, as the key to success of many therapeutics greatly depends on the development of novel technologies to improve and control the delivery of drugs.

## 4. Expert opinion

Erythrocyte carrier systems have unique properties and are useful as specific targeting agents conferring the prolonged and sustained action of the encapsulated drug while causing minimal immunological or other side effects. RBC carriers, in fact, provide a safe, physiological carrier system model, which can conventionally be used to obtain the sustained release of the encapsulated molecule or one of its products and serve as a model for targeted delivery strategies, particularly to macrophage cells.

As erythrocytes are carriers of biological origin, they have the disadvantage of a greater variability and a lesser standardisation in their preparation compared with other carrier systems. A further problem could be represented by the storage of the loaded erythrocytes for long periods of time (months); consequently, we prepare autologous loaded erythrocytes when they are requested.

Although erythrocytes have been proposed for different uses in medicine and several studies have already been carried out in *in vitro* and *in vivo* animal models, strategies for human use remain very limited due to the difficulties involved in storage, exposure to contamination and the absence of validated industrial preparation procedures. Over the last few years, in the attempt to overcome these problems, some steps forward have been taken and a new procedure as well as new blood-processing equipment have been developed. Prompted by our experience in erythrocyte-based delivery, we designed and built a new apparatus with which autologous RBCs can be loaded with a nondiffusible prodrug, while maintaining all the products pyrogen-free and sterile. This new procedure allows the processing of small volumes of autologous blood to be reinfused into the same donor, thus avoiding the risk of transmitting infections. The availability of a new procedure for the processing of small volumes of autologous blood to be reinfused, and the development of new biomedical applications of engineered RBCs for the delivery of therapeutics, make us optimistic about the future of engineered RBCs.

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### Bibliography

Papers of special note have been highlighted as either of interest (•) or of considerable interest (••) to readers.

1. DE LOACH JR, WAY JL: *Carrier and bioreactor red blood cells for drug delivery and targeting.* Elsevier, Tarry-Town, NY, USA (1994).
2. SPRANDEL U, WAY JL: *Erythrocytes as drug carriers in medicine.* Plenum Press, New York, USA and London, UK (1997).
3. MAGNANI M, DE LOACH JR: *The use of resealed erythrocytes as carriers and Bioreactors.* Plenum Press, New York, USA and London, UK (1992).
4. MAGNANI M, ROSSI L, BRANDI G, SCHIAVANO GF, MONTRONI M, PIEDIMONTE G: Targeting antiretroviral nucleoside analogues in phosphorylated form to macrophages: *in vitro* and *in vivo* studies. *Proc. Natl. Acad. Sci.USA* (1992) **89**(14):6477-6481.

•• **Describes the procedure by which loaded erythrocytes can be selectively targeted to macrophages.**

5. MAGNANI M, DE FLORA A: *Red blood cell aging.* Plenum Press, New York, NY, USA (1991).
6. KRAVTZOFF R, DESBOIS I, CHASSAIGNE M *et al.*: *In vivo* activity of L-asparaginase entrapped into human and mouse red blood cells. In: *Advances in the bioscences.* R Green, JR De Loach (Eds),

Pergamon Press, Oxford, UK (1991):127-137.

7. BRUGGEMANN U, ROUX EC, HANNIG J, NICOLAU C: Low-oxygen-affinity red cells produced in a large-volume, continuous-flow electroporation system. *Transfusion* (1995) **35**(6):478-486.
8. BAX BE, BAIN MD, FAIRBANKS LD, WEBSTER AD, CHALMERS RA: *In vitro* and *in vivo* studies with human carrier erythrocytes loaded with polyethylene glycol-conjugated and native adenosine deaminase. *Br. J. Haematol.* (2000) **109**(3):549-554.
9. MAGNANI M, FAZI A, MANGANI F, ROSSI L, MANCINI U: Methanol detoxification by enzyme-loaded erythrocytes. *Biotechnol. Appl. Biochem.* (1993) **18**(3):217-226.
10. BYUN HM, SUH D, YOON H *et al.*: Erythrocyte ghost-mediated gene delivery for prolonged and blood-targeted expression. *Gene Ther.* (2004) **11**(5):492-496.
11. ROSSI L, SERAFINI S, MAGNANI M: Red blood cell loading: a selection of procedures. In: *Erythrocyte Engineering for Drug Delivery and Targeting.* M Magnani (Ed.), Kluwer Academic/Plenum Publishers, New York, NY, USA (2003):1-18.
12. LIZANO C, PEREZ MT, PINILLA M: Mouse erythrocytes as carriers for coencapsulated alcohol and aldehyde dehydrogenase obtained by electroporation *in vivo* survival rate in circulation, organ distribution and ethanol degradation. *Life Sci.* (2001) **68**(17):2001-2016.
13. FRANCO RS, BARKER R, MAYFIELD G, SILBERSTEIN E, WEINER M: The *in vivo* survival of human red cells with low oxygen affinity prepared by the osmotic pulse method of inositol hexaphosphate incorporation. *Transfusion* (1990) **30**(3):196-200.
14. IHLER GM: Entrapment of DNA and fluorescent compounds in erythrocyte carriers by endocytosis. *Bibl. Haematol.* (1985) **51**:127-133.
15. KAY MM, GOODMAN SR, SORENSEN K *et al.*: Senescent cell antigen is immunologically related to band 3. *Proc. Natl. Acad. Sci.USA* (1983) **80**(6):1631-1635.
16. CHIARANTINI L, ROSSI L, FRATERNALE A, MAGNANI M: Modulated red blood cell survival by membrane protein clustering. *Mol. Cell. Biochem.* (1995) **144**(1):53-59.
17. POSTIC B, HORVATH JA, TALWANI R: Antiviral treatment of human immunodeficiency virus infection: update for 2003. *J. S. C. Med. Assoc.* (2003) **99**(6):148-153.
18. GARTNER S, MARKOVITS P, MARKOVITZ DM, KAPLAN MH, GALLO RC, POPOVIC M: The role of mononuclear phagocytes in HTLV-III/LAV infection. *Science* (1986) **233**(4760):215-219.
19. MAGNANI M, GIOVINE M, FRATERNALE A *et al.*: Red blood cells as a delivery system for AZT. *Drug Deliv.* (1995) **2**:57-61.
20. BENATTI U, GIOVINE M, DAMONTE G *et al.*: Azidothymidine homodinucleotide-loaded erythrocytes and bioreactors for slow delivery of the antiretroviral drug azidothymidine. *Biochem. Biophys. Res. Commun.* (1996) **220**(1):20-25.
21. FRATERNALE A, ROSSI L, MAGNANI M: Encapsulation, metabolism and release of 2-fluoro-ara-AMP from human erythrocytes. *Biochem. Biophys. Acta.* (1996) **1291**(2):149-154.
22. ROSSI L, BRANDI G, SCHIAVANO GF *et al.*: Heterodimer-loaded erythrocytes as bioreactors for slow delivery of the antiviral drug azidothymidine and the antimycobacterial drug ethambutol. *AIDS Res. Hum. Retroviruses.* (1999) **15**(4):345-353.
23. MAGNANI M, CASABIANCA A, FRATERNALE A *et al.*: Synthesis and targeted delivery of an azidothymidine homodinucleotide conferring protection to macrophages against retroviral infection. *Proc. Natl. Acad. Sci.USA* (1996) **93**(9):4403-4408.
24. ROSSI L, BRANDI G, SCHIAVANO GF *et al.*: Macrophage protection against human immunodeficiency virus or herpes simplex virus by red blood cell-mediated delivery of a heterodinucleotide of azidothymidine and acyclovir. *AIDS Res. Hum. Retroviruses.* (1998) **14**(5):435-444.
25. ROSSI L, SERAFINI S, CAPPELLACCI L *et al.*: Erythrocyte-mediated delivery of a new homodinucleotide active against human immunodeficiency virus and herpes simplex virus. *J. Antimicrob. Chemother.* (2001) **47**(6):819-827.
26. FRANCHETTI P, ROSSI L, CAPPELLACCI L *et al.*: Inhibition of HIV-1 replication in macrophages by red blood cell-mediated delivery of a heterodinucleotide of azidothymidine and 9-*(R)*-2-(phosphono methoxypropyl)adenine. *Antivir. Chem. Chemother.* (2001) **12**(3):151-159.
27. FRANCHETTI P, ABU SHEIKHA G, CAPPELLACCI L *et al.*: A new acyclic heterodinucleotide active against human immunodeficiency virus and herpes simplex virus. *Antiviral. Res.* (2000) **47**(3):149-158.
28. SERAFINI S, ROSSI L, ANTONELLI A *et al.*: Drug delivery through phagocytosis of red blood cells. *Transfus. Med. Hemother.* (2004) **31**:92-101.
29. ROSSI LS, SERAFINI S, FRANCHETTI P *et al.*: Targeting nucleotide dimers containing antiviral nucleosides. *Curr. Med. Chem. Anti-Infective Agents* (2005) (In press).
30. D'ASCENZO M, ANTONELLI A, CHIARANTINI L, MANCINI U, MAGNANI M: Red blood cells as a glucocorticoids delivery system. In: *Erythrocytes as Drug Carriers in Medicine.* U Sprandel, JL Way (Eds), Plenum Press, New York, NY, USA (1997):81-88.
31. CRINELLI R, ANTONELLI A, BIANCHI M, GENTILINI L, SCARAMUCCI S, MAGNANI M: Selective inhibition of NF-κB activation and TNF-α production in macrophages by red blood cell-mediated delivery of dexamethasone. *Blood Cells Mol. Dis.* (2000) **26**(3):211-222.
32. ROSSI L, SERAFINI S, CENERINI L *et al.*: Erythrocyte-mediated delivery of dexamethasone in patients with chronic obstructive pulmonary disease. *Biotechnol. Appl. Biochem.* (2001) **33**(2):85-89.
    - **The first clinical application of loaded RBCs by using the new developed apparatus 'Red Cell Loader'.**
33. ROSSI L, CASTRO M, D'ORIO F *et al.*: Low doses of dexamethasone constantly delivered by autologous erythrocytes slow the progression of lung disease in cystic fibrosis patients. *Blood Cells Mol. Dis.* (2004) **33**(1):57-63.
    - **A pilot study in cystic fibrosis comparing the efficacy of drug delivery using RBCs versus conventional therapy.**
34. MAGNANI M, ROSSI L, D'ASCENZO M, PANZANI I, BIGI L, ZANELLA A: Erythrocyte engineering for

drug delivery and targeting. *Biotechnol. Appl. Biochem.* (1998) **28**(1):1-6.

•• **Describes the apparatus developed for the encapsulation of drugs in human erythrocytes under blood-banking conditions (Red Cell Loader).**

35. DE LOACH JR: Dialysis method for entrapment of proteins into resealed red blood cells. In: *Methods Enzymol.* R Green, KS Widder (Eds), Academic Press, San Diego, CA, USA (1987):235-242.

36. SPRANDEL U: Erythrocytes as carrier for therapeutics enzymes. An approach towards enzyme therapy of inborn errors of methabolism. In: *Bibliotheca haematological (Volume 51).* A Hassing (Ed.), Karger, Basel (1985):7-14.

37. IHLER GM, GLEW RH, SCHNURE FW: Enzyme loading of erythrocytes. *Proc. Natl. Acad. Sci.USA* (1973) **70**(9):2663-2666.

38. MAGNANI M, LAGUERRE M, ROSSI L *et al.*: Acetaldehyde dehydrogenase-loaded erythrocytes as bioreactors for the removal of blood acetaldehyde. *Alcohol. Clin. Exp. Res.* (1989) **13**(6):849.

39. ROSSI L, BIANCHI M, MAGNANI M: Increased glucose consumption by human and mice erythrocytes overloaded with hexokinase and glucose oxidase. In: *Resealed erythrocytes as carriers and bioreactors.* R Green, JR De Loach (Eds), Pergamon Press, Oxford, UK (1991):169-179.

40. BAX BE, BAIN MD, WARD CP, FENSOM AH, CHALMERS RA: The entrapment of mannose-terminated glucocerebrosidase (Alglucerase) in human carrier erythrocytes. *Biochem. Soc. Trans.* (1996) **24**(3):441S.

41. GOLDSMITH JC, ROER ME, ORRINGER EP: A new treatment strategy for hemophilia B: incorporation of factor IX into red cell ghosts. *Am. J. Hematol.* (1979) **7**(2):119-125.

42. EICHLER HG, SCHNEIDER W, RABERGER G, BACHER S, PABINGER I: Erythrocytes as carriers for heparin. Preliminary *in vitro* and animal studies. *Res. Exp. Med.* (1986) **186**(6):407-412.

43. OREKHOVA NM, AKCHURIN RS, BELYAEV AA, SMIRNOV MD, RAGIMOV SE, OREKHOV AN: Local prevention of thrombosis in animal arteries by means of magnetic targeting of aspirin-loaded red cells. *Thromb. Res.* (1990) **57**(4):611-616.

44. FLYNN G, HACKETT TJ, MCHALE L, MCHALE AP: Encapsulation of the thrombolytic enzyme, brinase, in photosensitized erythrocytes: a novel thrombolytic system based on photodynamic activation. *J. Photochem. Photobiol. B.* (1994) **26**(2):193-196.

45. DELAHOUSSE B, KRAVTZOFF R, ROPARS C: Use of erythrocytes as a new route of administration of fibrinolytic agents. In: *Erythrocytes as drug carriers in medicine(Volume 5).* U Sprandel, JL Way (Eds), Plenum Press, New York, NY, USA (1997):35-42.

46. ROSSI L, BRANDI G, MALATESTA M *et al.*: Effect of listeriolysin O-loaded erythrocytes on Mycobacterium avium replication within macrophages. *J. Antimicrob. Chemother.* (2004) **53**(5):863-866.

47. DE CHASTELLIER C, LANG T, THILO L: Phagocytic processing of the macrophage endoparasite, *Mycobacterium avium*, in comparison to phagosomes which contain *Bacillus subtilis* or latex beads. *Eur. J. Cell. Biol.* (1995) **68**(2):167-182.

48. ANTONELLI A, CRINELLI R, BIANCHI M, CERASI A, GENTILINI L, SERAFINI G, MAGNANI M: Efficient inhibition of macrophage TNF-α production upon targeted delivery of K48R ubiquitin. *Br. J. Haematol.* (1999) **104**(3):475-481.

49. FRATERNALE A, CASABIANCA A, ORLANDI C *et al.*: Macrophage protection by addition of glutathione (GSH)-loaded erythrocytes to AZT and DDI in a murine AIDS model. *Antiviral Res. (*2002) **56**(3):263-272.

50. PALAMARA AT, GARACI E, ROTILIO G *et al.*: Inhibition of murine AIDS by reduced glutathione. *AIDS Res. Hum. Retroviruses.* (1996) **12**(14):1373-1381.

51. FRATERNALE A, CASABIANCA A, ROSSI L *et al.*: Erythrocytes as carriers of reduced glutathione (GSH) in the treatment of retroviral infections. *J. Antimicrob. Chemother.* (2003) **52**(4):551-554.

52. EGHOLM M, BUCHARDT O, CHRISTENSEN L, BEHRENS C, FREIER SM, NIELSEN PE: PNA hybridizes to complementary oligonucleotides obeying the Watson-Crick hydrogen-bonding rules. *Nature* (1993) **365**(6446):566-568.

53. WITTUNG P, KAJANUS J, EDWARDS K *et al.*: Phospholipid membrane permeability of pepRtide nucleic acid. *FEBS lett.* (1995) **365**(1):27-29.

54. CHIARANTINI L, CERASI A, FRATERNALE A *et al.*: Inhibition of macrophage iNOS by selective targeting of antisense PNA. *Biochemistry* (2002) **41**:8471-8477.

55. ARIMA H, SAKAMOTO T, ARAMAKI Y, ISHIDATE K, TSUCHIYA S: Specific inhibition of nitric oxide production in macrophages by phosphorothioate antisense oligonucleotides. *J. Pharm. Sci.* (1997) **86**(10):1079-1084.

56. MUZYKANTOV VR, MURCIANO JC, TAYLOR RP, ATOCHINA EN, HERRAEZ A: Regulation of the complement-mediated elimination of red blood cells modified with biotin and streptavidin. *Anal. Biochem.* (1996) **241**(1):109-119.

57. ZALTZMAN AB, VAN DEN BERG CW, MUZYKANTOV VR, MORGAN BP. Enhanced complement susceptibility of avidin-biotin-treated human erythrocytes is a consequence of neutralization of the complement regulators CD59 and decay accelerating factor. *Biochem. J.* (1995) **307**(3):651-656.

58. MUZYKANTOV VR, SMIRNOV MD, SAMOKHIN GP. Avidin attachment to biotinylated erythrocytes induces homologous lysis via the alternative pathway of complement. *Blood* (1991) **78**(10):2611-2618.

59. MURCIANO JC, MEDINILLA S, ESLIN D, ATOCHINA E, CINES DB, MUZYKANTOV V: Prophylactic fibrinolysis through selective dissolution of nascent clots by tPA-carrying erythrocytes. *Nat. Biotechnol.* (2003) **21**(8):891-896.

60. GANGULY K, KRASIK T, MEDINILLA S *et al.*: Blood clearance and activity of erythrocyte-coupled fibrinolytics. *J. Pharmacol. Exp. Ther.* (2005) **312**(3):1106-1113.

61. MUZYKANTOV VR, SAKHAROV DV, SMIRNOV MD, SAMOKHIN GP, SMIRNOV VN: Immunotargeting of erythrocyte-bound streptokinase provides local lysis of a fibrin clot. *Biochem. Biophys. Acta.* (1986) **884**(2):355-362.

62. IHLER G, LANTZY A, PURPURA J, GLEW RH: Enzymatic degradation of uric acid by uricase-loaded human erythrocytes. *J. Clin. Invest. (*1975) **56**(3):595-602.

63. MAGNANI M MANCINI U, BIANCHI M, FAZI A: Comparison of

uricase-bound and uricase-loaded erythrocytes as bioreactors for uric acid degradation. *Adv. Exp. Med. Biol.* (1992) **326**:189-194.

64. HAMIDI M, TAJERZADEH H: Carrier erythrocytes: an overview. *Drug Deliv.* (2003) **10**(1):9-20.

65. MISHRA PR, JAIN NK: Folate conjugated doxorubicin-loaded membrane vesicles for improved cancer therapy. *Drug Deliv.* (2003) **10**(4):277-282.

66. GUTIERREZ MILLAN C, ZARZUELO CASTANEDA A, SAYALERO MARINERO ML, LANAO JM: Factors associated with the performance of carrier erythrocytes obtained by hypotonic dialysis. *Blood Cells Mol. Dis.* (2004) **33**(2):132-140.

67. MILLAN CG, MARINERO ML, CASTANEDA AZ, LANAO JM. Drug, enzyme and peptide delivery using erythrocytes as carriers. *J. Control. Release* (2004) **95**(1):27-49.

68. GOTHOSKAR AV: Resealed erythrocytes: a review. *Pharm. Thechnol.* (2004) **1**:140-158.

69. MAGNANI M, ROSSI L, FRATERNALE A *et al.*: Erythrocyte-mediated delivery of drugs, peptides and modified oligonucleotides. *Gene Ther.* (2002) **9**(11):749-751.

70. MAGNANI M: *Erythrocyte Engineering for Drug Delivery and Targeting*. Kluwer Academic/Plenum Publishers, New York, NY, USA (2003).

- **Shows the therapeutic potential of erythrocytes as carriers for drugs.**

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