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Review Article

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Resealed erythrocytes: An engineering approach for drug delivery and drug targeting

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ABSTRACT

The main objective of this review is to explore the various features, drug loading technology and biomedical application of resealed erythrocytes. Resealed erythrocyte becomes more popular because it has ability to circulate throughout the body, biocompatibility, zero order release kinetics of drugs, reproducibility and ease of preparation. Erythrocytes are biocompatible, biodegradable, possess long circulation half-lives and can be loaded with a variety of biologically active compounds using various chemical and physical methods (hypotonic dilution, hypotonic hemolysis, electro-insertion, entrapment by endocytosis, hypoosmotic lysis). These drug carriers are rapidly taken up from blood by macrophages of reticuloendothelial system (RES) present in liver, lung and spleen of the body. Wide varieties of drugs like Anti-inflammatory, steroidal and chemotherapeutic agents are seen to have reduced side effects upon incorporation into these carriers. Carrier erythrocytes are prepared by collecting blood sample from the organism & separating erythrocytes from plasma. By using various methods the cells are broken & the drug is entrapped into the erythrocytes, finally they are resealed and the ultimate carrier is referred to as "Resealed Erythrocytes". The morphology, isolation techniques, properties and methods of drug loading are highlighted in this paper along with the characterization and applications of resealed erythrocytes, which hopefully put some light for researchers working in this area.

Keywords: Resealed Erythrocytes, Carriers, Encapsulation, RES, Phagocytosis.

INTRODUCTION

Erythrocytes, the most abundant cells in the human body, have potential carrier capabilities for the delivery of drugs. Erythrocytes are biocompatible, biodegradable, possess very long circulation half-lives and can be loaded with a variety of chemically and biologically active compounds using various chemical and physical methods. Application of erythrocytes as promising slow drug release or site-targeted delivery systems for a variety of bioactive agents from different fields of therapy has gained [1,2,3]. Now days we have aimed at development of drug delivery systems which enhance the drug targeting along with high therapeutic advantages for safe and effective management of diseases. The targeting process of drug can be approaches by either chemical modification or by appropriate carrier. There are many drug delivery carriers has been investigated presently like nonoparticle, microspheres, lipid vesicular carrier, microemulsion, pharmacosomes, ethosomes, cellular carrier and macromolecule. The targeted or site-specific drug delivery is a very promising goal because it provides one of the most effective ways to improve

the therapeutic index (TI) of drug whilst devoiding its potential interaction with non-targeted tissue. There are different carriers has been used for the drug targeting among which cellular carrier offer a greater potential advantages related to its biodegradability, biocompatibility, self degradability & it also offers high drug loading capacity [4, 5, 6].

ERYTHROCYTES

Erythrocytes are also known as red blood cells (RBCs), they are highly specialized oxygen carrier in the body which transported via the circulatory system. They taken oxygen in the lungs or gills and release it while squeezing through the capillaries. These cells cytoplasm is rich in haemoglobin, an iron containing biomolecules that can bind oxygen & also responsible for the blood red colour [7]. The red blood cells develop in the bone marrow & circulate for about 100-120 days in the body until their components are recycled by macrophages. The processes of erythrocyte production within the body are called as erythropoiesis; the erythrocytes are produced in red bone marrow under the regulation of a hemopoeitic hormone known as erythroprotein [8].

RESEALED ERYTHROCYTES

Resealed erythrocytes are the part of parental control release formulation, RBCs have been used extensively studies for their potential carrier & capability for delivery of drug loaded microsphere. In this carrier erythrocytes are prepared and the blood sample is collect from the organism of interest, the erythrocytes are separating from the plasma, then the entrapping drug in the erythrocytes & resealing the cellular carriers. Hence the overall process is based on the response of these cells under osmotic condition. Through the process of reinjection, the drug loaded erythrocytes provide slow circulating depots & target the drug to a disease tissue organ [9, 10].

STRUCTURE AND ITS PHYSIOLOGY

Erythrocytes are the most abundant cells in the human body (~5.4 million cells/mm^3 blood in a healthy male and ~4.8 million cells/mm^3 blood in a healthy female). Erythrocytes are like biconcave discs with an average diameter of 7.8 µm, a thickness of 2.5 µm in periphery, 1 µm in the center, and a volume of 85–91 μm^3**.** Due to flexible biconcave shape it enables erythrocytes to squeeze through narrow capillaries, which may be only 3µm wide [10,11,12]. Plasma membrane of this is strong and flexible, which allows them to deform without rupturing as they squeeze through narrow capillaries. RBCs lack a nucleus and other organelles and can neither reproduce nor carry on extensive metabolic activities. RBCs are highly specialized for their oxygen transport function, because their mature RBCs have no nucleus, all their internal space is available for oxygen transport; the shape of RBCs also facilitates its function. A biconcave disc has a much greater surface area for the diffusion of gas molecules into and out of the RBCs than would say a sphere or a cube. Each RBC contains about 280 million hemoglobin molecules. The red blood cells membrane, a semi permeable components of the cell, associated with energy metabolism in the maintenance of the permeability characteristic of the cell of various cations (Na^+, K^{++}) and anions (Cl^-**,** HCO_3^-) [13, 14, 15].

✓ **Erythrocytes as carrier in two ways**

Targeting particular tissues

For targeting, only the erythrocyte membrane is used. This is obtained by splitting the cell in hypotonic solution and after introducing the drug into the cells, allowing them to reseal into spheres. Such erythrocytes are called Red cell ghosts.

For prolonged release of drugs

Alternatively, erythrocytes can be used as a continuous or prolonged release system, which provide prolonged drug action. There are different methods for encapsulation of drugs within erythrocytes. They remain in the circulation for prolonged periods of time (up to 120 days) and release the entrapped drug at target site with slow and steady rate [7].

✓ **Erythrocytes Isolation**

- Firstly blood is collected into heparinized tubes by vein puncture.
- It is withdrawn from cardiac/sphlenic puncture (in small animal) and through veins (in large animals) in a syringe containing a drop of anti-coagulant to prevent coagulation of blood sample.
- Now whole blood is centrifuged at 2500 rpm for 5min. at 4 $\pm 1^0$C in a refrigerated centrifuge.
- Now it's time to carefully removed, the serum and buffy coats and the packed cells wash three times with phosphate buffer solution (pH=7.4).

- Then washed erythrocytes are diluted with phosphate buffer solution (PBS) and stored at 4^oC for as long as 48 hrs before use.
- There are various types of mammalian erythrocytes have been used for drug delivery, such as erythrocytes of mice, pigs, dogs, goats, monkeys, chicken, rats, and rabbits [7,16,17,18].

✓ **Properties of resealed erythrocytes**

- Low leaching /leakage of drug should take place before target site.
- Drug should be released at target site in a sustained manner.
- The drug should be of optimum size & shape and it should be permit the passage through capillaries minimum leakage of drug should take place.
- It should have ability to carry broad spectrum of drug.
- It should have compatible to blood and should have low toxic effect.
- The carrier system should have appreciable storage stability, it should have specific physiochemical ability by desired target site could be recognized [6,19].

✓ **Encapsulation Requirements**

There is need of following requirements for encapsulation-

- Nonpolar molecules can be entrapped in erythrocytes in salts form. Ex- Tetracycline Hydrochloride salt can be loaded in bovine RBCs.
- The lipohilic molecule might be entrapped in erythrocytes by absorbing over other molecules.
- The charged molecule is remaining same the longer than uncharged molecule; the size of molecule entrapped is a important factor when the molecule is smaller than sucrose and larger than β-galactosidase.
- There are two types of polar in the dialyzed erythrocyte are present, one set of pares exists at all times in the dialyzed cell and another set of pares appears and disappears constantly [20,21].

MERITS OF RESEALED ERYTHROCYTES [6, 22]

- They are natural product of body, which are biodegradable in nature.
- The entrapment of drug does not require the chemical modification of drugs to be entrapped.
- They are non-immunogenic in action and can be targeted to disease tissue/organ.
- They facilitate incorporation of protein, nucleic acid in eukaryotic cells by cell infusion with RBC.
- Considerable uniform size and shape of carrier,
- Relatively inert intracellular environment can be encapsulated in a small volume of cells.
- Possible to maintain steady-state plasma concentration, decrease fluctuation in concentration.
- Fluctuations in concentration decreases after attaining steady-state plasma concentration.
- It protect organism against toxic effect of drug (e.g. antineoplastics).
- Prolong the systemic activity of drug by residing for a longer time in the body.
- The lack of occurrence of undesired immune response against encapsulated drug.
- As compared to that of normal erythrocytes it has longer life span in circulation.

DEMERITS OF RESEALED ERYTHROCYTES [9, 22]

- The main problem with this drug carrier is that they remove in vivo by RES, which limits their usefulness as drug carriers and in some cases it may cause toxicological problems.
- The storage of the loaded erythrocytes is a further problem provided that there are viable cells and need to survive in circulation for a long time upon re-entry to the host body.
- There may be chances of clumping of cells and dose dumping.
- Several molecules may alter the physiology of the erythrocyte.

METHODS FOR DRUG LOADING

1. Hypo-osmotic lysis
   a) Dilution method
   b) Preswelling method
   c) Dialysis method
   d) Osmoticlysis method
2. Membrane perturbation method

3. Electro encapsulation method
4. Endocytosis method
5. Lipid fusion method
6. Electric cell fusion method

1. Hypo-osmotic lysis method: These are divided into four types are as follows-

✓ Dilution method
✓ Preswelling method
✓ Dialysis method
✓ Osmotic lysis method

Dilution method: The first simplest and fastest method investigated was hypotonic dilution for the loading of chemicals into erythrocytes. According to this method, a volume of packed erythrocytes is diluted with 2–20 volumes of aqueous solution of a drug; the tonicity of solution is then maintained by adding a hypertonic buffer. After that, the resultant mixture is centrifuged, the supernatant is discarded and the pellet is washed with isotonic buffer solution. The major demerit of this method is its low entrapment efficiency and a considerable loss of hemoglobin and other cell components, which reduces the circulation half-life of the loaded cells. These are phagocytosed by RES macrophages and can be used for targeting RES organs. This dilution is used for loading enzymes such as β-galactosidase, β-glucosidase, asparginase, and arginase & bronchodilators such as salbutamol [22].

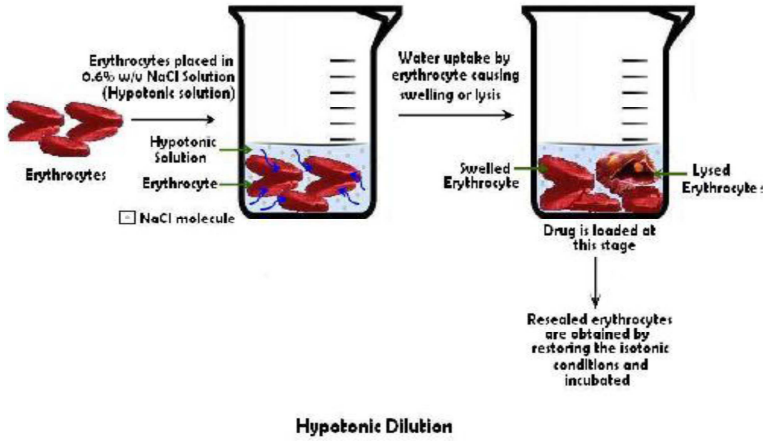

Figure.1: Hypotonic dilution method

Preswelling method: It is based upon initial controlled swelling in a hypotonic buffered solution. This mixture is centrifuged at low *g* values. The supernatant is discarded and the cell fraction is brought to the lysis point by adding 100–120 µL portions of an aqueous solution of the drug to be encapsulated. After that the mixture is centrifuged between the drug-addition steps. The tonicity of a cell mixture is restored at the lysis point by adding a calculated amount of hypertonic buffer. The lysis point is detected by the disappearance of a distinct boundary between the cell fraction and the supernatant upon centrifugation. Lastly the cell suspension is incubated at 37 ^{0}C to reanneal the resealed erythrocytes [1,10,23].

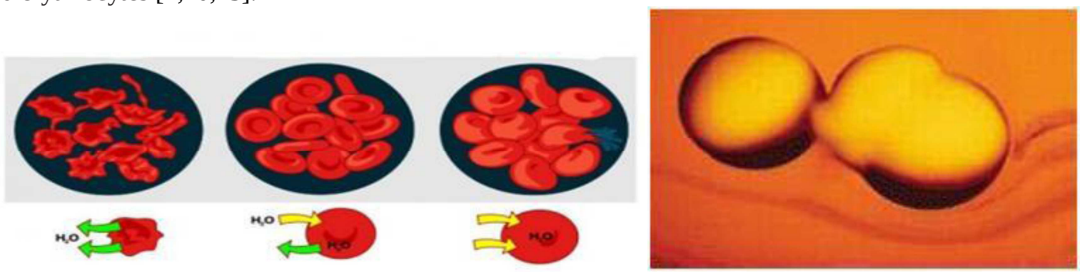

Figure.2: Hypotonic preswelling

Hypotonic dialysis method: This method was first reported by Klibansky in 1959 and was used in 1977 by Deloach and Ihlerand Dale for loading of enzymes and lipids. There are many methods are based on the principle of semi

permeable dialysis membrane which maximizes the intracellular: extracellular volume ratio for macromolecules during lysis and resealing. A desired hemocrit is achieves in this process by mixing of erythrocyte suspension and drug solution. The mixture is placed into dialysis tubing and then both ends of tube are tied with thread. An air bubble of nearly 25% of the internal volume is left in the tube. The tube is placed in the bottle containing 100ml of swelling solution. The bottle is placed at 4^oC for the desire lysis time. The contents of the dialysis tubing are mixed by shaking the tube using the strings. Then dialysis tube is placed in 100 ml of resealing solution. After that the loaded erythrocytes thus obtained, then washed with cold phosphate buffer at 4°C. A good entrapped efficiency is obtained in this [24,25]. It has been used for loading enzymes such as β-galactosidase, glucoserebrosidase, asparginase, inositol hexaphosphatase as well as drugs such as gentamicin, adriamycin, pentamidine and uramycin, interlukin-2, desferroxamine and human recombinant erythropoietin [22].

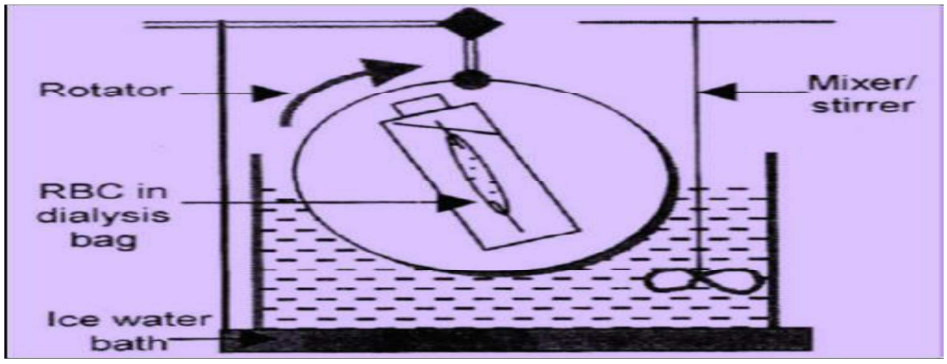

Figure.3: Hypotonic Dialysis technique

Osmotic lysis method: It is also known as the osmotic pulse method. If erythrocytes are incubated in solutions of a substance with high membrane permeability, the solute will diffuse into the cells because of the concentration gradient and it is followed by an influx of water to maintain osmotic equilibrium. Chemicals including urea solution, polyethylene glycol and ammonium chloride have been used for isotonic hemolysis. Finally suspension was diluted with isotonic-buffered drug solution and cells were separated & resealed at 37^0C [20].

2. Membrane perturbation method: It is based on the increase in membrane permeability of erythrocytes when the cells are exposed to certain chemicals that the permeability of erythrocytic membrane increases upon exposure to polyene antibiotic such as amphotericin B. In1980, this method was used successfully by Kitao and Hattori to entrap the antineoplastic drug daunomycinin human and mouse erythrocytes used halothane for the same purpose. However these methods have induced irreversible destructive changes in the cell membrane and hence are not very famous [6].

3. **Electro-encapsulation method:** It is also known as electroporation method, which is based on using transient electrolysis leading to generate pores that produce desirable membrane permeability for drug loading into erythrocytes. It involves suspending of erythrocytes in an isotonic buffer in an electrical discharge chamber. It has a capacitor in an external circuit which is charged to a definite voltage and then discharged within a definite time interval through cell suspension to produce a square-wave potential. In 1980, it was successfully used to entrap the antineoplastic drug daunomycin in human and mouse erythrocytes. This method also induces irreversible destructive changes in the cell membrane and hence is not very popular [13, 26, 27].

4. Entrapment by Endocytosis: It was reported by Schrier in 1975. Endocytosis involves the addition of one volume of washed packed erythrocytes to nine volumes of buffer containing 2.5 mM ATP, 2.5mM $MgCl_2$ and 1mM $CaCl_2$ followed by incubation for 2 min. at room temperature. The pores created by this method are resealed by using 154 mM of NaCl and incubation at 37°C for 2 min. The vesicle membrane separates endocytosed material from cytoplasm & protecting it from the erythrocytes. Various drugs are used in this technique primaquine, 8 aminoquinolones, vinblastine, chlorpromazine, phenothiazine, hydrocortisone, tetracaine, & vitamin A [6, 9].

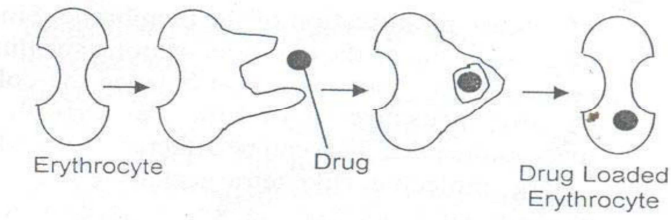

Figure.4: Entrapment by Endocytosis

5. Lipid fusion method: Lipid fusion method involves lipid vesicle containing a drug can be directly fused to human erythrocytes; it leads to an exchange with a lipid-entrapped drug. Hence this method is used for entrapping inositol monophosphate which helps to improve the O_2 carrying capacity of RBCs [9].

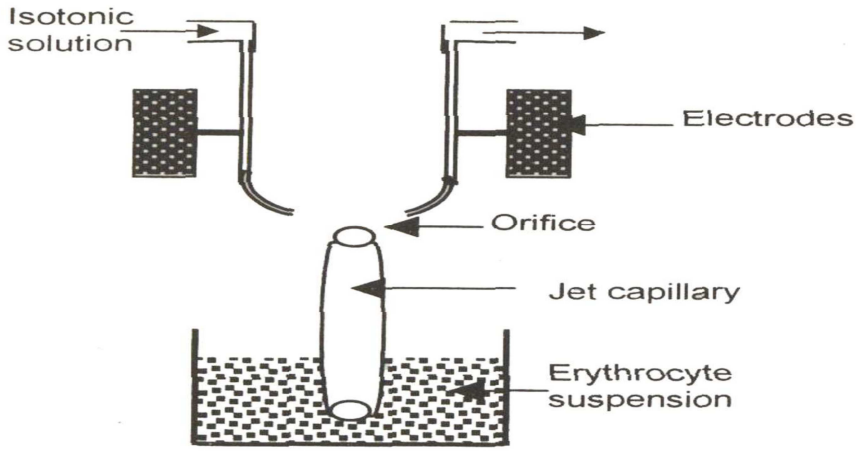

Figure.5: Lipid diffusion method

6. **Electric cell fusion method:** It involves initial loading of drug molecules into erythrocyte ghosts followed by adhesion of these cells to target cells. The fusion process is promoted by the application of an electric pulse, which causes the release of an entrapped molecule. The loading of a cell-specific monoclonal antibody into an erythrocyte ghost is better example of this method. An antibody against a specific surface protein of target cells can be chemically cross-linked to drug-loaded cells that would direct these cells to desired cells [6].

ROUTES OF ADMINISTRATION

It was reported that survival of cells in circulation to the cells administered by intra peritoneal injection is equivalent to i.v. injection. The subcutaneous route for slow release of entrapped agent, reported that the loaded cell released encapsulated molecules at the injection site. Generally resealed erythrocyte during experimentation has been administered to the laboratory animals intravenously through cardinal vein. The scientist De Loach utilized subcutaneous route for slow release of entrapped agents and evaluated the disposition of the interleukin-2 in mice receiving a subcutaneous injection. Talwar (1993) have been proposed erythrocyte based nasal delivery of propranolol [7, 24].

Table: 1 Various Parameters and techniques used for characterization [1]

PARAMETER	TECHNIQUES USED
1. Physical	Transition electron microscopy, optical microscopy, Scanning electron microscopy, phase contrast microscopy
Size, shape, surface morphology	
Drug release	Diffusion cell dialysis
Drug content	Deproteinization of cell membrane followed by assay of drug, radiolabelling.
Surface electrical potential spectroscopy	Zeta potential determination by photon correlation [PCS]
Vesicle size & size distribution	Transmission electron microscopy, Optical microscopy
Surface pH	pH sensitive probes
Deformity	Capillary method
2. Biological	
Pyrogenicity	LAL test, Rabbit method
Sterility	Sterility testing method
Toxicity	Toxicity testing method
3. Cellular	
% Hb content	Deproteinization of cell membrane followed by haemoglobin assay.
Cell volume	Laser light scattering
% cell recovery	Neubaur chamber, hematological analyzer
%Osmotic fragility	Stepwise incubation with isotonic to hypotonic saline solutions and determination of drug and hemoglobin assay.
Turbulent shock	Dilution with distilled water & estimation of drug and hemoglobin
Erythrocyte sedimentation rate	Determine ESR technique

APPLICATIONS OF RESEALED ERYHROCYTES

In-Vitro Applications

Phagocytosis cells have been used for *in vitro* to facilitate the uptake of enzymes by phagolysosomes. The enzyme content within carrier RBC could be visualized with the help of cytochemical technique. The most frequent *in vitro* application of RBC is that of micro-injection in which protein or nucleic acid was injected into eukaryotic cells by fusion process. Similar in case of antibody, where antibody molecules are introduced using erythrocytic carrier system & it immediately diffuse throughout the cytoplasm [24].

In-vivo applications

1) Slow drug release: Erythrocytes have been used as circulating depots for the sustained delivery of antineoplastic, antiparasitics, veterinary antiamoebics, vitamins, steroids, antibiotics and cardiovascular drugs mechanism involve for drug release is accumulation of erythrocytes in lymph nodes upon subcutaneous administration followed by hemolysis to release the drugs [24, 28].

2) Drug targeting: Drug delivery should be site specific and target oriented to exhibit maximal ther apeutic index with minimum adverse effects. It acts as drug carriers and targeting tools as well. It can be used to target RES organs as well as non RES organs. To target organs of mononuclear phagocytic system/ RES Surface modified erythrocytes are used because the change in the membrane is recognized by macrophages [7, 29].

3) Treatment of hepatic tumors: Antineoplastic drugs such as methotrexate, bleomycin, asparginase and adriamycin have been successfully delivered by erythrocytes to treat hepatic tumors. Daunorubicin diffuse rapidly from the cells upon loading and hence pose a problem which can be overcome by covalently linking daunorubicin to the erythrocytic membrane using glutaraldehyde orcisaconitic acid as a spacer [10].

4) Removal of RES iron overloads: To treat excess iron accumulated desferrioxamine-loaded erythrocytes have been used because of multiple transfusions to thalassemic patients. This drug targeting to the RES is very beneficial because the aged erythrocytes are destroyed in RES organs, which results in an accumulation of iron in these organs [7, 30].

5) Carriers for enzymes: For this purpose enzymes can be injected into the blood stream to replace a missing or deficient enzyme in metabolic disorders or to degrade toxic compounds accumulated in the blood due to a disease such as, environmental, lysosomal storage disorders such as gaucher's disease, hyperarginiaemia, hyperuricaemia, hyperphenyl- alaninaemia and kidney failure are only few examples of metabolic disorders that can be treated by administration of enzymes [20].

Table: 2 Resealed for delivery of enzyme [13]

Name of enzymes	Purpose
L-Asparaginase	To treat Acute Lymphoblastic Leukemia
Aminolevulinate dehydratase	To treat adolescent patient suffering from lead poisoning

Table: 3 Resealed erythrocytes for other than RES organ targeting [13]

Approaches	Types of drugs
Magnet-responsive Erythrocyte Ghosts	Encapsulation of small paramagnetic particles into erythrocytes
Photosensitized Erythrocytes	Methotrexate photosensitized by exposure to a haematoporphyrin derivative.
Antibody Anchored Erythrocytes (Immunoerythrocytes)	Antibody coating of resealed drug carrier erythrocytes
Ultrasound Mediated Delivery of Erythrocytes loaded drug(s)	colloidal particles and red blood cells

Table: 4 Resealed erythrocytes in RES Targeting [13]

Treatment/Diseases	Name of Drug(s)	Purpose
Lysosomal storage diseases	C-glucuronidase, Lysosomal enzymes, 13-galactosidase and 6-giucosidase	It is used to deliver lysosomal enzymes and drugs to lysosomes of the erythrophagocytic cells.
Gaucher'sDisease	Glucocerebrosidase	By using this loaded cells survived for 10 days in treated patient and no adverse reactions were found with respect to blood counts, blood pressure and renal functions.
Liver tumors	Anticancer drugs like Bleomycin, Adriamycin, Carboplatin, Gentamycin, etc.	Used to target hepatic carcinomas
Parasitic Diseases	immunoglobulin-G coated erythrocytes, Pentamidine loaded	treatment of parasitic diseases by targeting drug in which the parasite resides in the organs of RES.
Removal of toxic substances	Murine carrier erythrocytes containing bovine rhodanese thiosulphate	Used to antagonize the lethal effects of potassium cyanide in mice or antagonism of cyanide intoxication.

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