

THE PHARMA INNOVATION

Resealed Erythrocytes as a Carrier for Drug Targeting: A Review

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Erythrocytes have been the most interesting carrier and have found to possess great potential in drug targeting. Resealed erythrocytes are gaining more popularity because of their ability to circulate throughout the body, biocompatibility, zero order release kinetics, reproducibility and ease of preparation. Most of the resealed erythrocytes used as drug carriers are rapidly taken up from blood by macrophages of reticuloendothelial system (RES), which is present in liver, lung, and spleen of the body. The aim of the present review is to focus on the various features, drug loading technology and biomedical application of resealed erythrocytes.

Keyword: Resealed Erythrocytes, Carrier Drug Targeting

INTRODUCTION: Present pharmaceutical scenario is aimed at development of drug delivery systems which maximize the drug targeting along with high therapeutic benefits for safe and effective management of diseases¹. Targeting of an active biomolecule from effective drug delivery where pharmacological agent directed specifically to its target site. Drug targeting can be approached by either chemical modification or by appropriate carrier². Various carriers have been used for the drug targeting among which cellular carriers offer a greater potential advantage related to its biodegradability, non-pathogenicity, non-immunogenicity, biocompatibility, self-degradability along with high drug loading efficiency³. Leukocytes, platelets and erythrocytes have been proposed as cellular carrier systems⁴.

Erythrocytes:

Red blood cells (also referred to as erythrocytes) are the most common type of blood cells and the vertebrate organism's principal means of delivering oxygen (O₂) to the body tissues via the blood flow through the circulatory system. They take up oxygen in the lungs or gills and release it while squeezing through the body's capillaries. These cells' cytoplasm is rich in hemoglobin, an iron-containing biomolecule that can bind oxygen and is responsible for the blood's red color. In humans, mature red blood cells are flexible biconcave disks that lack a cell nucleus and most organelles. 2.4 million new erythrocytes are produced per second⁵.

Resealed Erythrocytes: Such drug-loaded carrier erythrocytes are prepared simply by collecting blood samples from the organism of interest, separating erythrocytes from plasma, entrapping drug in the erythrocytes, and resealing the resultant cellular carriers⁶. Hence, these carriers are called resealed erythrocytes. The overall process is based on the response of these

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cells under osmotic conditions⁷. Upon reinjection, the drug-loaded erythrocytes serve as slow circulating depots and target the drugs to a reticuloendothelial system (RES)⁸.

Morphology and physiology of erythrocytes:

Erythrocytes are the most abundant cells in the human body (~5.4 million cells/mm³ blood in a healthy male and ~4.8 million cells/mm³ blood in a healthy female). Erythrocytes are biconcave discs with an average diameter of 7.8 μ m, a thickness of 2.5 μ m in periphery, 1 μ m in the center, and a volume of 85–91 μ m³^[11]. The flexible, biconcave shape enables erythrocytes to squeeze through narrow capillaries, which may be only 3 μ m wide.

Advantages of resealed erythrocytes as drug carriers: The resealed erythrocytes should have the following advantages:^[9,10,11,12,13]

- Their biodegradability with no generation of toxic products.
- The considerably uniform size and shape of the carrier.
- Relatively inert intracellular environment.
- Prevention of degradation of the loaded drug from inactivation by endogenous chemicals.
- The wide variety of chemicals that can be entrapped.
- The modification of pharmacokinetic and pharmacodynamic parameters of drug.
- Attainment of steady-state plasma concentration decreases fluctuations in concentration.
- Protection of the organism against toxic effects of drugs (e.g. antineoplastics).
- They are able to circulate throughout the body and facilities for separation, handling, transfusion, and working with erythrocytes the availability of the techniques
- The prevention of any undesired immune response against the loaded drug
- Their ability to target the organs of the RES.

- The possibility of ideal zero-order drug-release kinetics.
- The lack of occurrence of undesired immune response against encapsulated drug.
- The large quantity of drug that can be encapsulated within a small volume of cells ensures dose sufficiency.
- A longer life span in circulation as compared with other synthetic carriers.^[19,35,36] and optimum conditions may result in the life span comparable to that of normal erythrocytes^[33, 37,38]
- Easy control during life span ranging from minutes to months.^[19]
- A decrease in side effects of drugs.^[17,29,32]
- A considerable increase in drug dosing interval with drug residing in therapeutic window region for longer time periods.^[17,29,31,33]

Isolation of erythrocytes:

- Blood is collected into heparinized 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 $4 \pm 1^{\circ}$ C in a refrigerated centrifuge.
- The serum and buffy coats are carefully removed and packed cells washed three times with phosphate buffer saline (pH=7.4).
- The washed erythrocytes are diluted with PBS and stored at 4° C until used.
- Various types of mammalian erythrocytes have been used for drug delivery, including erythrocytes of mice, cattle, pigs, dogs, sheep, goats, monkeys, chicken, rats, and rabbits. To isolate erythrocytes, blood is collected in heparinized tubes by venipuncture. Fresh whole blood is typically used for loading purposes because the encapsulation

efficiency of the erythrocytes isolated from fresh blood is higher than that of the aged blood. Fresh whole blood is the blood that is collected and immediately chilled to 4 °C and stored for less than two days. The erythrocytes are then harvested and washed by centrifugation. The washed cells are suspended in buffer solutions at various hematocrit values as desired and are often stored in acid-citrate-dextrose buffer at 4 °C for as long as 48 h before use. Jain and Vyas have described a well-established protocol for the isolation of erythrocytes [13]. Erythrocyte ghost can be used as adenosine triphosphate (ATP) [14]. Entrapment of dextran (molecular weight 10–250 kDa) and loading of drugs in erythrocytes was reported separately.

- Their biocompatibility, particularly when autologous cells are used, hence no possibility of triggered immune response.

Methods of drug loading in resealed erythrocytes: Several methods can be used to load drugs or other bioactive compounds in erythrocytes, including physical (e.g., electrical pulse method) osmosis-based systems, and chemical methods (e.g., chemical perturbation of the erythrocytes membrane). Irrespective of the method used, the optimal characteristics for the successful entrapment of the compound requires the drug to have a considerable degree of water solubility, resistance against degradation within erythrocytes, lack of physical or chemical interaction with erythrocyte membrane, and well-defined pharmacokinetic and pharmacodynamic properties. The several methods for loading drug in erythrocytes are giving in follows:

Hypotonic hemolysis: [16, 17]

This method is based on the ability of erythrocytes to undergo reversible swelling in a hypotonic solution. Erythrocytes have an exceptional capability for reversible shape changes with or without accompanying volume change and for reversible deformation under stress. An increase in volume leads to an initial

change in the shape from biconcave to spherical. This change is attributable to the absence of superfluous membrane; hence, the surface area of the cell is fixed. The cells assume a spherical shape to accommodate additional volume while keeping the surface area constant. The volume gain is 25–50%. The cells can maintain their integrity up to a tonicity of 150 mos m/kg, above which the membrane ruptures, releasing the cellular contents. At this point (just before Cell lysis), some transient pores of 200–500 Å are generated on the membrane. After cell lysis, cellular contents are depleted. The remnant is called an erythrocyte ghost. The principle of using these ruptured erythrocytes as drug carriers is based on the fact that the ruptured membranes can be resealed by restoring isotonic conditions. Upon incubation, the cells resume their original biconcave shape and recover original impermeability.

Use of red cell loader: [18]

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 h 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 processed erythrocytes had normal survival in vivo. The same cells could be used for targeting by improving their recognition by tissue macrophages.

Hypotonic dilution: [19]

Hypotonic dilution was the first method investigated for the encapsulation of chemicals into erythrocytes and is the simplest and fastest. 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. The major drawbacks of this method include low entrapment efficiency and a considerable loss of hemoglobin and other cell components. This reduces the circulation half-life of the loaded cells. These cells are readily phagocytosed by RES macrophages and hence can be used for targeting RES organs. Hypotonic dilution is used for loading enzymes such as galactosidase and glucosidase, asparaginase, and arginase, as well as bronchodilators such as salbutamol.

Hypotonic preswelling: [20, 21, 22]

This method was developed by Rechsteiner in 1975 and was modified by Jenner et al. for drug loading. The technique 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 Ltrs 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 37°C 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, asparaginase, cyclophosphamide, 1-antitrypsin, methotrexate, insulin, metronidazole, levothyroxine, enalaprilat, and isoniazid.

Hypotonic dialysis: [23, 24, 25]

This method was first reported by Klibansky in 1959 and was used in 1977 by DeLoach and Ihler, and Dale for loading enzymes and lipids. Several methods are 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. The medium is agitated slowly for 2 h. 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. The use of standard hemodialysis equipment for loading a drug in erythrocytes was reported by Roper et al. 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 led to the concept of “continuous flow dialysis,” which has been used by several other researchers.

The loaded cells exhibit the same circulation half-life as that of normal cells. Also, this method has high entrapment efficiency on the order of 30–50%, cell recovery of 70–80%, high-loading capacity and is amenable to automation with control of process variables. The drawbacks include a long processing time and the need for special equipment. This method has been used for loading enzymes such as galactosidase, glucosylbromidase, asparaginase, inositol hexaphosphatase, as well as drugs such as gentamicin, adriamycin, pentamidine and furamycin, interleukin-2, desferrioxamine, and human recombinant erythropoietin.

Isotonic osmotic lysis:

This method, also known as the osmotic pulse method, involves isotonic hemolysis that is achieved by physical or chemical means. The isotonic solutions may or may not be isoionic. 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 hemolysis. However, this method also is not immune to changes in membrane structure composition. In 1987, Francoet al. developed a method that involved suspending erythrocytes in an isotonic solution of dimethyl sulfoxide (DMSO). The suspension was diluted with an isotonic-buffered drug solution. After the cells were separated, they were resealed at 37⁰ C.

Chemical perturbation of the membrane ^[26]:

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. In 1980, this method was used successfully to entrap the antineoplastic drug daunomycin in human and mouse erythrocytes. However, these methods induce irreversible destructive changes in the cell membrane and hence are not very popular.

Electro-insertion or electro encapsulation ^[27, 28, 29]:

In 1973, Zimmermann tried an electrical pulse method to encapsulate bioactive molecules. Also known as electroporation, the method is based on the observation that electrical shock brings about irreversible changes in an erythrocyte membrane. In 1977, Tsong and Kinosita suggested the use of transient electrolysis to generate desirable membrane permeability for drug loading. The erythrocyte membrane is opened by a dielectric breakdown. Subsequently, the pores can be resealed by incubation at 37⁰ C in an isotonic medium.

The procedure involves suspending erythrocytes in an isotonic buffer in an electrical discharge chamber. 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. The characteristic pore diameter created in the membrane depends upon the intensity of electric field, the discharge time, and the ionic strength of suspending medium. The colloidal macromolecules contents of the cell may lead to cell lysis because of the increase in osmotic pressure. This process can be prevented by adding large molecules (e.g., tetrasaccharide stachyose and bovine serum albumin) and ribonucleose. One advantage of this method is a more uniform distribution of loaded cells in comparison with osmotic methods. The main drawbacks are the need for special instrumentation and the sophistication of the process. Entrapment efficiency of this method is 35%, and the life span of the resealed cells in circulation is comparable with that of normal cells. Various compounds such as sucrose, urease, methotrexate, isoniazid, human glycoporphin, DNA fragments, and latex particles of diameter 0.2 μ m can be entrapped within erythrocytes by this method. Mangal and Kaur achieved sustained release of a drug entrapped in erythrocytes with the use of electroporation.

Entrapment by endocytosis: ^[30]

This method was reported by Schrier et al. in 1975. Endocytosis involves the addition of one volume of washed packed erythrocytes to nine volumes of buffer containing 2.5 mM ATP, 2.5 mM MgCl₂, and 1mM CaCl₂, 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 37⁰C for 2 min. The entrapment of material occurs by endocytosis. The vesicle membrane separates endocytosed material from cytoplasm thus protecting it from the erythrocytes and vice-versa. The various candidates entrapped by this method include primaquine and related 8-amino-quinolines, vinblastine, chlorpromazine and related phenothiazines, hydrocortisone, propranolol, tetracaine, and vitamin A.

Loading by electric cell fusion ^[31]: 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 ^[32]: 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%).

In vitro storage ^[33]: The success of resealed erythrocytes as a drug delivery system depends to a greater extent on their in vitro storage. Preparing drug-loaded erythrocytes on a large scale and maintaining their survival and drug content can be achieved by using suitable storage methods. However, the lack of reliable and practical storage methods has been a limiting factor for the wide-spread clinical use of the carrier erythrocytes. The most common storage media include Hank's balanced salt solution and acid-citrate-dextrose at 4°C. Cells remain viable in terms of their physiologic and carrier characteristics for at least 2 weeks at this temperature. The addition of calcium-chelating agents or the purine nucleosides improve circulation survival time of cells upon reinjection. Exposure of resealed erythrocytes to membrane stabilizing agents such as dimethyl sulfoxide, dimethyl, 3, 3-di-thio-bispropionamide, gluteraldehyde, toluene-2-4-diisocyanate followed by lyophilization or sintered glass filtration has been reported to enhance their stability upon storage. The resultant powder was

stable for at least one month without any detectable changes. But the major disadvantage of this method is the presence of appreciable amount of membrane stabilizers in bound form that remarkably reduces circulation survival time. Other reported methods for improving storage stability include encapsulation of a prodrug that undergoes conversion to the parent drug only at body temperature, high glycerol freezing technique, and reversible immobilization in alginate or gelatin gels.

Applications of Resealed erythrocytes:

Resealed erythrocytes have several possible applications in various fields of human and veterinary medicine. Such cells could be used as circulating carriers to disseminate a drug within a prolonged period of time in circulation or in target-specific organs, including the liver, spleen, and lymph nodes. A majority of the drug delivery studies using drug-loaded erythrocytes are in the preclinical phase. In a few clinical studies, successful results were obtained ^[34, 35, 36].

- **Slow drug release:** ^[37] 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.

Routes of administration include intravenous, which is the most common, followed by subcutaneous, intraperitoneal, intranasal, and

oral. Studies regarding the improved efficacy of various drugs given in this form in animal models have been published. Examples include an enhancement in anti-inflammatory effect of corticosteroids in experimentally inflamed rats, increase in half-life of isoniazid, levothyroxine, cytosine arabinoside, and interleukin- 2, prolongation of plasma half-life of erythropoietin from 30 min to 35 h in mice, and can increase in mean survival time of mice with experimental hepatoma after injecting methotrexate loaded erythrocyte. Thalassemic patients, because of multiple blood transfusions, are prone to hemosydrrosis, a disease state associated with an excess storage of iron. This state is treated using SC or IV injections of iron-chelating compound desferrioxamine, which causes severe adverse effects in case of multiple injections. This agent was loaded on to erythrocytes and the performance of these cells upon reinjection was observed and found to be promising. This therapeutic method is approved in the United States as regular management tool of hemosydrrosis since 1984.

- **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 macrophages [38]. However; resealed erythrocytes also can be used to target organs other than those of reticuloendothelial (RES).
- **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. The

various approaches to modify the surface characteristics of erythrocytes include

- **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, glucuronidase, galactosidase. The disease caused by an accumulation of glucocerebrosides in the liver and spleen can be treated by glucocerebrosidase-loaded erythrocytes.
- **Treatment of hepatic tumors:** [39] Hepatic tumors are one of the most prevalent types of cancer. Antineoplastic drugs such as methotrexate, bleomycin, asparaginase, 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 [40] as a spacer. The resealed erythrocytes loaded with carboplatin show localization in liver [41].
- **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.

- **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. The release of nucleotides requires conversion of these moieties to purine or pyrimidine bases. Resealed erythrocytes have been used to deliver deoxycytidine derivatives, recombinant herpes simplex virus type 1 (HSV-1) glycoprotein B, azidothymidine derivatives, azathioprene, acyclovir, and fludarabine phosphate. [42]
- **Enzyme therapy:** [43, 44, 45] Enzymes are widely used in clinical practice as replacement therapies to treat diseases associated with their deficiency (e.g., Gaucher's disease, galactosuria), degradation of toxic compounds secondary to some kind of poisoning (cyanide, organophosphorus), and as drugs. The problems involved in the direct injection of enzymes into the body have been cited. One method to overcome these problems is the use of enzyme-loaded erythrocytes. These cells then release enzymes into circulation upon hemolysis act as a "circulating bioreactors" in which substrates enter into the cell, interact with enzymes, and generate products or accumulate enzymes in RES upon hemolysis for future catalysis.

CONCLUSION: The use of resealed erythrocytes looks promising for a safe and sure delivery of various drugs for passive and active targeting. However, the concept needs further optimization to become a routine drug delivery

system. The same concept also can be extended to the delivery of biopharmaceuticals and much remains to be explored regarding the potential of resealed erythrocytes.

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