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A review article on resealed erythrocytes: A pharmaceutically engineered approach to targeted drug delivery

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Abstract
Carrier erythrocytes, resealed erythrocytes loaded by a drug or other therapeutic agents, have been introduced extensively in recent years for both temporally and spatially controlled delivery of a wide variety of drugs and other bioactive agents owing to their specified degree of biocompatibility, biodegradability and a series of other potential advantages. Carrier erythrocytes have been evaluated in thousands of drug administration in humans proving safety and efficacy of the treatments. Biopharmaceuticals, therapeutically significant peptides and proteins, nucleic acid-based biological, antigens and vaccines, are among the recently focused pharmaceuticals for being delivered using carrier erythrocytes. Erythrocytes mediated drug delivery has been reported with therapeutic enzymes and antiviral agents to maximize therapeutic performance, reduce the side effects, as circulating depots for controlled drug release, drug targeting, treatment of parasitic diseases, hepatic tumors, removal of toxic agents etc. In this review, isolation of carrier erythrocytes, methods of drug loading, methods and clinical applications of resealed erythrocytes were presented.

Keywords: Erythrocytes, isolation of erythrocytes, methods of drug loading in RES, application of RES, advantage of RES, disadvantages of RES

Introduction
Basic features of Erythrocytes: 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 in healthy female). These are biconcave disc shaped cells with an average diameter of 7.5 microns, a thickness of 2.0 microns in periphery, 1 microns in the center, and a volume of 85–91 mm^3 (Fig.1). The flexible, biconcave shape enables erythrocytes to squeeze through narrow capillaries, which may be only 3 microns wide.

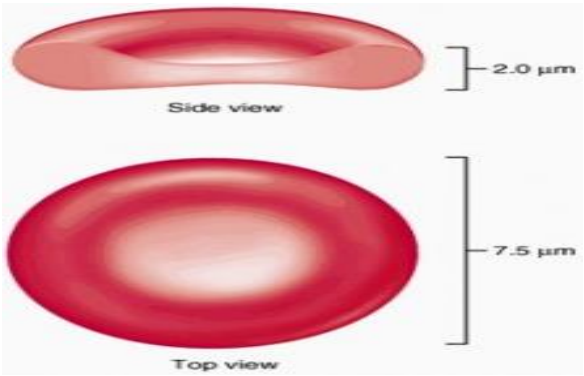

Fig 1: Structure of erythrocytes

Mature erythrocytes are very simple in structural appearance. They do not have the nucleus and other cell organelles. Their plasma membrane consist of haemoglobin, which is a heme-containing protein that is responsible for O_2–CO_2 binding inside the erythrocytes. The main role of erythrocytes is the transport of O_2 from the lungs to tissues and the CO_2 produced in tissues back to lungs. Thus erythrocytes are a highly specialized O_2 carrier system in the body. Erythrocytes live only about 120 days because of their attrition or depletion on their plasma membranes as they squeeze through the narrow blood capillaries [3, 4].

Composition of Electrolyte in Erythrocyte's: The concentration of K^{+} (potassium) and Na^{+}(sodium) differ in that the electrolyte potassium is more in erythrocytes and sodium in plasma. The osmotic pressure of the interior of the erythrocytes is equal to that of plasma and termed as isotonic. Changes in the osmotic pressure of the medium surrounding the red blood cells change and manipulated the morphology and tonicity of the cells. If the medium is hypotonic, water diffuses into the cells and they get swells and eventually looses all their haemoglobin content and may burst. On the other hand, if the medium is hypertonic, they will shrink and become irregular in appearance [5].

Haematocrit Value and Erythrocytes Sedimentation Rate: The haematocrit is the percent volume occupied by the cells and is determined by simple centrifugation of the blood. When blood, in the presence of some anticoagulant is centrifuged the cells settle down to the bottom of the tube, while the plasma rises at the top.

The erythrocytes are often characterized in terms of haematocrit value that is the fraction of erythrocytes portion to total blood. The haematocrit value is a parameter that indicates both the number and the size of the erythrocytes. The erythrocytes are also characterized by erythrocyte sedimentation rate (ESR).When the blood mixed with an anticoagulants and keep for some time, the erythrocytes form aggregates and settle down under the force of gravity alone, the rate at which this settling occurs is known as erythrocytes sedimentation rate. The volume of clear plasma above the sediment erythrocyte at end of 1 hr determines the erythrocyte sedimentation rate [5].

Isolation of Erythrocytes

- Blood is collected into heparin zed tubes by venipuncture.
- Blood is withdrawn from cardiac /splenic puncture (in small animal) and through veins (in large animals) in a syringe containing a drop of anti coagulant.
- The whole blood is centrifuged at 2500 rpm for 5 min. at 4±10ºC
- The serum and buffy coats are carefully removed and packed cells washed three times with phosphate buffer saline (pH=7.4). 4±10ºC in a refrigerated centrifuge.
- The washed erythrocytes are diluted with PBS and stored at 4°C until used [6, 7].

Advantages of Erythrocytes in Drug Loading

1. A remarkable degree of biocompatibility, particularly when the autologous cells are used for drug loading.
2. Complete biodegradability and the lack of toxic product(s) resulting from the carrier biodegradation.
3. Considerable protection of the organism against the toxic effects of the encapsulated drug, e.g. antineoplasts.
4. Remarkably longer life-span of the carrier erythrocytes in circulation in comparison to the synthetic carriers. In the optimum condition of the loading procedure, the life-span of the resulting carrier cells may be comparable to that of the normal erythrocytes.
5. An easily controllable life-span within a wide range from minutes to months.
6. Desirable size range and the considerably uniform size and shape.
7. Protection of the loaded compound from inactivation by the endogenous factors.
8. Possibility of targeted drug delivery to the RES organs.
9. Relatively inert intracellular environment
10. Availability of knowledge, techniques, and facilities for handling, transfusion, and working with erythrocytes
11. Possibility of ideal zero-order kinetics of drug release.
12. Wide variety of compounds with the capability of being entrapped within the erythrocytes.
13. Modification of the pharmacokinetic & pharmacodynamic parameters of the drug.
14. Remarkable decrease in concentration fluctuations in steady state in comparison to the conventional methods of drug administration, which is a common advantage for most of the novel drug delivery systems.
15. Considerable increase in drug dosing intervals with drug concentration in the safe and effective level for a relatively long time [8-17].

Disadvantages

1. The major problem encountered in the use of biodegradable materials or natural cells as drug carriers is that they are removed *in vivo* by the RES as result of modification that occurred during loading procedure in cells. This, although expands the capability to drug targeting to RES, seriously limits their life-span as long-circulating drug carriers in circulation and, in some cases, may pose toxicological problems.
2. The rapid leakage of certain encapsulated substances from the loaded erythrocytes.
3. Several molecules may alter the physiology of the erythrocyte.
4. Given that they are carriers of biological origin, encapsulated erythrocytes may present some inherent variations in their loading and characteristics compared to other carrier systems
5. Possible contamination due to the origin of the blood, the equipment used and the loading environment. Rigorous controls are required accordingly for the collection and handling of the erythrocytes [18-22].

Methods of loading in Resealed Erythrocytes

Hypotonic hemolysis method

1. Hypotonic dilution method
2. Hypotonic Pre-swelling
3. Hypotonic dialysis
4. Isotonic osmotic lysis

- Chemical perturbation of the membrane
- Electro-insertion or electro encapsulation
- Entrapment by endocytosis

1. Loading by electric cell fusion
2. Loading by lipid fusion.

Hypotonic Hemolysis: This method is based on the ability of erythrocytes to undergo reversible swelling in a hypotonic solution. The four variations of the procedure have been described in Table 1 [23].

Table 1: Comparison of various hypoosmotic lysis methods

Methods	%Loading	Advantages	Disadvantages
Dilution Method	1-8 %	Fastest and simplest especially for low molecular weight drugs	Entrapment efficiency is very less (1-8 %).
Dialysis Method	30-45 %	Better *in vivo* survival of erythrocyte	Time consuming heterogeneous Size distribution
Preswell Dilution	20-70 %	Good retention of cytoplasm *In vivo*	-----
Isotonic Osmotic lysis	----	Better *in vivo* surveillance	Impermeable only to large molecule, time consuming

Use of Red Cell Loader: Novel method was developed for entrapment of nondiffusible drugs into erythrocytes. They developed a piece of equipment called a "red cell loader". With as little as 50 ml of a blood sample, different biologically active compounds were entrapped into erythrocytes within a period of 2 hours at room temperature under blood banking conditions. The process is based on two sequential hypotonic dilutions of washed erythrocytes followed by concentration with a hemofilter and an isotonic resealing of the cells. There was 30% drug loading with 35–50% cell recovery. The same cells could be used for targeting by improving their recognition by tissue macrophages.

1. **Hypotonic dilution:** In this method, a volume of packed erythrocytes is diluted with 2–20 volumes of aqueous solution of a drug. The solution tonicity is then restored by adding a hypertonic buffer. The resultant mixture is then centrifuged, the supernatant is discarded, and the pellet is washed with isotonic buffer solution. This reduces the circulation half life of the loaded cells. These cells are readily phagocytized by RES macrophages and hence can be used for targeting RES organs. Hypotonic dilution is used for loading enzymes such as galactosidase and glucosidase, asparginase.
2. **Hypotonic Preswelling:** The technique is based upon initial controlled swelling in a hypotonic buffered solution. This mixture is centrifuged at low *g* The supernatant is discarded and the cell fraction is brought to the lysis point by adding 100–120 Liters portions of an aqueous solution of the drug to be encapsulated. The mixture is centrifuged between the drug-addition steps. The lysis point is detected by the disappearance of a distinct boundary between the cell fraction and the supernatant upon centrifugation.

The tonicity of a cell mixture is restored at the lysis point by adding a calculated amount of hypertonic buffer. Then, the cell suspension is incubated at 37ºC to reanneal the resealed erythrocytes. Such cells have a circulation half life comparable to that of normal cells. This method is simpler and faster than other methods, causing minimum damage to cells. Drugs encapsulated in erythrocytes using this method include propranolol, asparginase, cyclo-pohphamide [24].

1. **Hypotonic Dialysis:** This method is based on the principle that semipermeable dialysis membrane maximizes the intracellular: extracellular volume ratio for macromolecules during lysis and resealing. In the process, an isotonic, buffered suspension of erythrocytes with a hematocrit value of 70–80 is prepared and placed in a conventional dialysis tube immersed in 10–20 volumes of a hypotonic buffer (2). The medium is agitated slowly for 2 hrs. The tonicity of the dialysis tube is restored by directly adding a calculated amount of a hypertonic buffer to the surrounding medium or by replacing the surrounding medium by isotonic buffer. The drug to be loaded can be added by either dissolving the drug in isotonic cell suspending buffer inside a dialysis bag at the beginning of the experiment or by adding the drug to a dialysis bag after the stirring is complete.

In this method, the erythrocyte suspension and the drug to be loaded were placed in the blood compartment and the hypotonic buffer was placed in a receptor compartment. This lead to the concept of "continuous flow dialysis," which has been used by several other researchers**.** This method has been used for loading enzymes such as galactosidase, glucoserebrosidase as well as drugs such as gentamicin Adriamycin, pentamidine, interlukin-2 and human recombinant erythropoietin [25].

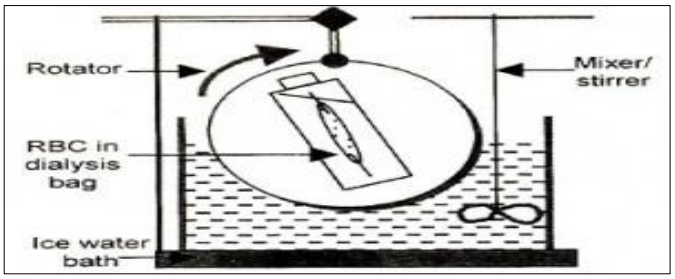

Fig 2: Schematic presentation of erythrocyte dialyzer apparatus for entrapping protiens into erythrocytes using dialysis method.

Isotonic Osmotic Lysis: This method, also known as the osmotic pulse method, involves isotonic hemolysis that is achieved by physical or chemical means. 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. This process is followed by an influx of water to maintain osmotic equilibrium. Chemicals such as urea solution, polyethylene glycol and ammonium chloride have been used for isotonic heamolysis. The suspension was diluted with an isotonic-buffered drug solution. After the cells were separated, they were resealed at 37ºC [25].

- **Chemical perturbation of the Membrane:** This method is based on the increase in membrane permeability of erythrocytes when the cells are exposed to certain chemicals. Permeability of erythrocytic membrane increases upon exposure to polyene antibiotic such as amphotericin B. However, these methods induce irreversible destructive changes in the cell membrane and hence they are not very popular [26].
- **Electro-insertion or Electro-Encapsulation:** The procedure involves suspending erythrocytes in an isotonic buffer in an electrical discharge chamber (3). A capacitor in an external circuit is charged to a definite voltage and then discharged within a definite time interval through cell suspension to produce a square-

wave potential. The optimum intensity of an electric field is between 1–10 kW/cm and optimal discharge time is between 20–160.

An inverse relationship exists between the electric-field intensity and the discharge time. The compound to be entrapped is added to the medium in which the cells are suspended from the commencement of the experiment. This process can be prevented by adding large molecules (e.g. bovine serum albumin) and ribonucleose. Various compounds such as sucrose, urease, methotrexate, isoniazid, human glycophorin, DNA fragments [27].

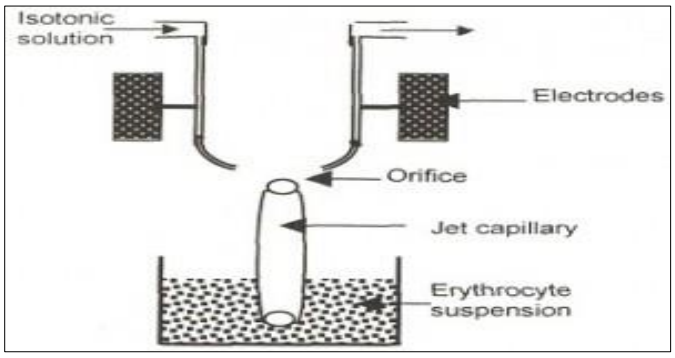

Fig 3: Electro encapsulation technique

- **Entrapment by Endocytosis:** Endocytosis involves the addition of one volume of washed packed erythrocytes to nine volumes of buffer containing 2.5 mM ATP, 2.5 mM $MgCl_2$, 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 37ºC for 2 min. The entrapment of material occurs by endocytosis. The various candidates entrapped by this method include primaquine and related 8–amino–quinolines, vinblastine, chlorpromazine, hydrocortisone and the vitamin.

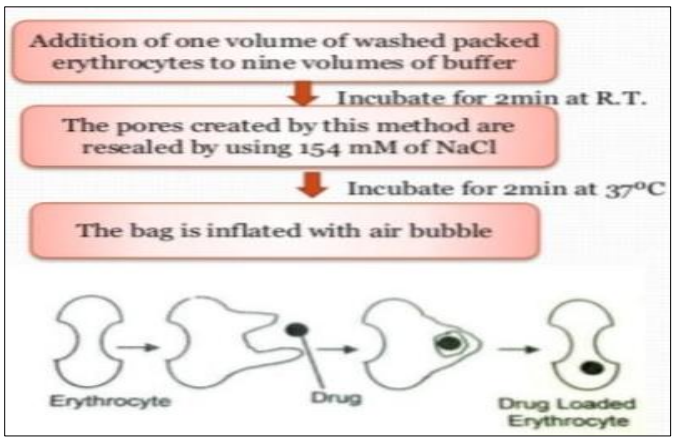

Fig 4: Entrapment by endocytosis

Loading by Electric Cell Fusion: This method involves the initial loading of drug molecules into erythrocyte ghosts followed by adhesion of these cells to target cells. The fusion is accentuated by the application of an electric pulse, which causes the release of an entrapped molecule. An example of this method is loading a cell-specific monoclonal antibody into an erythrocyte ghost. 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.

Loading by Lipid Fusion: Lipid vesicles containing a drug can be directly fused to human erythrocytes, which lead to an exchange with a lipid entrapped drug. This technique was used for entrapping inositol monophosphate to improve the oxygen carrying capacity of cells. However, the entrapment efficiency of this method is very low (1%) [28-31].

Release mechanism of Loaded Drugs: There are mainly three ways for a drug release from the erythrocyte carriers

- **Phagocytosis**: By the process of phagocytosis normally erythrocyte cells removed from the blood circulation. The degree of cross linking determines whether liver or spleen will preferentially remove the cells.
- **Diffusion through the membrane of the Cells**: Diffusion through the membrane depends on the drug molecule penetrate through a lipid bilayer i.e. bioactive compound have lipid solubility.
- **Using a Specific Transport System**: Most of the drug molecules enter cells by a specific membrane protein system because the carriers are proteins with many properties analogous to that of enzymes [32].

***In-vitro* characterization of Loaded Erythrocytes**

1. **Cell counting and Cell Recovery:** This involves counting the number of red blood cells per unit volume of whole blood, usually by automated counting. Red cell recovery may be calculated on the basis of the differences in the hematocrit and the volume of the suspension of erythrocytes before and after loading. The goal is to minimize the loss during the encapsulation procedure to maximize cell recovery.
2. **Morphological Aspect:** The morphological examination of these ghost erythrocytes is undertaken by comparison with untreated erythrocytes using either transmission (TEM) or scanning (SEM) electron microscopy. By means of electron microscopy observation may be made of the morphological changes in the erythrocytes induced by osmosis-based encapsulation methods, when they are subjected to solutions of different osmolality.
3. **Osmotic Fragility:** Osmotic fragility is a test to detect abnormal fragility of red blood cells. Untreated or loaded erythrocytes are tested by exposure to hypotonic solutions, making them swell, in order to determine the relative fragility of the red cells.
4. **Osmotic Shock:** Far 0.5 study, erythrocyte suspension (1 ml, 10%) were diluted with H_2O (5 ml) and centrifuge at 3000 rpm far 15 minute. The supernatant was estimated far %Hb release spectrophotometrically.
5. **Turbulence Shock:** Turbulence shock enables an evaluation to be made of the stability of the loaded erythrocytes against the turbulence stress exerted by the cells against in-vivo circulation turbulence. The test is performed by the method of Deloach *et al*. whereby the suspension of cells is passed several times through a 22-gauge needle.
6. **Haemoglobin Release:** The content of hemoglobin of the erythrocytes may be diminished by the alterations in the permeability of the membrane of the red cells during the encapsulation procedure. Furthermore, the relationship between the rate of hemoglobin and the rate of drug release contributes to interpreting the mechanisms involved in the release of the substance encapsulated from the erythrocytes. The hemoglobin leakage is tested

using a red cell suspension by recording the absorbance of supernatant at 540 nm on a spectrophotometer [33-39].

Application of Resealed Erythrocytes

1. Slow drug release
2. Drug targeting
3. Targeting RES organs
4. Targeting the liver, Enzyme deficiency/ replacement therapy
5. Treatment of hepatic tumors
6. Treatment of parasitic diseases
7. Removal of RES iron overload
8. Removal of toxic agents
9. Targeting organs other than those of RES
10. Delivery of antiviral agents
11. Enzyme therapy
12. Improvement in oxygen delivery to tissues

7. **Slow Drug Release:** Erythrocytes have been used as circulating depots for the sustained delivery of antineoplastics, antiparasitics, veterinary antiamoebics, vitamins, steroids, antibiotics and cardiovascular drugs. The various mechanisms proposed for drug release include passive diffusion, specialized membrane associated carrier transport, phagocytosis of resealed cells by macrophages of RES, subsequent accumulation of drug into the macrophage interior, followed by slow release, accumulation of erythrocytes in lymph nodes upon subcutaneous administration followed by hemolysis to release the drug.
8. **Drug Targeting:** Ideally, drug delivery should be site-specific and target-oriented to exhibit maximal therapeutic index with minimum adverse effects. Resealed erythrocytes can act as drug carriers and targeting tools as well. Surface-modified erythrocytes are used to target organs of mononuclear phagocytic system/ Reticuloendothelial system because the changes in the membrane are recognized by macrophage.
9. **Targeting Reticuloendothelial System (RES) organs:** Damaged erythrocytes are rapidly cleared from circulation by phagocytic Kupffer cells in liver and spleen. Resealed erythrocytes, by modifying their membranes, can therefore be used to target the liver and spleen.
10. **Targeting the liver Enzyme deficiency/ Replacement Therapy:** Many metabolic disorders related to deficient or missing enzymes can be treated by injecting these enzymes. However, the problems of exogenous enzyme therapy include a shorter circulation half life of enzymes, allergic reactions, and toxic manifestations. These problems can be successfully overcome by administering the enzymes as resealed erythrocytes. The enzymes used include-glucosidase, glucoronidase, galactosidase. The disease caused by an accumulation of glucocerebrosides in the liver and spleen can be treated by glucocerebrosidase- loaded erythrocytes.
11. **Treatment of Hepatic Tumors:** Hepatic tumors are one of the most prevalent types of cancer. Antineoplastic drugs such as methotrexate, bleomycin, asparginase, and adriamycin have been successfully delivered by erythrocytes. Agents such as daunorubicin diffuse rapidly from the cells upon loading and hence pose a problem. This problem can be overcome by covalently linking daunorubicin to the erythrocytic membrane using gluteraldehyde or cisaconitic acid as a spacer. The resealed erythrocytes loaded with carboplatin show localization in liver.
12. **Treatment of Parasitic Diseases:** The ability of resealed erythrocytes to selectively accumulate within RES organs make them useful tool during the delivery of antiparasitic agents. Parasitic diseases that involve harboring parasites in the RES organs can be successfully controlled by this method. Results were favorable in studies involving animal models for erythrocytes loaded with antimalarial, antileishmanial, and antiamoebic drugs.
13. **Removal of Reticuloendothelial System (RES) iron overload:** Desferrioxamine-loaded erythrocytes have been used to treat excess iron accumulated because of multiple transfusions to thalassemic patients. Targeting this drug to the RES is very beneficial because the aged erythrocytes are destroyed in RES organs, results in an accumulation of iron in these organs.
14. **Removal of Toxic Agents:** Cannon *et al*., reported inhibition of cyanide intoxication with murine carrier erythrocytes containing bovine rhodanase and sodium thiosulfate. Antagonization of organophosphorus intoxication by resealed erythrocytes containing a recombinant phospho-diestrase also has been reported.
15. **Targeting organs other than those of Reticuloendothelial System (RES):** Recently, resealed erythrocytes have been used to target organs outside the RES. The Various approaches include: Entrapment of paramagnetic particles along with the drug entrapment of photosensitive material, the use of ultrasound waves, antibody attachment to erythrocyte membrane to get specificity of action.
16. **Delivery of Antiviral Agents:** Several reports have been cited in the literature about antiviral agents entrapped in resealed erythrocytes for effective delivery and targeting. Because most antiviral drugs are nucleotides or nucleoside analogs, their entrapment and exit through the membrane needs careful consideration. Nucleosides are rapidly transported across the membrane whereas nucleotides are not and thus exhibiting prolonged release profiles.
17. **Enzyme Therapy:** 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 likewise, environmental, lysosomal storage disorders such as Gaucher's disease, hyperarginiaemia, hyperuricaemia and kidney failure are only few examples of metabolic disorders that can be treated by administration of enzymes.
18. **Improvement in Oxygen Delivery to tissues:** Hemoglobin is the protein responsible for the oxygen-carrying capacity of erythrocytes. Under normal conditions, 95% of hemoglobin is saturated with oxygen in the lungs, whereas under physiologic conditions in peripheral blood stream only 25% of oxygenated hemoglobin becomes deoxygenated. Thus, the major fraction of oxygen bound to hemoglobin is recirculated with venous blood to the lungs [4].

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