Resealed Erythrocytes: Potential Carrier for the Delivery of Drugs and Drug-Loaded Microspheres

[1]Ms. Suvarna Bhadane, [2]Ms. Manisha Moda, [3]Mr. Vijay Modale, [4]Ms.Swathi Paskanti

[1]Assistant professor, [2,3,4]Student of B. Pharmacy
Department of Quality Assurance
Ideal College of Pharmacy & Research, Bhal Kalyan (E) Thane, India.

Abstract: **Erythrocytes have been extensively studied for their potential carrier capabilities for the delivery of drugs and drug-loaded microspheres. 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 carriers. Hence, these carriers are called *resealed erythrocytes*. The overall process is based on the response of these cells under osmotic conditions. Upon reinjection, the drug-loaded erythrocytes serve as slow circulating depots and target the drugs to a Reticulo Endothelial system (RES).**

***Key words*- Erythrocytes, Reticulo Endothelial system, isolation, Biomedical Applications**

1. INTRODUCTION [1]

Morphology and Physiology of Erythrocytes

Erythrocytes are the most abundant cells in the human body (about 5.4 million cells/mm3 blood in a healthy male and about 4.8 million cells/mm3 blood in a healthy female). These cells were described in human blood samples by Dutch Scientist Lee Van Hock in 1674. In the 19th century, Hope Seyler identified hemoglobin and its crucial role in oxygen delivery to various parts of the body. Erythrocytes are 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 µm3. The flexible, biconcave shape enables erythrocytes to squeeze through narrow capillaries, which may be only 3 µm wide. Mature erythrocytes are quite simple in structure. They lack a nucleus and other organelles. Their plasma membrane encloses hemoglobin, 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. Because a nucleus is absent, all the intracellular space is available for O_2 transport. Also, because mitochondria are absent and because energy is generated anaerobically in erythrocytes, these cells do not consume any of the oxygen they are carrying.

Erythrocytes live only about 120 days because of wear and tear on their plasma membranes as they squeeze through the narrow blood capillaries. Worn-out erythrocytes are removed from circulation and destroyed in the spleen and liver (RES), and the breakdown products are recycled. The process of erythrocyte formation within the body is known as *erythropoiesis*. In a mature human being, erythrocytes are produced in red bone marrow under the regulation of a hemopoietic hormone called *erythropoietin*.

2. SOURCE AND ISOLATION OF ERYTHROCYTES [1, 2]

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 4^0C 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 4^0C for as long as 48 h before use. Jain and Vyas have described a well-established protocol for the isolation of erythrocytes.
In 1953, Gardos tried to load erythrocyte ghost using adenosine triphosphate (ATP). In 1959,Marsden and Ostting reported the entrapment of dextran (molecular weight 10–250 kDa). In 1973, the loading of drugs in erythrocytes was reported separately by Ihler et al. and Zimmermann. In 1979, the term *carrier erythrocytes* was coined to describe drug-loaded erythrocytes.

3. ADVANTAGES OF ERYTHROCYTES AS DRUG CARRIERS [3, 4]

Advantages include:

- There biocompatibility, particularly when autologous cells are used, hence no possibility of triggered immune response
- There 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)
Other advantages are:
● There ability to circulate throughout the body
● The availability of the techniques and facilities for separation, handling, transfusion, and working with erythrocytes
● The prevention of any undesired immune response against the loaded drug
● There 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 and optimum conditions may result in the life span comparable to that of normal erythrocytes
● Easy control during life span ranging from minutes to months
● A decrease in side effects of drugs
● A considerable increase in drug dosing interval with drug residing in therapeutic window region for longer time periods

4. METHODS OF DRUG LOADING [5, 6, 7]

Several methods can be used to load drugs or other bioactive compounds in erythrocytes, including physical (e.g., electricalpulse 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.

A. Hypotonic Hemolysis. 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 about 25–50%. The cells can maintain their integrity up to a tonicity of approximately 150mosm/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.

B. Use of Red Cell Loader. Magnani et al. developed a novel method 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 approximately 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.

C. Hypotonic Dilution. 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 a 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 β-galactosidase and β-glucosidase, asparginase, and arginase, as well as bronchodilators such as salbutamol.

D. Hypotonic Preswelling. 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 µL 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 ^{0}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, asparginase, cyclopohphamide, cortisol-21-phosphate, ω1-antitrypsin, methotrexate, insulin, metronidazole, levothyroxine, enalaprilat, and isoniazid.

E. Hypotonic Dialysis. This method was first reported by Klibansk 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 was 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 β- galactosidase, glucoserebrosidase, asparginase, inositol hexaphosphatase, as well as drugs such as gentamicin, adriamycin, pentamidine and furamycin, interlukin-2, desferroxamine, and human recombinant erythropoietin.

F. 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 resistant to changes in membrane structure composition. In 1987, Franco et 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 37 ^{o}C.

G. 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. In 1973, Deuticke et al. showed that the permeability of erythrocytic membrane increases upon exposure to polyene antibiotic such as amphotericin B. In 1980, this method was used successfully by Kitao and Hattori to entrap the antineoplastic drug daunomycin in human and mouse erythrocytes. Lin et al. used halothane for the same purpose. However, these methods induce irreversible destructive changes in the cell membrane and hence are not very popular.

H. Electro-Insertion or Electroencapsulation. 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 37 ^{o}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 µs. 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 glycophorin, DNA fragments, and latex particles of diameter 0.2 µm 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.

I. Entrapment by Endocytosis. 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 37 ^{o}C 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.

J. 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.

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

5. IN VITRO CHARACTERIZATION [13]

The in vivo performance of resealed erythrocytes is affected to a great extent by their biological properties. Hence, in vitro characterization forms an important part of studies involving such cellular carriers. Table I summarizes the various evaluation parameters and the techniques applied for their determination.

The morphology of erythrocytes decides their life span after administration. Light microscopy reveals no observable change in resealed cells but in few cases spherical erythrocytes (spherocytes) are detected. Scanning electron microscopic studies have shown that a majority of the cells maintain their biconcave discoid shapes after the loading procedure, and few stomatocytes—a form of spherocytes with an invagination in one point—are formed. In some cases, cells of smaller size (microcyte) are also observed.

Shape change (deformability) is another factor that affects the life span of the cells. This parameter evaluates the ease of passage of erythrocytes through narrow capillaries and the RES. It determines the rheological behavior of the cells and depends on the viscoelasticity of the cell membrane, viscosity of the cell contents, and the cellular surface-to-volume ratio. The deformability is measured by passage time of definite volume of cells through capillary of 4µm diameter or polycarbonate filter with average pore size of 45 µm. Another indirect approach is to evaluate chlorpromazine induced shape changes turbid metrically.

The osmotic fragility of resealed erythrocytes is an indicator of the possible changes in cell membrane integrity and the resistance of these cells to osmotic pressure of the suspension medium. The test is carried out by suspending cells in media of varying sodium chloride concentration and determining the hemoglobin released. In most cases, osmotic fragility of resealed cells is higher than that of the normal cells because of increased intracellular osmotic pressure.

The turbulence fragility is yet another characteristic that depends upon changes in the integrity of cellular membrane and reflects resistance of loaded cells against hemolysis resulting from turbulent flow within circulation. It is determined by the passage of cell suspension through needles with smaller internal diameter (e.g., 30 gauge) or vigorously shaking the cell suspension. In both cases, hemoglobin and drug released after the procedure are determined. The turbulent fragility of resealed cells is found to be higher.

Routine clinical hematological tests also can be carried out for drug-loaded cells, including mean corpuscular volume, mean corpuscular hemoglobin, mean corpuscular hemoglobin content. Studies have shown that the average size and hemoglobin content of resealed cells is lower than that of normal cells.

Drug content of the cells determines the entrapment efficiency of the method used. The process involves deproteinization of packed, loaded cells (0.5 mL) with 2.0 mL acetonitrile and centrifugation at 2500 rpm for 10 min. The clear supernatant is analyzed for the drug content.

The most important parameters for evaluation of resealed erythrocytes is the drug release pattern. Hemoglobin is also invariably released because drug release involves the loss of cell membrane integrity indicating hemolysis. On the basis of the various in vitro release experiments carried out on these cells, three general drug release patterns are observed:

● The rate of drug release is considerably higher than that of hemoglobin. In other words, drug diffuses readily. Such a pattern is shown by lipophilic drugs, including methotrexate, phenytoin, dexamethasone, primpquin, and vitamin B12. Cell lysis is not essential for the release of such drugs.

● The rate of drug release is comparable to that of hemoglobin. This indicates that cell lysis is essential for drug release and drug can not be released by mere diffusion. Polar drugs such as gentamicin, heparin, and enalaprilat, and enzymes such as asparginase, peptides, including urogasterone and l-lysine-l-phenylalanine follow such pattern.

● The rate of drug release lies between the above mentioned two extremes; for example, propranolol, isoniazid, metronidazole, and recombinant human erythropoietin.

The two factors that determine the drug release pattern are size and polarity of the drug molecule. The release rate can be modified by cross-linking cell membrane with gluteraldehyde, which results in a slower drug release. This can also be achieved by entrapping biodegradable prodrug such as o-acetyl propranolol, o-pivaloyl propranolol, cortisol-21- phosphate, prednisolone-21-sodium succinate, and cytosine arabinoside monophosphate. The complexation of a drug with macromolecules such as dextran and albumin also retard the release rate.

6. In vitro storage [9, 12, 14]

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 ^{o}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.

7. IN VIVO LIFE SPAN AND IMMUNOLOGICAL CONSEQUENCES [14]

The efficacy of resealed erythrocytes is determined mainly by their survival time in circulation upon reinjection. For the purpose of sustained action, a longer life span is required, although for delivery to target-specific RES organs, rapid phagocytosis and hence a shorter life span is desirable. The life span of resealed erythrocytes depends upon its size, shape, and surface electrical charge as well as the extent of hemoglobin and other cell constituents lost during the loading process.
The various methods used to determine in vivo survival time include labeling of cells by 51Cr or fluorescent markers such as fluorescin isothiocyanate or entrapment of 14C sucrose or gentamicin.
The circulation survival kinetics of resealed erythrocytes show typical bimodal behavior with a rapid loss of cells during the first 24 h after injection, followed by a slow decline phase with a half life on the order of days or weeks. The early loss accounts for _15–65% loss of total injected cells.
The erythrocytic carriers constructed of red blood cells of mice, cattle, pigs, dogs, sheep, goats, and monkeys exhibit a comparable circulation profile with that of normal unloaded erythrocytes. On the other hand, resealed erythrocytes prepared from red blood cells of rabbits, chickens, and rats exhibit relatively poor circulation profile.
Erythrocytes loading using hypotonic haemolysis followed by isotonic resealing; dialysis and electroencapsulation methods appear to sustain normally in circulation. A bimodal type of survival kinetics is observed: a rapid loss of cells during first 24 h followed by much slower loss afterwards. The early loss possibly accounts for removal of nearly 15% of total cell population; this represents the cells that are severely damaged during the loading procedures. The second phase has half-life of orders of weeks for different mammalian erythrocytes.
It is observed that erythrocyte carriers constructed of autologous sources do not elicit immunological consequences. However, it is customary to realize that during loading process; some antigenic impurities may get entrapped resulting in immunological manifestations. The erythrocytic carriers constructed of red blood cells of mice, cattle, pigs, dogs, sheep, goats, and monkeys exhibit a comparable circulation profile with that of normal unloaded erythrocytes. On the other hand, resealed erythrocytes prepared from red blood cells of rabbits, chickens, and rats exhibit relatively poor circulation profile.

8. BIOMEDICAL APPLICATIONS OF RESEALED ERYTHROCYTES [8]

A. Erythrocytes As Carriers For Enzymes: 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. These cells release enzymes into circulation upon haemolysis and act as "circulating bioreactors" in which substrates enter the cells, interact with enzymes, and generate products or accumulate enzymes in RES upon hemolysis for future catalysis. The first report of successful clinical trials of the resealed erythrocytes loaded with enzymes for replacement therapy is that of ß-glucoserebrosidase for the treatment of Gaucher's disease. The disease is characterized by inborn deficiency of lysosomal ß-glucoserebrosidase in cells of RES thereby leading to accumulation of ßglucoserebrosides in macrophages of the RES. The most important application of resealed erythrocytes in enzyme therapy is that of asparginase loading for the treatment of pediatric neoplasms. This enzyme degrades aspargine, an amino acid vital for cells. This treatment prevents remission of pediatric acute lymphocytic leukemia. Other enzymes used for loading resealed erythrocytes include urease, galactose-1-phosphate uridyl transferase, uricase and acetaldehyde dehydrogenase.

B. Erythrocytes for Drug Targeting: A drug delivery should ideally be site specific and target oriented in order to exhibit maximal therapeutic index and minimum side and toxic effects. It has been observed that osmotically loaded erythrocytes can act as a drug carrier in systemic circulation. Whereas chemically surface modified erythrocytes are targeted to organs of mononuclear phagocytic system because of changes incorporated in the membrane that are recognized by macrophage cells.

C. Drug Targeting to Reticuloendothelial System: The damaged erythrocytes are quickly removed from circulation by phagocytic Kupffer cells located in liver and spleen. Though, resealed erythrocytes have been proposed for passive auto-vectorization to reticuloendothelial system where modified surface characteristics impart them the selectivity and specificity towards target cells.

Various approaches for modifying the circulatory and tissue uptake parameters of resealed erythrocytes to make them site specific and target oriented are adopted.

i) Surface modification with antibody: Circulatory half life is shortened on coating resealed erythrocytes with antibodies. Lightly coated resealed erythrocytes cause a reduction of half life from 27 days to several minutes and majority of erythrocytes are sequestered by spleen macrophages. On the other handheavily modified cells cause a similar reduction incirculating half life while in this case; the majority of erythrocytes are sequestered by liver macrophages and as a result accommodated in these organs.

ii) Surface modification involving carbohydrates: Enzymatically modified cell surface carbohydrates of erythrocytes exhibit a different biodistribution as compared to their plain version. Sialidase, an enzyme that removes sialic acid from external glycoprotein and glycolipids causes significant reduction in the circulatory half life of erythrocytes and make them prone to macrophage uptake especially in liver and spleen.

iii) Surface modification with sulphydryls: External sulphydryls of erythrocytes can be oxidized to varying degrees and thus circulatory half life can be manipulated from a normal value of 27 days to several min (8 min).

D. Drug Targeting To Liver [9, 12]

.**i) Treatment of Liver Tumors:** Anticancer agents encapsulated in erythrocytes can be used for targeting hepatic carcinomas. Various agents such as bleomycin, Adriamycin, carboplatin, gentamycin, and methotrexate have been tried. Erythrocyte membrane bound daunorubicin has also been used as a delivery system in anticancer treatment.

ii) Treatment of parasitic diseases: The ability of resealed erythrocytes to selectively accumulate within the RES rich organs makes them versatile drug carriers for the delivery of antiparasitic agents. Parasitic diseases in which the parasite resides in reticuloendothelial system have been effectively treated with resealed erythrocytes. Pentamidine, primaquine phosphate and metronidazole have been successfully utilized for treatment of leshmaniasis, malaria and extraintestinal amoebiasis on experimental laboratory models.

iii) Removal of RES iron overload: In iron overload resulting from repeated blood transfusion, reticuloendothelial cells are the primary and the major sites of iron accumulation. Iron chelating drug (desferrioxime) has been entrapped in erythrocytes, for promoting excretion of iron that is present as intracellular ferritin and haemosiderin deposit. The drug forms soluble chelates and depletes the depots.

iv) Removal of toxic agents: A relatively new approach was employed to antagonize organophosphorus intoxication by using resealed carrier erythrocytes containing a recombinant phosphotriesterase. This enzyme has been reported to hydrolyze many organophosphorus compounds, including paraoxon, a potent cholinesterase inhibitor.

Paraoxon is rapidly hydrolyzed by this enzyme to pnitro phenol and diethyl phosphate. Incorporation of phosphotriesterase within resealed Murine erythrocytes was accomplished by hypotonic dialysis. Addition of paraoxon to reaction mixtures containing these resealed erythrocytes loaded with phosphotriesterase resulted in rapid hydrolysis of paraoxon. Hydrolysis of paraoxon does not occur when these carrier erythrocytes contain no phosphotriesterase. These in vitro studies suggest that carrier erythrocytes may be developed as an effective approach for prophylactic and therapeutic antagonism of organophosphorus intoxication.

E. Targeting To Sites Other Than Res Rich Organs: Resealed erythrocytes have the ability to deliver a drug or enzyme to the macrophage-rich organs, this is, unfortunately the premium limitation of this delivery system. Organ targeting other than RES has been tried recently with resealed erythrocytes.

i) Magnet-responsive erythrocyte ghosts: Magnetic erythrocytes have been reported as an intelligent version for site specific biophysically modulated targeting strategy. The encapsulation of small paramagnetic particles into erythrocytes might allow their localization to a particular location under the influence of external magnetic field. The in vivo target specificity and selectivity of the drug loaded magnetoresponsive erythrocytes was evaluated by assessing drug content at target site and other cutaneous and subcutaneous parts.

ii) Photosensitized erythrocytes: This system was used firstly for delivery of methotrexate in tumor therapy. Erythrocytes were loaded with methotrexate and photosensitized by subsequent exposure to a haematoporphyrin derivative.

Following their accumulation at the site these were photoirradiated for effective photodynamic effect. Thus a combination of chemotherapy and photodynamic therapy could be a useful modality in the treatment of tumors of body located at site other than RES predominant organs.

iii) Ultrasound mediated delivery of erythrocytes loaded drugs: The application of ultrasound to thin shelled micro bubbles flowing through small microvessels produces vessel wall ruptures in vivo. The endothelial barrier limits many intravascular drug and gene delivery vehicles. Microvessel ruptures caused by insonification of microbubbles in vivo provides a non invasive means for delivering engineered red blood cells across the endothelial lining of barriers to the target tissue.

F. Delivery of Antiviral Agents: Antiviral agents are encapsulated in the resealed erythrocytes for effective delivery and targeting. Because of rapid plasma elimination and toxicity of the most commonly used drugs, daily multiple drug therapies must often be continued throughout life, frequently causing major side effects and as a consequence poor patient compliance.

Therefore, alternative strategies that reduce the toxicity of the drugs and allow prolonged application intervals are needed. Both purine and pyrimidine nucleosides are transported extremely rapidly by facilitated diffusion.

Nucleotides however are not transported across the membrane and thus remain entrapped causing prolonged release profile.8 Release of nucleotides from erythrocytes thus requires conversion of nucleotides to purine or pyrimidine bases. The inter-conversion of non-transportable form of drug to the transportable form of drug can be brought about in two different ways:

• Normal erythrocyte enzymes can take part in the inter-conversion as in case of arabinoside cytosine triphosphate, where phosphatase present in erythrocyte removes phosphates from arabinoside cytosine triphosphate.

• Non erythrocytes enzymes could be conjugated or entrapped in the erythrocytes to perform interconversion as in the case of pyridoxine and pyridoxal, which are transported through facilitated diffusion. Once transported they can be phosphorylated and interconverted.

Thus erythrocytes loaded with antiviral drugs may act in vitro as bioreactors ensuring sustained release of these drugs.

G. Oxygen Deficiency Therapy: Resealed erythrocytes are also used in cases of oxygen deficiency where an improved oxygen supply is required as in the following cases:
- High altitude conditions.
- Small number of alveoli.
- Increased resistance to oxygen diffusion in the lungs.
- Reduction in oxygen transport capacity.
- Liver mediated detoxification processes.

In these cases, resealed erythrocytes can be used to improve the oxygen releasing capacity of the native erythrocytes. The underlying problem with normal erythrocytes is that 95% of haemoglobin is normally saturated with oxygen in the lungs whereas in physiological conditions in peripheral blood stream only about 25% of the oxygenated haemoglobin become deoxygenated. Thus the major fraction of oxygen bound to haemoglobin is recirculated with the venous blood to the lungs. The use of this bound fraction has been suggested for the therapy of oxygen deficiency. This is possible when oxygen release is facilitated and increased in the capillary system and its affinity towards haemoglobin is reduced. This can be achieved by the enhancement of the 2, 3-diphosphoglycerate level or its substitute in the erythrocytes. Inositol hexaphosphate loaded into erythrocytes binds irreversibly to the haemoglobin and reduces its oxygen binding thus releasing the same in the capillaries.

H. Delivery of Macromolecules [10, 11]

Biological functions of macromolecules such as DNA, RNA, and proteins are exploited for various cell biological applications. Hence, various methods are used to entrap these macromolecules into cultured cells (e.g., microinjection). In microinjection, erythrocytes are used as micro syringes for injection to the host cells.

The microinjection process involves culturing host eukaryotic cells *in vitro*. The cells are coated with fusogenic agent and then suspended with erythrocytes loaded with the compound of interest in an isotonic medium. Sendai virus (hem agglutinating virus of Japan, HVJ) or its glycoproteins or polyethylene glycol have been used as fusogenic agents. The fusogen causes fusion of co suspended erythrocytes and eukaryotic cells. Thus, the contents of resealed erythrocytes and the compound of interest are transferred to host cell.

This procedure has been used to microinject DNA fragments, arginase, proteins, nucleic acids, ferritin, latex particles, bovine and human serum albumin, and enzyme thymidine kinase to various eukaryotic cells.

Advantages of this method include quantitative injection of materials into cells, simultaneous introduction of several materials into a large number of cells, minimal damage to the cell, avoidance of degradation effects of lysosomal enzymes and simplicity of the technique.

Disadvantages include a need for a larger size of fused cells, thus making them amenable to RES clearance, adverse effects of fusogens and unpredictable effects on cell resulting from the co-introduction of various components. Hence, this method is limited to mainly cell biological applications rather than drug delivery.

9. Novel Approaches [15]

i) Erythrosomes. These are specially engineered vesicular systems that are chemically cross-linked to human erythrocytes' support upon which a lipid bilayer is coated. This process is achieved by modifying a reverse-phase evaporation technique. These vesicles have been proposed as useful encapsulation systems for macromolecular drugs.

ii) Nanoerythrosomes. These are prepared by extrusion of erythrocyte ghosts to produce small vesicles with an average diameter of 100 nm. Daunorubicin was covalently conjugated to nanoerythrosomes using gluteraldehyde spacer. This complex was more active than free daunorubicin alone, both in vitro and in vivo.

10. 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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