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Engineering erythrocytes for the modulation of drugs' and contrasting agents' pharmacokinetics and biodistribution☆

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ABSTRACT

Pharmacokinetics, biodistribution, and biological activity are key parameters that determine the success or failure of therapeutics. Many developments intended to improve their *in vivo* performance, aim at modulating concentration, biodistribution, and targeting to tissues, cells or subcellular compartments. Erythrocyte-based drug delivery systems are especially efficient in maintaining active drugs in circulation, in releasing them for several weeks or in targeting drugs to selected cells. Erythrocytes can also be easily processed to entrap the desired pharmaceutical ingredients before re-infusion into the same or matched donors. These carriers are totally biocompatible, have a large capacity and could accommodate traditional chemical entities (glucocorticoids, immunossuppresants, *etc.*), biologics (proteins) and/or contrasting agents (dyes, nanoparticles). Carrier erythrocytes have been evaluated in thousands of infusions in humans proving treatment safety and efficacy, hence gaining interest in the management of complex pathologies (particularly in chronic treatments and when side-effects become serious issues) and in new diagnostic approaches.

Contents

1. Introduction

In order to achieve a therapeutic success, the plasma concentration of the majority of drugs, including both small chemical entities and biologics (*i.e.* recombinant proteins, antibodies and their derivatives, and nucleic acid-based therapeutics), should be within the therapeutic window and should result in a proper tissue distribution. Mainly owing to this reason, our understanding should no longer be limited to the mechanisms governing drug absorption, distribution, metabolism and excretion, but should also consider the compartmentalization of these processes and the interaction of the active substance with its

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delivery system and with specific and non-specific targets. In fact, *in vivo* fate of a drug is determined by its physicochemical properties, by the interaction of the same with the selected drug delivery system and in addition by the physiological and/or pathological features of the body receiving the treatment.

Administration of several active substances by conventional formulations and dosage forms may lead to their rapid clearance resulting in short plasma half-life, which is frequently not effective and/or does not exhibit sufficient *in vivo* pharmacological activities.

Limited plasma half-life of most therapeutic agents is commonly due to their susceptibility to degradation by liver and kidney enzymes, fast renal clearance by glomerular filtration (connected to hydrophilic properties as well as small size of the drug), recognition and subsequent processing by the reticuloendothelial system (RES) and/or to their potential immunogenicity [1]. These problems commonly lead to poor bioavailability and reduced *in vivo* activity which further limits the clinical applications and the therapeutic index of several drugs [2,3]. Solutions to these problems cannot be found in either frequent administrations or high dose to achieve the required therapeutic efficacy, since these are associated with significant side effects, reduced quality of life and compliance, besides the inconvenient economic burden, which decreases the benefit to risk ratio [4]. Instead, in order to ensure safety and efficacy, drugs should preferentially be delivered selectively to their target site and at an optimal concentration. In fact, lack of selectivity in biodistribution sometimes leads to unwanted side effects, particularly for drugs that have severe cytotoxicity and/or induce drug resistance [5].

1.1. Intravascular contrasting agents

As well as for therapeutics, clinical success of *in vivo* diagnostics depends on various parameters: pharmacokinetic, short and long-term tolerability in the body, safety and functionality in the desired organ, cell targeting. The most common imaging modalities include Magnetic Resonance Imaging (MRI) [6], Computed Tomography (CT) [7], Single Photon Emission Computed Tomography (SPECT) [8], Positron Emission Tomography (PET) [9], Ultrasound (US) [10] and Optical Imaging (OI) (bioluminescence and fluorescence) [11], as well as combinations of the same. Contrast agents are used to enhance visibility of specific tissues by increasing the signal-to-noise ratio referred to surrounding tissues, and therefore to provide clear discrimination between normal and pathological regions in areas of interest [12,13]; however, the optimum performance of contrast agents has not yet been achieved since there is always a competition between their desired distributions in specific organs and their highly active clearance mechanisms [14]. In fact, the amount and distribution pattern in different organs and tissues, and the rate of recognition and removal from the body [15] should be considered important criteria that determine how long a contrast agent takes to reach the signal peak and the retention time of the signal after its intravenous injection [16].

Knowing these parameters is crucial to enhance the expected functionality and to mediate the fate of a contrast agent in the body [17].

1.2. The emergence of cell-based delivery systems

A possible and interesting solution to overcome restrictions related to the use of drugs and contrast agents lies in the exploitation of cell-mediated drug delivery systems (DDS) that is an emerging and promising strategy to address the above challenges. Numerous are the DDS based on circulating cells like recently described in detail by Su Y. et al. 2015 [18]. Among them, erythrocytes stand out to be the most appealing in order to improve the pharmacokinetics, biodistribution and pharmacodynamics of therapeutics or diagnostics.

Inherent biochemical and biophysical properties of red blood cells (RBCs or erythrocytes) make them an ideal drug delivery platform: RBC-based therapies have a significant advantage over alternative technologies in terms of half-life, stability, versatility, safety and ease of manufacture. Thanks to their remarkable long life-span in circulation they act as potential reservoirs for a slow, controlled and sustained release of valuable payloads, performing both as passive carriers (*e.g.* proteins or diagnostics) or as active bioreactors due to the enzymatic systems that their possess (*e.g.* prodrugs) [19]. Prolonged retention time in blood can provide a chance to extend the duration of pharmacological activity both to increase the therapeutic action in circulation or to lengthen the period of imaging analysis mediated by contrast agents [5].

On the contrary, by exploiting the natural fate of RBCs, it is possible to quicken the removal of drug-loaded RBCs from circulation with the aim of rapidly and selectively targeting drugs to macrophages, leading to a change in the pharmacokinetics of the molecule of interest. The use of drug-loaded RBCs was first reported in 1973 for enzyme replacement therapy of inborn errors of metabolism [20] and since then, significant progress has been made as shown by the abundant literature produced in the last decades on red blood cells as drug delivery system and the most important results have been collected in several and influential reviews [21–25]. This strategy is based on the possibility to transiently open pores on the RBC membrane that can be easily crossed by non-diffusible drugs that will remain confined within the RBCs once pores close.

Among the available loading procedures, hypotonic dialysis allows a high percentage of cell recovery (range 50–80% according to experimental conditions, [26]) which, in addition, show a normal glycolytic activity, ATP and 2,3-BPG content [27]. Moreover, the recovered RBCs show morphological features quite similar to those observed in native cells with the presence of some microcytic and hypochromic cells [28]; the observed decrease in MCH and MCHC values [26] might affect the total capability of these cells to transport oxygen that could be estimated to range from 75 to 85% the original capacity. However, this should not have an impact on blood functions since the amount of loaded cells usually re-infused in patients represents a very small fraction of the total RBCs in circulation and thus the reduction of function would not be clinically relevant. Moreover, regarding survival of the red blood cells processed by hypotonic dialysis, erythrocyte mean half-life has been reported to be 28 days, that is within the normal range of 19–29 days [29,30].

Loading procedures have been used *in vitro* and *in vivo* for the encapsulation of many substances (*e.g.* anti-inflammatory, antimicrobial agents, enzymes, polypeptides, antibodies, therapeutic proteins, antitumor drugs, oligonucleotides, contrasting agents, *etc.*) in order to modulate their pharmacokinetics. In this review, we focus our attention on a strategy to overcome the problems of actual therapeutics and diagnostics concerning *in vivo* half-life extension, modulation of bioavailability and biodistribution profile in order to maximize the therapeutic efficacy and to minimize the amount of administered drug as well as the frequency and cost of therapeutic interventions. An additional review in this same issue of ADDS by Vladimir Muzykantov will describe additional strategies able to address the payload to selected sites within the vascular system [31].

2. Therapeutics: main limitations to their use and RBC-based strategy to overcome them

2.1. Enzymes as therapeutic agents

Recombinant proteins hold increasing potential as therapeutic agents and are already successfully used as very efficient drugs in the treatment of many pathophysiological conditions. Namely, recombinant proteins are used to treat endocrine disorders, to combat various cancers, to alleviate autoimmune diseases, as active pharmaceutical ingredients in many vaccines [32] and as replacement therapy for the treatment of enzyme deficiency diseases or as a degradation system of toxic metabolites or compounds secondary to some kind of poisoning. The main drawbacks of biopharmaceutical proteins are their suboptimal

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physicochemical and pharmacokinetic (PK) properties. Main limitations for their systemic use by intravenous administration are physicochemical instability, limited solubility, proteolytic inactivation by serum enzymes, relatively short elimination by glomerular filtration (molecules with a molecular mass below 5 kDa and unbound to plasma proteins can pass the filter completely, [33]), iper-sensitivity reactions and tissue toxicity. Additionally, they are prone to be recognized by the immune system, resulting in the generation of neutralizing antibodies, following their introduction in the body when not autologous. These shortcomings result at best in a too short circulating half-life. Due to the obvious advantages of long-acting protein drugs, strategies to prolong their plasma half-life are highly on demand. Many technologies have been developed during the last decade focusing on improvement of characteristics of the protein drugs to gain the desired PK properties. They include amino acid manipulation, fusion to FC region of immunoglobulin domains, conjugation with serum proteins (albumin) or coupling with synthetic polymers (PEGylation) [34], which are able to reduce immunogenicity and proteolytic instability, thus extending half-life of therapeutic proteins to varying degrees. A number of studies have demonstrated that PEGylation of enzymes (a process by which polyethylene glycol chains are attached to protein drugs) results in reduced RES uptake and prolonged circulation half-life, improving enzymatic stability by a steric hindrance of proteolytic enzymes and decreasing renal excretion by increasing molecular mass of proteins [35].

Although polyethylenglycol (PEG) exhibits several properties that are of relevance for pharmaceutical application (*e.g.*, lack of toxicity, fast clearance from the body, high solubility) [36,37], PEG itself can be highly immunogenic thus compromising the duration of pharmacologic activity of protein drugs [38]. In fact, immunogenicity and relatively rapid inactivation require frequent patient treatments to maintain therapeutic blood index. This need induces the production of high levels of antibody titers making this approach invasive and expensive. Alternatively, an attractive option to improve protein plasma half-life could be their delivery inside RBCs that does not show the limitations of synthetic carriers: being endogenous cells they are safe and biocompatible, particularly when the autologous ones are used. In fact, clinical studies suggest that RBCs are ideal carriers for enzymes in circulation, able to shield the loaded proteins from premature proteolytic degradation and inactivation by immune system, hiding them from the physiological environment [39], particularly when repeated administrations are needed [40–45].

RBCs loaded with therapeutic proteins can be employed as delivery system in different types of application: approaches of enzyme replacement therapy (ERT) to treat inherited metabolic disorders, removal of undesired metabolites from the bloodstream (detoxification) [46,47] or depletion of target amino acids involved in cancer cell proliferation. In Fig. 1 is illustrated the process by which enzymes entrapped into RBCs metabolize cell membrane-permeating substrates in every of these conditions.

The first category includes some inborn errors of metabolism involving enzyme deficiencies such as adenosine deaminase (ADA) deficiency, in which the absence of this enzyme induces an accumulation of its substrates (adenosine, Ado, and 2′-deoxyadenosine, dAdo), leading to preferential intracellular phosphorylation of dAdo to deoxyadenosine triphosphate (dATP). The progressive increase of these purine nucleotides' metabolites until toxic concentrations causes a lymphotoxic effect and severe combined immunodeficiency (SCID). Stem cell transplantation from human leukocyte antigen (HLA)-identical sibling donors is usually the first-line treatment option. As an alternative ADA gene therapy is emerging [48]. For patients without suitable donors, enzyme replacement therapy (ERT) with PEG-ADA is available [49–53], but the slight improvement of ADA plasma half-life (from 30 min to 72 h, [50]) and evocation of immune response by neutralizing anti-ADA and anti-PEG antibodies require frequent intramuscular injections, making treatment invasive and expensive. Therefore, therapeutic approach based on ADA-encapsulated autologous RBCs [43] could bring benefit for all these patients, thanks to the ability of loaded erythrocyte to maintain sustained ADA activity permitting the degradation of plasma dAdo (which is able to permeate the RBC membrane). In fact, carrier ADA-erythrocytes provided *in vivo* metabolic and clinical effect

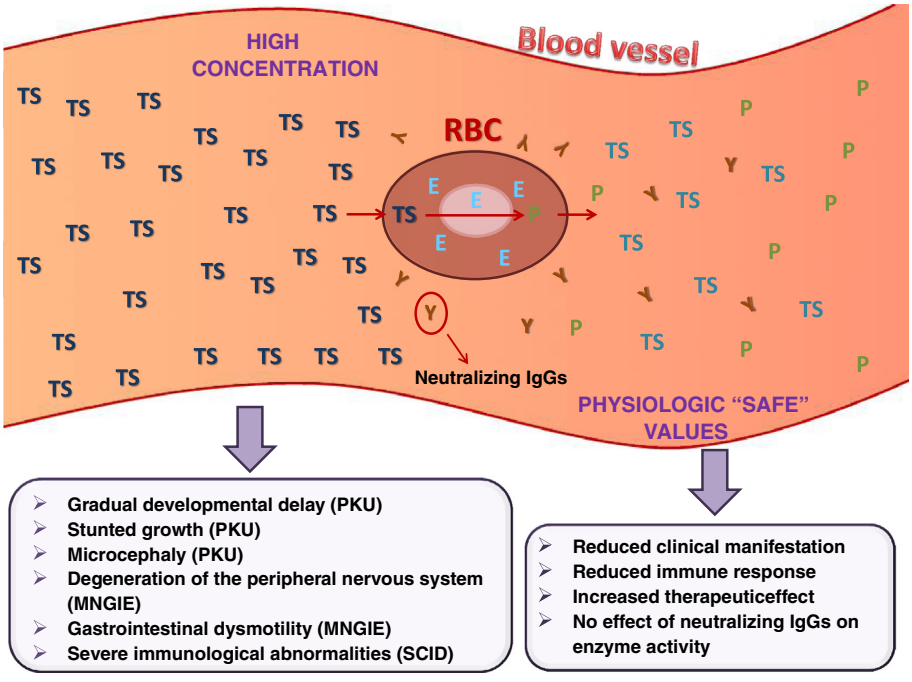

Fig. 1. Enzyme-loaded red blood cells at work. The picture shows how enzyme-loaded red blood cells are able to reduce high levels of toxic or unwanted substrates to physiological «safe» values. Moreover, circulating enzyme-neutralizing IgGs have no effect on enzyme activity thanks to the internalization of the therapeutic enzyme inside the cell. TS: toxic substrate (*i.e.* phenylalanine; adenosine and deoxyadenosine; thymidine and deoxyuridine); E: therapeutic enzyme (*i.e.* phenylalanine ammonia lyase, thymidine phosphorylase, adenosine deaminase); P: product of the intracellular reaction; PKU: phenylketonuria; MNGIE: mitochondrial neurogastrointestinal encephalomiopathy syndrome; SCID: severe combined immunodeficiency.

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reducing dATP concentration and increasing absolute lymphocyte counts [45].

In the same field can be placed another congenital enzyme deficiency, the mitochondrial neurogastrointestinal encephalomyopathy (MNGIE), a rare autosomal recessive metabolic disorder caused by mutations in the nuclear TYMP gene encoding for the enzyme thymidine phosphorylase (TP); such alterations cause a plasma and tissue accumulation of thymidine and deoxyuridine, which in turn generates imbalances within the mitochondrial nucleotide pools, responsible for mitochondrial dysfunction [54–56]. Recently, enzyme replacement therapy by repeated administrations of erythrocyte-encapsulated recombinant TP successfully reduced plasma nucleoside levels, resulting in marked daily decrease of urinary thymidine and deoxyuridine excretion with consequent significant clinical improvements [57]. Although available, stem cell transplantation was found of limited benefit and suitable only for selected patients [58].

Another inherited metabolic disease that can take advantage from ERT is phenylketonuria (PKU), characterized by an absence or deficiency of the hepatic enzyme phenylalanine hydroxylase (PAH), which is responsible for the increase in blood phenylalanine (L-Phe) concentration to neurotoxic levels, thus impairing cognitive development. The aim of treatment is to lower plasma phenylalanine levels to the recommended therapeutic range to prevent developmental delay and support normal growth. The non-mammalian enzyme phenylalanine ammonia lyase (PAL), that converts phenylalanine to ammonia and trans-cinnamic acid, is a potential substitute for the native PAH protein. In initial preclinical trials in rodent models of PKU, the free form of recombinant PAL has been tested by both oral [59–61] or subcutaneous administration [59,62] yielding a decrease in L-Phe levels for a short period of time. However, this is not a viable long-term option because the reduction is hampered by clearance of the enzyme through proteolysis and activation of immune response [63]. To optimize the ability of this therapeutic protein, modified forms of PAL have been developed by PEGylation processes. Sarkissian et al. [63,64] illustrated how blood L-Phe levels were slightly lowered by oral administration of rPAL-PEG in PAH-deficient mice and, to a larger extent, by weekly subcutaneous administrations of this PEGylated protein over 3 months. Nowadays a concluded Phase I study (NCT00925054) on a single dose of subcutaneously injected rPAL-PEG has been successful in prolonging enzyme plasma half-life and in reducing its immunogenicity but not to achieve the complete neutralization of the latter (with the production of antibodies both against PEG and rPAL, causing some moderate hypersensitivity adverse events, even though the treatment was generally fairly well tolerated) [65]. Currently in progress Phase II (NCT01212744, NCT00925054, NCT01560286) and Phase III (NCT01889862 and NCT01819727) studies concerning repeated administrations for the evaluation of efficacy and safety are confirming what previously observed including adverse events, like hypersensitivity reactions in injection site and the development of anti-drug and anti-PEG antibodies [65–72].

These problems could have in addition the effect of altering the PEG-conjugate biodistribution and bioavailability by acting on the complex clearance rate [73] not only of PAL but also of all other PEG-conjugate derivatives that a patient in need should eventually take for reasons independent from PKU. Administration through red blood cells as delivery system could therefore represent a valid and viable alternative to overcome such immunogenicity and bioavailability issues. RBC-mediated enzyme replacement therapy for PKU has been proposed for the first time in 1990 by Sprandel et al., in a short-term study demonstrating the effectiveness of this approach [74].

In a recent work, Rossi et al. [26] have demonstrated *in vitro* the feasibility of rPAL encapsulation into RBCs by a procedure of hypotonic dialysis, isotonic resealing and "reannealing", and the capacity of these loaded RBCs to provide a therapeutically relevant concentration of enzyme able to lower and maintain blood L-Phe levels as near as possible to physiologic values. In a second phase conducted on a murine model of phenylketonuria, the multiple treatment (at 9–10 day-intervals) with the most suitable dose of rPAL-loaded RBCs confirmed *in vivo* the long-term ability to restore L-Phe plasma pool in a safe range during the whole experiment (70 days). Moreover, evaluation of plasma antibodies revealed a growing increase in IgG titers; in fact, the immune response is effectively elicited due to antigen processing and presentation by macrophages during the natural process of RBC clearance from circulation. However, neutralizing IgGs had no effect on rPAL, thus confirming the validity of erythrocytes as protecting delivery system.

On the whole, the great potential of protein loaded-RBCs as ERT for the treatment of inborn metabolic diseases opens new perspectives for the development of therapies for other kinds of disorders, for example by using enzymes as antitumor drugs. In this context, clinical results up to now refer to the use of L-asparaginase for the treatment of acute lymphoblastic leukemia (ALL) and lymphoid malignancies. ALL cells have to rely solely on an extracellular source of L-asparagine (ASN) to maintain protein synthesis because of a limited expression of the enzyme asparagine synthetase (AS). As a consequence, systemic prolonged depletion of ASN by administration of asparaginase (enzyme that converts ASN to L-aspartic acid and ammonia) leads to impairment of protein biosynthesis and to the arrest of cancer cell cycle causing ALL cellular death [75]. L-asparaginase free supply presented some undesirable drawbacks such as short plasma half-life (8–24 h) and high rate of allergic reactions [76]. PEG-modified enzyme partially overcame the above mentioned inconveniences (half-life extension of 5.6 days), but showed other important side effects including liver and pancreas dysfunctions, neurological disorders and most commonly symptoms of intolerance. Thus, the encapsulation into RBCs has shown to positively modify pharmacokinetic parameters of the drug by enhancing protein half-life (up to 29 days) and to reduce adverse effects [41,77,78] as demonstrated in several preclinical studies on animal models (mice [79] and monkeys [80]).

This evidence was also revealed in pilot human clinical trials [77] in which plasma asparagine concentration rapidly decreased and remained to low levels for an extensive period of time improving the enzyme efficacy. Currently open randomized international Phase II clinical trial (dose-finding study) to evaluate the real clinical efficacy in the treatment of ALL (NCT01518517) confirms these recent findings. Moreover, even though the anti-L-asparaginase antibodies formation was observed in the patients' group treated with asparaginase-loaded RBCs, the enzyme was not prone to progressive neutralization by the immune response [40], due to the protective barrier provided by the erythrocyte membrane. Any clinical signs of hypersensitivity associated with antibody production and the maintenance of enzyme efficacy following repeated administrations of loaded RBCs are under investigation by Phase II/III in several clinical trials.

Besides the role as delivery system to clear undesired molecules from the bloodstream, erythrocytes could also target the entrapped proteins to the RES compartment. In fact, by accelerating the natural removal mechanism of senescent or damage red blood cells from circulation, various enzymes encapsulated into RBCs can be rapidly and selectively targeted to macrophages. This can be achieved by further submitting RBCs loaded with the drug of interest to a procedure of artificial membrane aging (by the use of zinc and bis(sulfosuccinimydil)suberate) promoting band 3 clustering which permits rapid recognition and phagocytosis by RES [81].

To this purpose, the licensed pharmacological glucocerebrosidase provides an ERT option in Gaucher's disease [82], a lysosomal storage disorder characterized by an accumulation of glucocerebroside in cells of the RES and bone marrow due to inherited deficiency of this enzyme. To date, only one known attempt demonstrated the safety of repeated administrations of glucocerebrosidase-loaded RBCs, showing benefit in liver function and relief of subjective symptoms [83].

By using this promising strategy of drug targeting through phagocytosis of RBCs, a number of different compounds can be efficiently delivered to macrophages for several purposes. Despite their central role in maintaining body homeostasis, macrophages are involved in harmful

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inflammatory reactions (*i.e.* rheumatoid arthritis), various autoimmune diseases (immunothrombocytopenia [84] or autoimmune hemolytic anemia [85]), they are responsible for the destruction of non-autologous grafted cells or material [86] and play also a key role as reservoirs of pathogens with macrophage tropism involved in human infections (HIV-1 [87], HSV-1 [88]). Thus, for all these pathological conditions, the system based on erythrocytes loaded with valuable molecules has proven effective either to directly induce *in situ* inhibition of viruses and microorganisms replication (like by loading the broad family of antiviral nucleoside analogs [89–93]) or to promote a specific and transient suppression of macrophage activities (like by loading bisphosphonates, such as clodronate [94–96]).

2.2. Glucocorticoid analogs

Glucocorticoids (GCs) are natural and synthetic analogs of the hormones secreted by the hypothalamic–anterior pituitary–adrenocortical (HPA) axis, which have anti-inflammatory activity and have been extensively used to treat various acute and severe inflammatory, immunological and allergic diseases [97]. Among GCs, dexamethasone (Dexa) is a highly potent and long-acting glucocorticoid used for more than 40 years in children, adolescents and adults with predominantly very great systemic GC activity and minimal or absent mineralocorticoid effects [98]. This steroid is employed primarily in short courses as rescue therapy for acute exacerbations, with chronic use reserved for those with severe diseases not adequately controlled by other medications. However, its terminal half-life of 3.5–4.5 h makes frequent administrations and high dosages necessary, causing a dangerous peak in plasma concentration eventually leading to toxic side effects [99], especially in long-term therapy. Therefore, prolonged treatments by systemic GCs are associated with unacceptable adverse effects that are widely known to occur in both a dose-dependent and duration–of-treatment manner. Side effects of GCs can relate endocrine/metabolic, cardiovascular, gastrointestinal, ophthalmic, musculoskeletal, immune functions and neuropsychiatric events.

Hence, it is understandable the importance of a drug delivery system able to modify the pharmacokinetics of Dexa so as to obtain a constant and effective drug concentration in circulation while avoiding the continuous oscillations above and below the therapeutic window responsible for the toxic effects and short duration of efficacy.

To this end, RBCs engineered to act as bioreactors for a controlled and sustained release of Dexa in bloodstream proved able to reduce the systemic toxicity of the drug simultaneously allowing the achievement of significant clinical results in the treatment of chronic inflammatory diseases and the improvement of therapeutic outcomes [100–105].

Previous laboratory experiences [106] demonstrated that Dexa can be encapsulated in erythrocytes in the form of a non-diffusible prodrug dexamethasone 21-phosphate (DSP), which is gradually converted by erythrocyte resident enzymes into the diffusible active principle Dexa and then released into the circulation by simple passive diffusion through cell membranes, as shown in Fig. 2. The slow dephosphorylation rate of DSP relies on the high Michaelis constant (Km) of the dephosphorylating RBC enzyme (mM range). In fact, the kinetics of drug release from the carrier erythrocytes depends both on Km of the intra-erythrocyte dephosphorylating enzyme and on the encapsulated prodrug concentration (from micromolar to 40–50 mM). The most important advantage derived from this DDS is the optimization of the drug pharmacokinetic profile: a single administration of DSP loaded-RBCs provides a therapeutic concentration *in vivo* for at least 21–28 days [101,102,104,105], while the conventional glucocorticoid treatments usually require a twice-a-day frequency of administration to maintain a drug concentration within therapeutic index. In detail, the low doses of DSP encapsulated into erythrocytes (about 10 mg/month/patient), at least one tenth the amount usually administered by therapeutic protocols, are enough to slow down the progression of the inflammatory diseases. Consequently, this long-term effect permits to prolong the time intervals between applications and to avoid the well-known corticosteroids' toxic side effects [103–105] related to dangerous peak plasma concentrations when administered in the free form. Despite the patients were exposed to less Dexa concentrations, these levels were high enough to allow the almost complete saturation of GC receptors and to bring clinical benefit. The treatment is well tolerated by the patients, which is important especially for those subjects affected by chronic inflammatory diseases who experience clinical efficacy also after years of treatment [101,107].

To make the transition from laboratory to clinics possible, a drug delivery company specialized in the development of drugs and diagnostics delivered through human red blood cells, EryDel S.p.A., developed an advanced technique for the encapsulation of DSP by a biomedical equipment named “Red Cell Loader” (RCL). This electromedical device, that works under pyrogen-free and sterile conditions [30,108] in conjunction with a disposable kit, processes a volume of blood in the order of 50 ml and completes the procedure within a couple of hours from blood collection.

Briefly, the procedure is based on two sequential dilutions of the washed red cells with two progressively more hypotonic working solutions leading to erythrocyte swelling and to the appearance of pores in their membranes which can now be crossed by the molecules of interest. A haemofilter is used to concentrate the swollen red cells before drug addition. The physiological osmotic pressure is then restored by a hypertonic solution able to close the membrane pores, thus resealing the erythrocytes and entrapping the drug inside. The non-encapsulated drug is then removed by extensive washing with physiological saline solution. A detailed description of this procedure and the main properties of the resulting processed cells can be found in Ref. 30, 108 and will be further reported elsewhere (Mambrini G. et al. in preparation). Recently, this equipment was used in a Phase II clinical study on patients with ataxia teleangiectasia (AT), after serendipity observation and limited studies in few patients showed beneficial effects of short-term treatment with betamethasone [109–112]. AT is a rare devastating neurodegenerative disease characterized by early onset ataxia, oculocutaneous teleangiectasias, immunodeficiency, recurrent infections, radiosensitivity and proneness to cancer [113], without available therapies. This study was performed to assess the effect of EryDex System (EDS, DSP encapsulated in autologous erythrocytes; property of EryDel S.p.A., www.erydel.com) on neurological symptoms of AT patients and proved to be promising as long-term steroid administration strategy, able to improve the neurological condition associated with the disease while minimizing the side effects of oral steroids' administrations.

The delivery of the corticosteroid Dexa by means of DSP encapsulation into autologous RBCs has received the designation of orphan drug by the European Medicinal Agency for the treatment of cystic fibrosis (*CF*) (EMEA/OD/039/04EU/3/04/ 230) and more recently for the treatment of Ataxia Telangiectasia (EU ODD: EU/3/13/1158 – EMA/OD/052/13); the latter was also granted by the US FDA (US ODD: 12–3732).

2.3. Immunosuppressant drugs: limitations related to pharmacokinetics and advantages to the use of erythrocytes as delivery system

Cyclosporine (CsA) and Tacrolimus (FK506) are among the most effective immunosuppressive agents for preventing or treating graft rejection in organ transplantation. Both are natural compounds produced, respectively, by the fungus *Tolypocladium inflatum* and the actinomycete *Streptomyces tsukubaensis* and, although they have different molecular structures, their molecular mechanisms and immunosuppressive and physiochemical properties are similar. Indeed, both belong to the class of “calcineurin inhibitors” and their activity results in the blocking of the dephosphorylation of the transcription factor “nuclear factor of activated T cells” (NF-AT), thus impeding the transcription of the most characterized IL-2 target gene [114]. At the molecular level, CsA and FK506 binding to calcineurin is mediated by two distinct cytosolic

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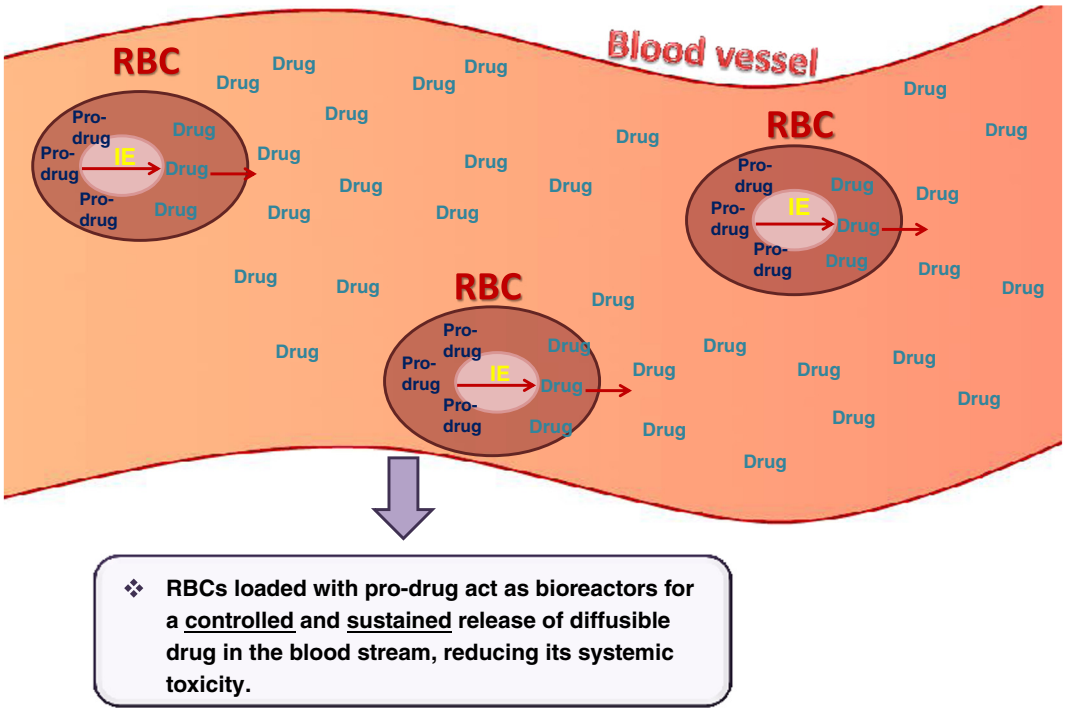

Fig. 2. Erythrocyte-mediated slow delivery. IE: intracellular enzyme; PRODRUG: pharmacologically inactive and non-diffusible form of the therapeutic compound that is metabolized into an active diffusible form inside the erythrocyte; DRUG: pharmacologically diffusible active principle that is slowly released in circulation.

proteins belonging to the immunophilin family, cyclophilin A (CypA) and the 12-kDa FK506 binding protein (FKBP12), respectively. Once drugs bind immunophilins, the resulting drug-protein duplex forms a ternary complex with calcineurin, thus inhibiting its activity.

The choice of CsA or FK506 in organ transplantation depends on immunological risk, recipient characteristics, concomitant immunosuppression and socio-economic factors. Generally, compared with CsA, FK506 shows a better side effect profile and increased long-term survival in patients but, unfortunately, it possesses a very narrow therapeutic window (5–20 ng/ ml whole blood 10–12 h post-dose). Indeed, FK506 is superior to CsA in improving graft survival and preventing acute rejection after kidney transplantation, but it increases post-transplant diabetes and neurological and gastrointestinal side effects [115]. Nevertheless, the Organ Procurement and Transplantation Network/ Scientific Registry of Transplant Recipient 2011 Annual Data Report shows that approximately 90% recipients transplanted in 2010 were initiated on Tacrolimus as part of their immunosuppression regimen and were continuing taking Tacrolimus 1 year after kidney transplantation [116].

However, CsA and FK506 are critical-dose drugs both having a narrow therapeutic index and exhibiting the desired therapeutic effect with acceptable tolerability only within a restricted range of blood concentrations [117]; furthermore, both immunosuppressant agents exhibit a high degree of intra- and inter-patient pharmacokinetic and pharmacodynamic variability, which increase the possibility of therapeutic failure if used in uniform doses in all patients, thereafter obliging physicians to a continuous, accurate and expensive therapeutic drug monitoring [118], especially because of the narrow therapeutic window.

The high inter-patient pharmacokinetic variability of both CsA [119] and FK506 [120,121] is caused by differences in the first-pass effect determined by drug metabolism and efflux by cytocrome P450 and the intestinal P-glycoprotein (P-gp) efflux pump, respectively [122], which are characterized by great individual variability. Intra-patient variations are supposed to be due to episodic absorptive variations caused by co-administered over-the-counter medications and/or a variety of foods in the diet but, whatever the reason, it is associated with increased treatment costs [123]. Although intravenous infusion bypasses adsorption and bioavailability issues, anaphylactic reactions induced by the vehicle utilized as emulsifiers to stabilize CsA and FK506 in aqueous solutions (Cremophor EL and PEG-60 hydrogenated castor oil, respectively), often compromise this form of parenteral administration.

Therefore, it is understandable how the highly variable pharmacokinetics of CsA and FK506 and, even more, their narrow therapeutic window, remain a great challenge for the optimization of immunosuppressant therapy and how the accurate drug blood concentration monitoring is necessary to control treatment efficacy and possible adverse effects. In respect of the latter, several assays have been developed for the quantification of the drugs in biological samples; in a routine clinical pathology laboratory, antibody-conjugated magnetic immunoassay (ACMIA) is the most common method providing accurate and reliable results consistent with those obtained with the commercially available liquid chromatography tandem mass spectrometry (LC–MS/MS) method which, however, requires more costs, organization and availability of skilled personnel [124]. More recently, an immune atomic force microscopy (AFM)-based method for the quantitative analysis of FK506 (but easy to use also for CsA detection), able to provide drug concentration closer to the immunosuppressive activity found in the patient's blood has been developed [125]; it might permit in the future a rapid, highly sensitive drug quantification with no need for large equipment or facilities.

If on the one hand, at the moment, therapeutic drug monitoring (TDM) challenge is under control and susceptible of improvement in the future, the limitations related to pharmacokinetics are still a non-exceeded challenge. RBCs might also in this case act as an effective delivery system capable of facilitating the use of critical-dose drugs such as immunosuppressants. Their exploitation in this direction is closely related to the physiological role played by RBCs when they are in the presence of the immunosuppressant agents CsA and FK506; indeed, after administration, either injected or absorbed into the body, these immunosuppressants are mainly and readily associated to RBCs. In detail, more than 70% CsA is bound to the erythrocyte peptidyl-prolyl

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cis-trans isomerase CypA with a total cell binding capacity of 43×10^{-5} nmol per 10^6 RBCs that corresponds to 2 μg CsA/ml RBC suspension at physiological hematocrit [126] and more than 80% (85.3 ± 1.5%) FK506 is bound to the two types of erythrocyte immunophilins, the above cited FKBP12 with peptidyl-prolyl cis-trans isomerase activity too and FKBP-13, a 13-kDa membrane associated protein, with a cell total binding capacity around 440 ng/ml of blood (1.0 ± 0.3 μg/ml packed RBCs), while only 0.46% FK506 is bound to lymphocytes [127–129]. The remaining percentages of the drugs are mainly associated with plasma proteins, with only little amounts represented by free, unbound active agents [130,131].

Starting from this physiological tendency of CsA and FK506 to be recruited by RBCs thanks to inner resident immunophilins, the idea arose to increase the amount of RBC-associated drugs by increasing the concentration of their binding proteins in order to modulate the immunosuppressant agents' pharmacokinetics. To this purpose, both recombinant human CypA and FKBP12 have been produced, loaded into human RBCs and tested for their ability to retain the corresponding diffusible drugs cyclosporine and Tacrolimus [132]. Immunophilins have been encapsulated in a dose-dependent way and higher protein entrapment corresponded to a higher content of intracellular immunosuppressant agents (Fig. 3); in detail, CypA-loaded RBCs showed a CsA binding capacity about 6-fold greater than unloaded cells (17.0 ± 3.2 μg CsA/ml RBCs at the higher loaded protein concentration) while FKBP12-loaded RBCs were able to retain a quantity of drug at least 11-fold greater than native cells (11.4 ± 2.9 μg FK506/ml RBCs at the higher amount of loaded protein tested), as shown by CsA and FK506 binding assay experiments. It is reasonable to think that these values may be further increased by adding more protein inside RBCs, since directly proportional results have always been obtained. Interestingly, FKBP12-loaded RBCs could be exploited for the transport of sirolimus (rapamycin) as well, since this immunophilin possesses high affinity also towards this immunosuppressant agent [133].

Three different modalities in the use of these engineered RBCs can be suggested:

a) Human autologous RBCs can be loaded *ex vivo* with the chosen immunophilin, incubated with the corresponding diffusible drug to permit its higher binding and then re-infused to the patient where the immunosuppressant agent will be slowly released in circulation; the drug released could eventually bind endogenous immunophilins physiologically present into native circulating RBCs. Thus, a single administration might ensure a drug concentration within the therapeutic window by overcoming bioavailability issues related to oral administration and adverse effects related to intravenous infusion.
b) RBCs can be loaded *ex vivo* with the chosen immunophilin and re-infused to the patient (where they will usually circulate for few months) who, in need, will receive orally or intravenously or by any acceptable modality, the corresponding diffusible compound (CsA or FK506); the drug, once in circulation, will reversibly bind the protein-loaded RBCs with higher affinity than the native erythrocytes. Therefore, the engineered RBCs will first bind the drug in circulation when its plasma concentration is high and, subsequently, release the drug when its plasma concentration decreases. In such a way, immunophilin-loaded RBCs will act as a reservoir for a slow drug delivery in circulation, greatly improving pharmacokinetic characteristics of the immunosuppressant drug.
c) Immunophilin-loaded RBCs could be used as an antidote for the treatment of patients that have accidentally received highly toxic CsA or FK506 concentrations. Tacrolimus, for example, is usually administered *per os*; however, in some clinical conditions such as nausea, vomiting, sedation or intubation, oral administration is not recommended and patients will be submitted to an intravenous treatment. Unfortunately, increased incidence of adverse and sometimes severe drug reactions have been associated with the intravenous administration in comparison to the oral one [134,135]. The increased risk of overexposure can be owed to unfortunate dose calculations, excessive oral to intravenous conversion rates, inappropriate dilutions for infusion and, finally, to the above mentioned hypersensitivity to polyoxyethylated hydrogenated castor oil used in the drug formulation [136]. All these conditions make the availability of an antidote able to readily remove the excess of free drug in circulation sorely needed. The aim can be easily reached through the proposed strategy since autologous FKBP12-loaded RBCs can be quickly prepared and re-infused (2 h-time required) in the patient where they will greedily bind Tacrolimus, thus lowering its toxic plasma concentration.

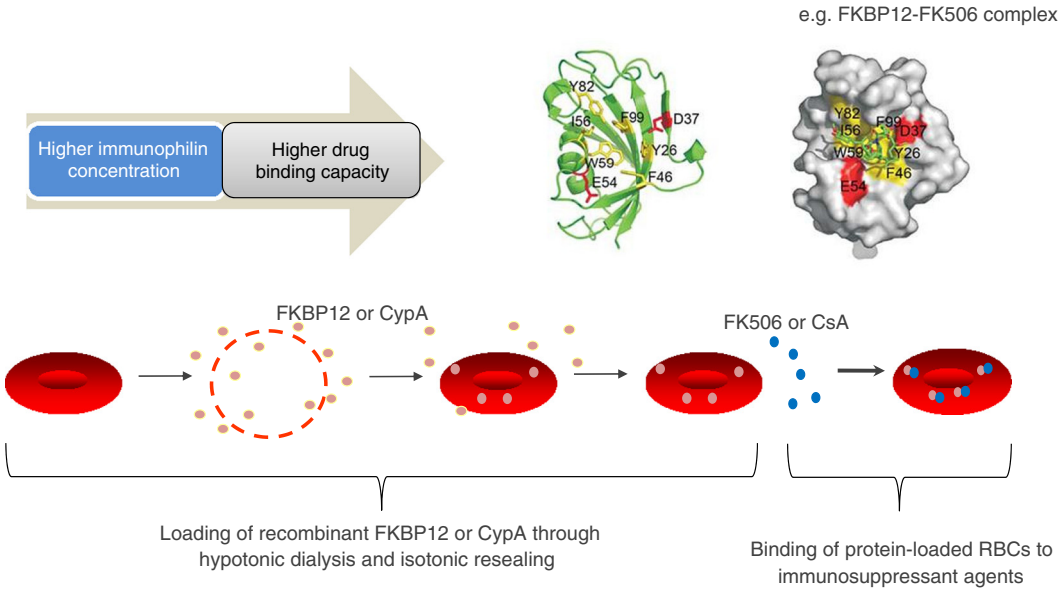

Fig. 3. Immunophilin-loaded RBCs as delivery system of the immunosuppressant agents tacrolimus (FK506) and cyclosporine (CsA). The recombinant immunophilins cyclophilin A (CypA) or 12-kDa FK506 binding protein (FKBP12) can be encapsulated into RBCs at different concentrations. Increasing protein concentrations correspond to increasing drug-binding capacities. The bound drug is subsequently released in circulation.

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Certainly, pharmacokinetic studies in animal models are needed to confirm the value of what suggested; however, if *in vivo* results will confirm these assumptions, physicians will benefit from a further help to deal with what is still a big problem in the management of organ transplantation patients.

Furthermore, the foregoing should be considered representative since it illustrates an intriguing strategy that allows encapsulating and retaining diffusible molecules inside red blood cells, otherwise not feasible with the classic loading procedure. It is a clear example of druggable proteins, a concept that could be extended to all human potentially druggable proteins or alternatively to protein domains present in human genome. Moreover, it could be possible to engineer the protein by site-specific mutagenesis, consequently increasing or decreasing its affinity for the therapeutic agent, with the aim to modulate the dissociation rate of the drug of interest, thus improving its pharmacokinetics. Alternatively, drugs themselves could be modified to acquire high affinity for immunophilins (either physiological or derived from engineered RBCs) to reduce their metabolism, improve their half-life and act as a slow and sustained delivery system [137].

3. Contrasting agents: main limitations to their use and RBC-based strategies to overcome them

3.1. Cyanine dyes and fluorescence imaging: pharmacokinetic issues and potential solutions in ophthalmology

Near-infrared fluorescence (NIRF) molecular imaging is emerging in many different medical fields as a noninvasive technique for disease imaging and tissue characterization. This is a non-ionizing, harmless, and highly sensitive diagnostic procedure that can potentially provide the sensitivity of nuclear medicine techniques, but without the limitations of usage due to radioactivity. Currently the only NIRF contrast agent approved by the United States Food and Drug Administration (FDA) for direct intravenous administration in medical diagnostics is Indocyanine Green (ICG) [138,139]. ICG is a water-soluble, amphiphilic tricarbocyanine dye with a molecular mass of 776 Da and the adsorption and emission maxima around 780 and 810 nm, respectively [140]. Due to its low toxicity (LD50 of 50–80 mg/kg) [141] and capacity to absorb and emit in the NIR spectral range, ICG is clinically used as a non-targeting NIRF contrast agent to guide biopsies [142–144], demark blood or lymphatic vasculatures and study liver function, cardiac output or ophthalmic perfusion [145]. Despite its many advantages, ICG use is still limited by several drawbacks: first of all, it aggregates easily into amphiphilic molecules in aqueous solutions to induce self-quenching and low quantum yields [146]; secondly, when administered in molecular form, ICG is rapidly cleared from the body with a very short plasma half-life of about 2–4 min [147–149], thus limiting the time available to image body regions during its vascular circulation. Since plasma levels fall to low values within minutes after intravenous administration, ICG is not so far a reasonable candidate for the clinical follow-up. The fast plasma clearance caused by rapid liver uptake challenges imaging performance, which requires fast systems capable of monitoring the rapidly changing concentration of the dye after administration. In addition, it often strongly binds to plasma proteins, showing negligible extravasation but leading to rapid agglomeration [141], and it undergoes oxidation, resulting in decreased absorption/emission and variability in the maximum absorption wavelength [150,151]. Finally, it is instable in aqueous solutions and prone to photo-bleaching under light exposure [148,152] and it lacks target moieties for molecular imaging [153]. As regards in particular the ophthalmology field, important applications for fluorescence imaging and fluorescent dyes as contrast agents have been developed. The ICG dye has an optimal fluorescent yield that allows the visualization of the ocular vascular bed during the angiographic exam of the retina. The ICG dye was first used in ophthalmology by Flower and Hochheimer in the early 1970s to image the choroidal circulation [154]. For the retina angiography, ICG is injected intravenously and is imaged as it passes through the ocular vessels; unfortunately, the ICG molecules are able to diffuse throughout the vessels, thus influencing the angiographic semiology. Moreover, inherent limitations in conventional angiography contribute to ignoring that the dynamics of plasma movement do not necessarily reflect the dynamics of erythrocyte movement, especially at the microvascular level where RBC movement is far more important to the circulation's metabolic efficiency than that of plasma. In fact, it is known that blood is a non-Newtonian fluid, *i.e.* a homogeneous mixture of two distinctly different non-Newtonian fluids: liquid (plasma) and particles (blood cells, especially RBCs). In conventional indocyanine green angiography of the ocular vasculatures, the observed fluorescence arises from dye molecules associated primarily with blood plasma, not erythrocytes. Even in capillaries, where they deform in order to pass through, erythrocytes cannot be seen in conventional angiogram images, hence metabolically significant phenomena such as vasomotion, which results in periodic suspension of erythrocyte movement through individual capillaries, cannot be directly visualized. Moreover, in conventional angiography, dye molecules leave the plasma and become associated with vessel walls, consequently those blood vessels rapidly exhibit steady-state fluorescence, thereby obscuring further visualization of blood movement. To address these intrinsic drawbacks of ICG for *in vivo* imaging, a potential approach is to encapsulate ICG into RBCs that provide increased stability, protection from non-specific plasma protein binding and prolonged circulation time [155]. By administering the contrast agent by means of RBCs, an important increase in its *in vivo* lifespan could be obtained, thus allowing ICG permanence in the blood vessels for a sufficient period of time to permit an adequate vascular imaging. In addition, ICG encapsulation within RBCs enables ICG concentration to be better evaluated so as to produce peak fluorescence, while avoiding plasma staining, thereby maintaining the high contrast needed for imaging individual cells in capillaries. Moreover, ICG-loaded RBCs are ideal to study dynamics of the particulate component of the blood in the ocular circulations under normal physiological conditions. Dye movement through the individual choriocapillary segments has never been observed, because they are beyond the spatial resolution of fundus cameras; therefore, it is unknown whether blood moves through every capillary segment in a given lobular area during blood ejection from its feeding arteriole upon each heartbeat. In contrast with fluorescent ICG-loaded RBCs, when viewing the angiogram sequences at video speed (25 images/s) or when playing subsets of image sequences in a loop-like fashion, it is relatively easy to track individual cells. In this way, in capillaries, it is also possible to visualize RBCs beside the plasma, because the engineered cells themselves emit fluorescence and the separation between adjacent RBCs exceeds the diffraction-limited resolution of the optics of the eye (7 μm for the perifoveal capillaries and 11 μm for the choriocapillaries). In fact, these conditions have been actually met by entrapping ICG dye in RBCs and re-injecting a small bolus of them; during transit to the ocular vasculatures, they are diluted in circulating blood to the extent that individual ICG-loaded RBCs were separated by more than 11 μm, making their visualization possible (Fig. 4A and B).

In addition, injections of ICG-loaded RBCs could be of particular relevance in the diagnosis of some retinal pathologies such as diabetic maculopathy, thanks to the possibility to evaluate vasomotion phenomenon in retinal capillaries. Vasomotion, propagating waves of alternating precapillary arteriolar contraction and dilation that result in periodic suspension of RBC movement through adjacent capillaries, has been observed in every other end-arteriolar vasculature studied so far. This phenomenon has been inferred to exist in the retinal capillaries, based on oscillations in retinal oxygen tensions (3–10 cycles/min) that correspond to brain microvascular vasomotion, but vasomotion-related RBC dynamics has never been directly observed in the primate eye. Vasomotion is an important mechanism for homogenous oxygen distribution in retinal tissue and that disturbances in it may underlie some retinal ischemic conditions, such as diabetic maculopathy. That being the case, eventually high-speed angiography using reinjection of

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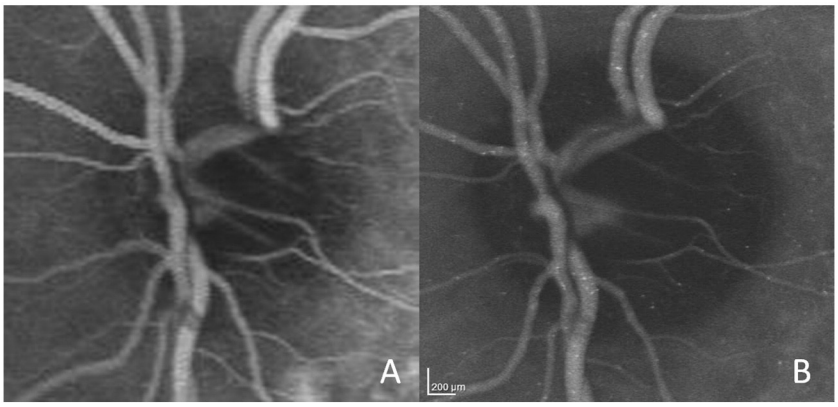

Fig. 4. Dye angiogram images of a normal human optic disk. A) Fluorescein angiogram (magnification 10×) and B) ICG-loaded RBC angiogram. The multiple hyper-fluorescent points seen on picture B correspond to the ICG-loaded circulating red blood cells.

autologous ICG-loaded RBCs would be a sensitive diagnostic tool for early detection of ischemic retinopathies and for monitoring progression and response to treatment. The feasibility of using ICG-loaded RBCs for diagnostic purposes has been successfully obtained in human in a small European pilot trial [156], which also demonstrated that the procedure is safe and well tolerated, meanwhile very easy to perform, thanks to the already mentioned "Red Cell Loader" equipment. In the discussion of the study, scientists did not report any severe adverse events after the intravenous injection of autologous ICG-loaded RBCs in humans and no allergic reaction was recorded even during the follow-up.

3.2. Superparamagnetic iron oxide (SPIO) nanoparticles as intravascular contrast agents: pharmacokinetic limitations and use of engineered RBCs to bypass them

Although the advances in nanotechnology and molecular cell biology have led to the improvement in stability and biocompatibility of nanoparticles, their half-life in blood circulation is still very short. The RES and renal clearance represent two major routes for the removal of nanoparticles from our body involving organs such as the liver, spleen and bone marrow that are rich in phagocytic cells able to engulf and digest nanoparticles. Studies have shown that the RES is responsible for the clearance of most nanoparticles larger than 10 nm, regardless of their shape and surface chemistry [157,158]. A portion of particles that are opsonized in blood circulation traffics to the spleen, while the remainder is sequestered in the Kupffer cells of the liver. The force for clearance is so strong that most nanoparticles administrated intravenously will be removed from the blood in as little as a few minutes to hours [159]. Size reduction and surface coating have been employed to increase the circulation time of superparamagnetic iron oxide (SPIO) nanoparticles used as intravascular contrast agents; however, the phagocytic cells limit the applicability of such compounds in the diagnostic applications, thus, the time window for bolus-based measurements is only a few minutes. Magnetic resonance angiography (MRA) using conventional gadolinium (Gd)-based molecular contrast agents suffers from poor physiological stability, uncontrollable pharmacokinetics, and a risk of toxicity. MRA studies using these molecular agents are also limited by short acquisition windows due to the rapid perfusion of molecules into extracellular compartments after first pass of aorta. Development of novel contrast agents overcoming low efficiency and poor biodistribution are urgently needed for highly efficient MRA. Because of good biocompatibility and excellent magnetic properties, iron oxide nanoparticles have been extensively explored especially as Magnetic Resonance Imaging (MRI) contrast agents, through controls over the size, composition and morphology [160].

These nanoparticles are referred to as superparamagnetic iron oxide particles (SPIO), ultrasmall superparamagnetic iron oxide particles (USPIO), very small superparamagnetic iron oxide particles (VSOP), monocrystalline iron oxide particles (MION) and cross-linked iron oxide (CLIO) depending on their size, crystalline structure, coating and higher order organization [161]. Within the SPIO family, two compounds are registered and approved: one compound goes under the name of Feridex® (Berlex) in the USA or Endorem® (size 80–150 nm, Guerbet) in Europe, and the second is Resovist® (size 62 nm, Schering) in Europe and Japan. In both cases, the clinical targets are liver and spleen since SPIOs are rapidly cleared from the blood by the RES of these organs. The particles are of medium size and coated with dextran (Feridex®, Endorem®) or an alkali-treated low molecular weight dextran, called carboxydextran (Resovist®) [162].

The principal effect of the SPIO particles is on T2* relaxation, and thus MR imaging is usually performed using T2/T2*-weighted sequences in which the tissue signal loss is due to the susceptibility effects of the iron oxide core. Enhancement on T1-weighted images can also be seen with the smaller Resovist®, however due to the high r2 relaxivity, Resovist® is more suited to T2/T2*-weighted imaging [163].

The differences between SPIO and USPIO are the longer blood half-life of the smaller particles and the lower T2 relaxivity of the latter. The USPIO particles' longer blood half-life of 1–2 h *versus* several minutes of the normal SPIO particles opens up new clinical indications, *e.g.* MRA and the targeting of organs other than spleen, liver or bone marrow. The only USPIO particle approved in the United States by the Food and Drug Administration (FDA) is ferumoxytol (Feraheme; AMAG Pharmaceuticals, Cambridge, Mass) for the treatment of iron-deficient anemia in adults with chronic kidney disease. Sigovan et al. demonstrated the feasibility of using ferumoxytol as a good first-pass MRA agent in imaging haemodialysis fistulas, with consistently superior image quality compared to non-enhanced TOF MRA [164]. However, ferumoxytol has recently been associated with severe anaphylaxis and at least 18 patients' deaths, and now it also carries an FDA black box warning (FDA Orders Stricter Warnings for Ferumoxytol (Feraheme), Medscape 2015, http://www.medscape.com/viewarticle/842309, [165]).

In this context, the development of innovative strategies able to prolong the contrast agents' residence time in blood is required. We focused our attention on the strategies based on the encapsulation of contrast agents in red blood cells (RBCs), which represent potential biomimetic and biocompatible carriers able to survive for days improving the biomedical and diagnostic applications in the detection of the vascular system.

The encapsulation of citrate-coated SPIO nanoparticles into RBCs by a hypotonic haemolysis method was proposed by Brähler et al. [166]

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and Stenberg et al. [167]. As for the loading procedure, RBCs and magnetite nanoparticles are incubated under stirring in hypo-osmotic lysing buffer and subsequently resealed by incubation in phosphate buffered saline. Although the resulting SPIO-loaded RBCs responded to the external magnetic field and relaxation times (T1 and T2) dramatically decreased, electron transmission analyses (TEM) have showed the magnetic nanoparticles to be not only distributed inside the cells but mostly concentrated in the cell membranes, both on the internal and external sides. This cell surface modification is a restriction for *in vivo* applications because it could activate the elimination of loaded cells by the immune system. Therefore, these SPIO-loaded RBCs showing iron oxides nanoparticles on cell surface are considered non-viable cells. It is known that intentional RBC membrane damages or modifications allow the specific targeting of carrier-RBCs to the Kupffer cells of the liver and other phagocytes of the RES. Hence, the circulation time of carrier RBCs strongly depends on membrane integrity and therefore on the applied loading procedure [168]. In 2008, Antonelli et al. [169] demonstrated that it is possible to encapsulate magnetic nanoparticles in human and murine RBCs without changing the main features of the natural cells using a procedure that permits a transient opening of cell membrane pores through a controlled hypotonic dialysis and successive isotonic resealing and reannealing of cells. Several SPIO nanoparticles, either commercially available or newly synthesized, have been evaluated for encapsulation into RBCs, and the results have shown that not all nanoparticles can be successfully loaded into erythrocytes, depending on different chemical–physical characteristics, such as different coating agents and size [170]. SPIOs should be monodispersed and sufficiently small so that they can readily pass into the RBCs when they become porous upon exposure to the hypotonic dialysis solution. The hypotonic solution induces a transient opening of pores with diameters between 50 and 200 nm in the RBC membrane [171], allowing particles to enter the cells. After resealing the pores in an isotonic buffer, particles are trapped inside the RBCs. The size of the particles is not restricted to SPIOs, such as Resovist® and Endorem®; the range of sizes also includes USPIOs, such as Sinerem®, which can be successfully loaded into RBCs. The total preparation procedure typically results in a cell recovery of loaded RBCs ranging from 60% to 70%, similar to that for unloaded RBCs, and with the same properties as those of native cells [169,170]. Nuclear magnetic resonance (NMR) measurements showed that SPIO-loaded RBCs, such as Resovist®- Endorem®- and Sinerem®-loaded RBCs, contain final iron concentrations ranging from 5.3 to 16.7 mM for human RBCs and from 1.4 to 3.55 mM for murine RBCs. The SPIO-loaded RBCs are attracted by an external magnetic field of 3000 G and they maintain for several days a relaxation rate (R) of about one order of magnitude higher than the value of control cells [169]. Cell integrity and morphology of these constructs did not change with respect to control cells. Furthermore, TEM analyses of SPIO-loaded RBCs revealed a homogeneous distribution of iron oxides into cell

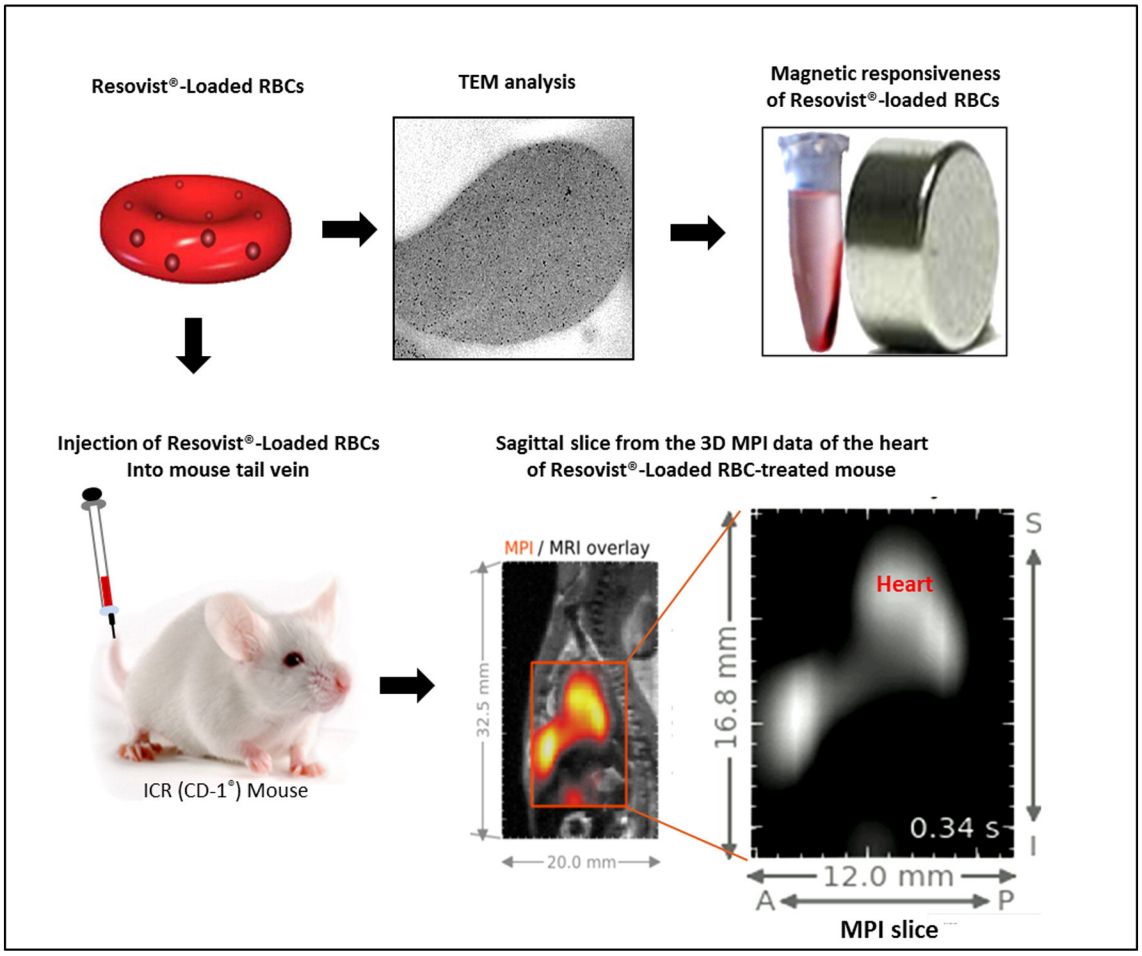

Fig. 5. Intravenous administration of murine Resovist®-loaded RBCs in mice and MPI acquisition. After intravenous injection of Resovist®-loaded RBCs, MPI of the blood pool of living mice is feasible up to several hours after injection with current MPI equipment, proving the efficacy of the RBC loading method proposed by Antonelli et al. [177,178] to increase the blood retention time of iron oxide nanoparticles *in vivo*. From the 3D MPI data acquired 3 h after Resovist®-loaded RBC injection, a sagittal slice through the position of the heart has been selected. The MPI images were interpolated to the MRI grid to be displayed as an overlay in orange color. The upper structure in the MPI image matches the heart, whereas the lower structures are found in the region of the liver and further down in the abdomen [179].

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cytoplasm, without any presence of aggregated particles on the outer side of the membrane. This result is very important to avoid RBC recognition and clearance by the immune system. Differently, other Authors in previous works have reported a technique that produced crenated ghost RBCs with limited stability [172,173]. Instead, SPIO-loaded RBCs proposed by Antonelli et al. [169,170] are still viable cells and once injected in the host are not removed by the phagocyte system, as demonstrated by *in vivo* experiments performed in mouse model [174]. These Authors showed that murine SPIO (or USPIO)-loaded RBCs intravenously injected in the mouse blood system stay in circulation longer than the free nanoparticle contrast agents. In fact, the blood retention time of murine Resovist®-loaded RBCs is longer (~12 days) than the free Resovist® (~1 h) administered at the same iron concentration [169,174]. The improved *in vivo* life span of iron oxide-based contrast agents could allow the same patient to be imaged on a number of occasions over time for MRI diagnosis. Recently, Antonelli et al. [175] have applied the loading procedure to the P904 contrast agent; this nanomaterial is efficiently loaded into human and murine RBCs and the amount of iron incorporated in the cells ranges from 1.5 to 12 mM. Moreover, Boni et al. have reported a relaxometric study on these P904-loaded RBCs, comparing the longitudinal (r1) and transverse (r2) relaxivity of these constructs, over a wide range of Larmor frequencies (0.01–300 MHz), with control samples of P904 nanoparticles dispersed in blood [176]. High r2 relaxivity together with a high r2/r1 ratio and a very long blood half-life make P904-loaded RBCs a promising blood-pool negative contrast agent for MR diagnostic applications. When encapsulated into RBCs, P904 contrast agent showed a higher survival in the mouse blood circulation than the free P904; the half-life is >72 h for P904-loaded RBCs and <2 h for the P904 free suspension. Further, the presence of P904-loaded RBCs in mouse circulation is still evident after 1 month from their injection, when T1 value returned to control values, while an equivalent amount of free P904 suspension injected in mice disappeared from circulation within few hours [175]. The use of SPIO-RBCs constructs could provide superior performance of diagnostic modalities such as MRI or multimodality imaging techniques, but in the next future, they could also be useful in Magnetic Particle Imaging (MPI), a novel 3D real-time imaging methodology introduced by Philips [177]. MPI is different from MRI since it is able to both directly visualize magnetic particles and to quantize iron oxide concentration rather than their effect on proton relaxation, that is the basis of MRI detection [178,179]. Unfortunately, the commercially available iron contrast agents that are currently being employed for MPI studies are not optimized, and this prevents current MPI studies from reaching optimal resolution and sensitivity. For example, it was estimated that only ~3% particles in Resovist® colloidal suspension exhibits an optimal nanoparticle diameter with a good MPI performance leading to significant signals. Moreover, the blood half-life of Resovist® limits its applicability for MPI, which requires a constant signal level over a longer time period. Recently, the use of SPIO–loaded RBCs in MPI has been investigated as blood pool trace material with longer blood retention time [180,181]. Rahmer et al. [182] reported the first *in vivo* MPI of mice using Resovist®-loaded RBCs. The suitability of Resovist® for RBC entrapment has already been established [169,174]. Despite the reduced sensitivity and resolution resulting from the size selection effect [180] and difficulties related to the application of the loading procedure to mice instead of humans, MPI *in vivo* experiments provided dynamic imaging of heartbeat in mice up to several hours after the administration of murine Resovist®-loaded RBCs. The concentration of iron carried by Resovist®-loaded RBCs in the mouse blood was determined by Magnetic Particle Spectroscopy (MPS). MPS spectra of blood samples withdrawn from mice treated with Resovist®-loaded RBCs demonstrated that these magnetic constructs can circulate in the mouse blood for many hours (3–24 h, Fig. 5) [182]. In this context, the use of longer blood half-time tracer materials would make MPI highly suitable for continuous imaging of the vessel tree or other blood-filled structures during interventional procedures and long-term monitoring of cardiovascular diseases.

4. Conclusions

After several years of *in vitro* and preclinical investigations, cell-based delivery systems have now left their infancy and in few cases entered clinical development, gaining the state of "orphan drugs" both by EMA and FDA. These applications are described in several original publications and some clinical data are close to submission to obtain final marketing approval, and are mentioned only shortly in this review. Instead, here we have tried to define areas where cell-based delivery systems, in our opinion, are more advantageous over other delivery systems. The selection is supported by a number of publications and by our personal experiences gained during more than 20 years of active research in the field.

Briefly, we can conclude that cell-based delivery systems, especially red blood cells as delivery system, have documented advantages in at least four areas:

1. Carrier of biologics, especially recombinant enzymes, which should circulate as long as possible. Cell-based delivery systems are able to keep into circulation fragile biologics, mainly recombinant enzymes, for several weeks, sometimes months, preserving their catalytic properties and preventing loss of function by induced inactivating antibodies.
2. Circulating reservoirs as slow drug delivery systems. The active drug is encapsulated into red blood cells in the form of a non-diffusible pro-drug (i.e dexamethasone 21-phosphate) that is converted by red cell resident enzymes into a diffusible drug (*i.e.* dexamethasone). As an alternative, a recombinant drug-binding protein or protein domain could be encapsulated to reversibly bind a diffusible drug (*i.e.* Tacrolimus).
3. Carrier of Infrared Fluorescent agents for angiography, preventing extravasation and binding to plasma proteins.
4. Carrier of nanoparticles, preventing opsonization and fast removal by the mononuclear phagocyte system especially in the liver and spleen.

For each of these areas we have provided non-exclusive and non-limiting examples with the only intention to prove with a real case the documented advantages of using red blood cells as delivery systems, and paving the way to solution of additional cases, that is when recombinant enzymes are to be used as therapeutic agents, when the blood concentration of a selected drug must be maintained within the therapeutic window for weeks or, finally, when infrared angiographic agents or nanoparticles must be kept into circulation to prevent extravasation and/or macrophage recognition and removal. Moreover, to date, no side-effects have been reported following infusions of loaded RBCs in patients [57,100–105,113]. It is noteworthy that up to now some thousands of treatments have already been performed, in few cases even for years on monthly bases, without adverse events. We are aware of many additional cases for which carrier erythrocytes and related technologies for the encapsulation of active agents are available but, instead of a long list of applications, we decided to focus on the most advanced ones, already in humans or at least at preclinical stage, to underline robustness of the approach and industrial feasibility. It is worth mentioning that two companies, Erytech Pharma in France and EryDel in Italy, have reached clinical developments and expect to submit the data for marketing authorizations in the near future. In conclusion, cell-based delivery systems have documented advantages, proved to be safe and to deliver the promise of a new technology capable of convenient solutions for old problems.

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