



Resealed Erythrocytes: A Novel Approach to Treat Chronic Diseases

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ABSTRACT

There are more than 30 drug delivery systems are present in this pharma innovative world. Now days the present scenario is focusing more towards the targeted drug delivery systems which is due to the increasing interest in taking of safe drugs and which is capable of reaching the drug in the desired target site with minimal side effects. In fact the main problem associated with systemic drug administration is essentially related to the bio-distribution of pharmaceuticals throughout the body. To overcome this problem and improving the patient compliance as well as efficiency we require a novel drug delivery through resealed erythrocytes is needed now a days. Resealed erythrocytes gaining more importance which is due to they have ability to circulate entire body and they are biocompatible, they show zero order release kinetics, complete biodegradability, lack of toxic product, controllable life span, decreasing drug side effects. And these have a significant impact on treating the chronic diseases like Cancer, Diabetes Mellitus, Rheumatoid Arthritis, HIV infection and Drug Addiction. In this comprehensive article the history of erythrocyte, carrier erythrocyte, some biological methods, isolation, *in vitro* and *in vivo* characteristics, delivery strategies, diagnostic and therapeutic applications and generation of resealed erythrocytes is reviewed.

Keywords: Resealed Erythrocytes, Erythrocytes, Erythrosomes, Red Blood Cells, Drug Carriers.

HISTORICAL BACKGROUND

Erythrocytes, mistaken for fat globules with the early available microscope of the Dutch microscopist and Leeuwenhoek were first described in the seventeenth century as particles "25,000 times smaller than a fine grain of sand"¹. A more precise description of these cells was given about hundred years later, by Howson finding these cells as flat discs rather than the globules. In the 19th century, Hoppe Seyler completed the Hünefeld's discovery of hemoglobin by identification of its crucial role in oxygen delivery to different tissues.² Reversible oxygenation was considered for a long time as the primary or even sole function of the red cell (along with CO₂ exchange) until the late twentieth century. However, now our understanding of erythrocyte function has broadened to include O₂, CO₂, hydrogen sulfide and nitric oxide exchange as well as immune clearance and, possibly clearance of other soluble blood components such as cytokines.¹

The first efforts for entrapment of chemicals in erythrocytes were made in 1953 by Gardos, who tried to load the "erythrocyte ghosts" by ATP.³ In 1959, Marsden and Ostling reported the entrapment of dextran with molecular weights of 10 to 250 KD in erythrocyte ghosts.⁴ Fourteen years later, the first reports on loading the erythrocyte ghosts by therapeutic agents for delivery purposes were published independently by Ihler.⁵ and Zimmerman⁶, and the term "carrier erythrocytes" was used for the first time in 1979 to describe the drug-loaded erythrocytes.⁷

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.

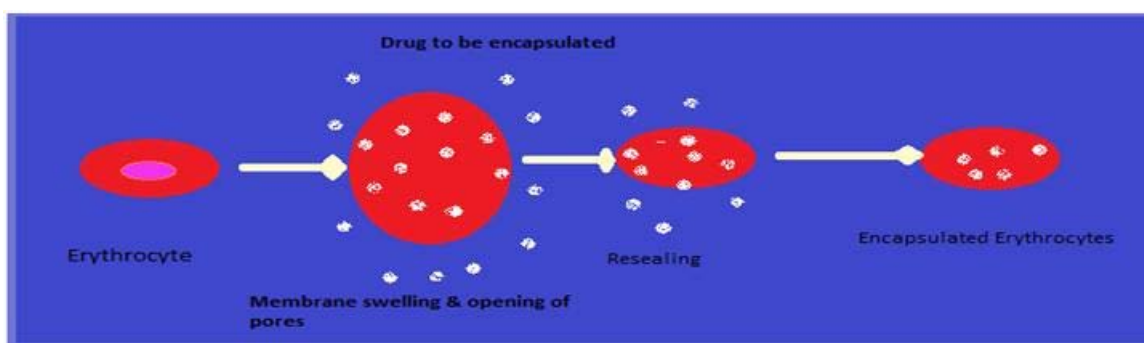


Figure 1: Process of Encapsulated Erythrocytes

MORPHOLOGY AND PHYSIOLOGY OF ERYTHROCYTES

Erythrocytes are the most abundant cells in the human body (5.4 million cells/mm in a healthy male and ~4.8 million cells/mm 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. 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:⁸⁻¹³

- 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 pharmacodynamics 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 ability 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 kinetics¹⁴.
- 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^{15, 16}.
- A longer life span in circulation as compared with other synthetic carriers^{19, 33, 34} and optimum conditions may result in the life span comparable to that of normal erythrocytes^{31, 35, 36}.
- Easy control during life span ranging from minutes to months¹⁹.
- A decrease in side effects of drugs^{17, 30}.

- A considerable increase in drug dosing interval with drug residing in therapeutic window region for longer time periods^{17, 29, 31}.

DISADVANTAGES

- They have a limited potential as carrier to non-phagocyte target tissue.
- Possibility of clumping of cells and dose dumping may be there.^{21, 22}



Figure 2: Erythrocytes

ERYTHROCYTES

Erythrocytes can be used as carriers in two ways:

1. Targeting particular tissue/organ

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

2. For continuous or prolonged release of drugs

Alternatively, erythrocytes can be used as a continuous or prolonged release system, which provide prolonged drug action. There are different methods for encapsulation of drugs within erythrocytes. They remain in the circulation for prolonged periods of time (up to 120 days) and release the entrapped drug at a slow and steady rate.²³

Isolation of erythrocytes

- ✚ Blood is collected into heparinized tubes by veinpuncture.
- ✚ 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 $4 \pm 1^\circ\text{C}$ 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 for as long as 48 h before use.
- ✚ 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.^{18, 24}

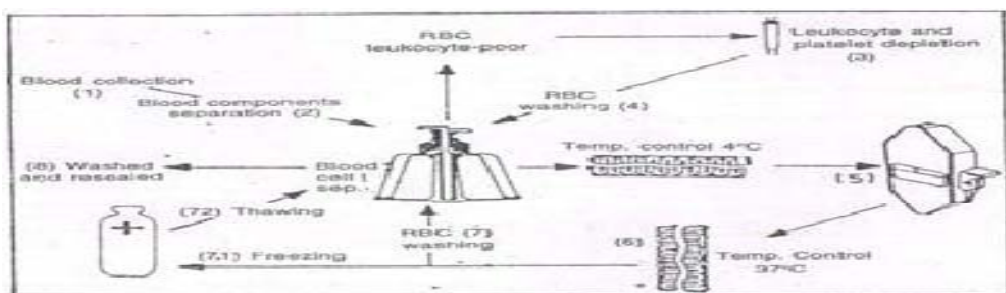


Figure 3: Isolation of Erythrocytes

Various condition and centrifugal force used for isolation of erythrocytes²⁵

Requirement for encapsulation

Variety of biologically active substance (5000-60,000dalton) can be entrapped in erythrocytes.

- Non-polar molecule may be entrapped in erythrocytes in salts. Example: Tetracycline Hydrochloride salt can be appreciably entrapped in bovine RBC.
- Generally, molecule should be Polar & Non polar molecule also been entrapped
- Hydrophobic molecules can be entrapped in erythrocyte by absorbing over other molecules.
- Once encapsulated charged molecule are retained longer than uncharged molecule. The size of molecule entrapped is a significant factor when the molecule is smaller than sucrose and larger than β -galactosidase.²⁶⁻²⁹

METHODS OF DRUG LOADING IN 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 pharmacodynamics properties.

Types

[A] Osmosis based methods

- 1] Hypotonic Hemolysis
- 2] Hypotonic Dilution
- 3] Hypotonic Dialysis
- 4] Hypotonic Pre-Swelling

B] Chemical perturbation of the membrane

C] Electro-insertion or electro encapsulation

D] Entrapment by endocytosis

[A] OSMOSIS BASED METHODS

1] Hypotonic Hemolysis

This method is based on the ability of erythrocytes to undergo reversible swelling in a hypotonic solution as shown in figure. Erythrocytes have an exceptional capability for reversible shape changes with or without accompanying volume change and for reversible deformation under the cells can maintain their integrity up to a tonicity of 150 mosm/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 Stress.

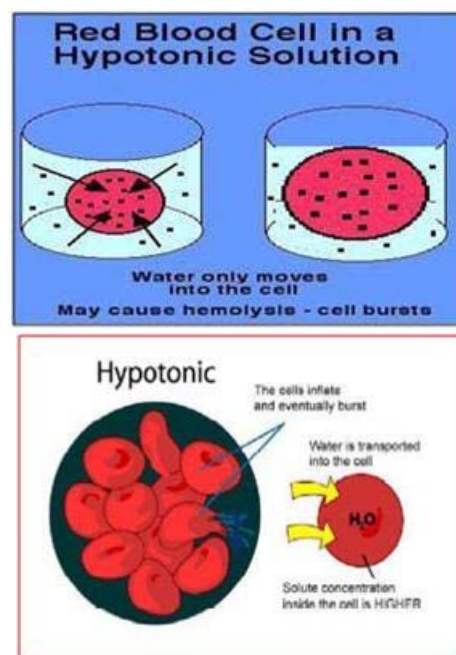


Figure 4: Hypotonic Hemolysis Method and Hypotonic Dilution Method

2] Hypotonic Dilution

The erythrocytes have little capacity to resist volume. This is the simplest and fastest method. In this a volume of packed erythrocytes is diluted with 2–20 volumes of aqueous solution of a drug. At an increase in volume above 50-75% of initial volume and in hypotonic solution the erythrocyte membrane ruptures and pores are created to entrapped drug. The solution tonicity is then restored by adding a hypertonic buffer.³⁰

3] Hypotonic Dialysis

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 semi permeable dialysis membrane maximizes the intracellular: extracellular volume ratio for macromolecules during lysis and resealing. In this process a desired hemocrit is achieved by mixing erythrocyte suspension and drug solution. This mixture is placed into dialysis tubing and then both ends of tube are tied with thread. An air bubble of nearly 25% of the internal volume is left in the tube. The tube is placed in the bottle containing 100ml of swelling solution. The bottle is placed at 4°C for the desired lysis time. The contents of the dialysis tubing are mixed intermittently by shaking the tube using the strings. The dialysis tube is then placed in 100 ml of resealing solution. The loaded erythrocytes thus obtained are then washed with cold phosphate buffer at 4°C. In this method a good entrapped efficiency is obtained.³¹

4] Hypotonic Pre - Swelling

As shown in Figure- 06 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 L portions of an aqueous solution of the drug to be encapsulated.³²

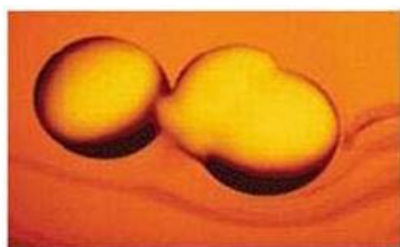


Figure 5: Hypotonic Pre - Swelling Method

B] 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 showed that the permeability of erythrocyte 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 these methods induce irreversible destructive changes in the cell membrane and hence are not very popular³⁰.

C] ELECTRO-INSERTION OR ELECTRO ENCAPSULATION

The method is based on the observation that electrical shock brings about irreversible changes in the erythrocyte membrane is opened by a dielectric breakdown. Subsequently, the pores can be resealed by incubation at 37°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 μ s. 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 experiment³⁰.

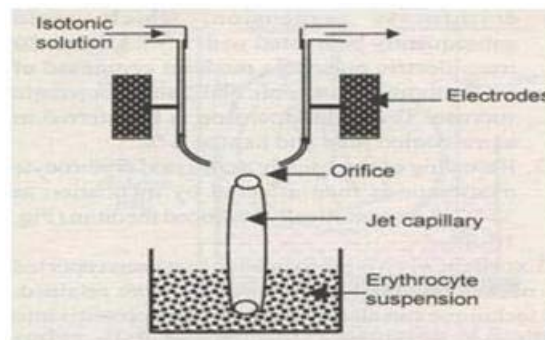


Figure 6: Electro-Insertion or Electro Encapsulation Method

An erythrocyte membrane 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.

Table 1: Different Methods of Loading in Resealed Erythrocytes

Methods	Loading	Advantage	Disadvantage
Dilution method	1-8%	Fastest and simplest for low molecular weight drugs	Entrapment efficiency is very less
Dialysis	30-45%	better in vivo survival of RBCs	Time consuming heterogeneous size distribution
Preswell dilution	20-70 %	Good retention of cytoplasm <i>in vivo</i>	–
Isotonic osmotic lysis	–	Better <i>in vivo</i> surveillance	Impermeable only to large molecule, time consuming

D] ENTRAPMENT BY ENDOCYTOSIS

This method was reported by Schrier in 1975. Endocytosis involves the addition of one volume of washed packed erythrocytes to nine volumes of buffer containing 2.5 mM ATP, 2.5mM 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 °C for 2 min. The entrapment of material occurs by endocytosis. The vesicle membrane separates endocytosis material from cytoplasm thus protecting it from the erythrocytes and vice-versa.

DRUG RELEASE CHARACTERISTICS OF LOADED ERYTHROCYTES

There are mainly three ways for a drug to efflux out from erythrocyte carrier's i.e. phagocytosis, diffusion through the membrane of the cell and use of specific transport system. The rate of diffusion depends upon the rate at which a particular molecule penetrates through a lipid bilayer it is greatest for molecule with high lipid solubility and gradually goes down with polarity or charged groups of the molecule. Prolongation of release could presumably be accomplished by entrapment of potent inhibitors of the appropriate transport protein along with the drug.

IN VITRO CHARACTERISATION

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.

A] Physical Characterization

1] Shape and Surface Morphology

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 (spherocytosis) 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 spherocytosis with an invagination in one point are formed. In some cases, cells of smaller size (microcyte) are also observed.

2] Drug content

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.

3] Deformability

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 4 μm diameter or polycarbonate filter with average pore size of 45 μm . Another indirect approach is to evaluate chlorpromazine induced shape changes turbidimetrically.

4] Drug Release

The most important parameters for evaluation of resealed erythrocytes are 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, primaquin 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 cannot be released by mere diffusion. Polar drugs such as gentamicin, heparin, enalapril and enzymes such as asparaginase peptides, including urogastrone and 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.

B] Cellular characterization

1] Osmotic Fragility

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.

2] Turbulent Fragility

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 gauges) 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.

3] Percent cell recovery

It can be determined by counting the number of intact cells per cubic mm of packed erythrocyte before and after loading the drug.

C] Biological characterization

It can be done by performing sterility test, pyrogen test using rabbit method and LAL test and toxicity test on animal.



IN VIVO CHARACTERISATION

The efficacy of resealed erythrocytes is determined mainly by their survival time in circulation upon reinjection. The various methods used to determine *in vivo* survival time include labeling of cells by ^{51}Cr or fluorescent markers such as fluorescein isothiocyanate or entrapment of ^{14}C 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 weeks³².

Route of administration

Usually resealed erythrocyte during experimentation has been administered to the laboratory animals intravenously through cardinal vein. DeLoach utilized subcutaneous route for slow release of entrapped agents. He evaluated the disposition of the interleukin -2 in mice receiving a subcutaneous injection. Recently Talwar (1993) have proposed erythrocyte based nasal delivery of propranolol.

In vitro storage

The success of resealed erythrocytes as a drug delivery system depends to a greater extent on their *in vitro* storage. 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, glutaraldehyde, toluene-2-4-diisocyanate followed by lyophilization or sintered glass filtration has been reported to enhance their stability upon storage. It can be also preserved by suspending cells in oxygenated HBBS contain 1% soft gelatin. The cells are well recovered after liquefying the gel by placing the tube in water bath at 37°C followed by centrifugation. Another method utilized for storage has been cryopreservation of RBCs in liquid nitrogen³³.

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

In-vitro applications

For *in vitro* phagocytosis cells have been used to facilitate the uptake of enzymes by phagolysosomes. Enzymes content within carrier RBC could be visualized with the help of cytochemical technique. The most frequent *in*

vitro application of RBC is that of micro-injection. A protein or nucleic acid was injected into eukaryotic cells by fusion process. Similarly, when antibody molecules are introduced using erythrocytic carrier system, they immediately diffuse throughout the cytoplasm.

In-vivo applications

- ✓ Targeting of bioactive agents to RES System
- ✓ Targeting to sites other than RES-rich Organs
- ✓ Erythrocytes as Circulating Bioreactors
- ✓ Erythrocytes as Carriers for Drugs
- ✓ Erythrocytes as Carriers for Enzymes
- ✓ Erythrocytes as Carriers for Proteins and Macromolecules.

Drug targeting to the RES organs

1] 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 drug³⁵.

2] Targeting to the liver

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 β -glucosidase, β glucuronidase and β -galactosidase³⁶.

3] Treatment of hepatic tumors

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 glutaraldehyde or cisaconitic acid as a spacer. The resealed erythrocytes loaded with carboplatin show localization in liver³³.



4] 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 antiamebic Drugs³⁶.

5] Removal of 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, which results in an accumulation of iron in these organs.

6] Removal of toxic agents

Cannon reported inhibition of cyanide intoxication with murine carrier erythrocytes containing bovinerhodanase and sodium thiosulfate. Antagonization of organophosphorus intoxication by resealed erythrocytes containing a recombinant phosphodiesterase also has been reported.

Drug targeting organs other than those of RES**7] Delivery of antiviral agents**

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.

8] Enzyme replacement therapy

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, organ phosphorus), 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. The first report of successful clinical trials of the resealed erythrocytes loaded with enzymes for replacement therapy is that of β -glucoserebrosidase for the treatment of Gaucher's disease. The disease is characterized by inborn deficiency of lysosomal β -glucoserebrosidase in cells of RES thereby

leading to accumulation of β -glucoserebrosides in macrophages of the RES³⁷.

9] Improvement in the oxygen delivering 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 reticulated with venous blood to the lungs. The use of this bound fraction has been suggested for the treatment of oxygen deficiency. 2, 3-Diphosphoglycerate (2,3-DPG) is a natural effectors of hemoglobin. The binding affinity of hemoglobin for oxygen changes reversibly with changes in intracellular concentration of 2,3-DPG. This compensates for changes in the oxygen pressure outside of the body, as the affinity of 2,3-DPG to oxygen is much higher than that of hemoglobin³⁵.

10] Microinjection of macromolecules

In microinjection, erythrocytes are used as microsyringes 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 (hemagglutinating virus of Japan HVJ) or its glycoproteins or polyethylene glycol have been used as fusogenic agents. The fusogen causes fusion of cosuspended 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 cells³⁸.

11] Applications of carrier Erythrocytes in delivery of biopharmaceuticals

The scientist can reported that, the potential applications of erythrocytes in drug delivery have been reviewed with a particular stress on the studies and laboratory experiences on successful erythrocyte loading and characterization of the different classes of biopharmaceuticals.

NOVEL APPROACHES**Erythroosomes**

Erythroosomes are specially engineered vesicular systems in which chemically cross-linked human erythrocyte cytoskeletons are used as support upon which a lipid bilayer is coated. This can be achieved by a modification procedure normally adopted for reverse phase evaporation. Erythroosomes are proposed as useful encapsulation system for drug delivery particularly for macromolecular drugs. Large (3, μ m diameter) mechanically stable proteoliposomes (erythroosomes)

were prepared in good yield by coating cross linked erythrocyte cytoskeletons with phosphatidyl choline.

Application

1. Pentamidine primaquine phosphate and metronidazole have been successfully utilized erythrocytes for the treatment of leishmaniasis, malaria on experimental models.
2. It can be serves as ideal carrier for antineoplastic drugs like bleomycin.
3. Vitamin and steroid have been encapsulated in RBCs.
4. It serves as a cellular sustain delivery system for in vivo administration of recombinant erythropoietin.
5. RBCs coated with recombinant interleukin-2 are reported to provide sustain release to allow low and nontoxic concentration of rIL-2 in circulation.

Nanoerythrocytes (nEs)

Nanoerythrocytes are vesicles prepared by the extrusion of RBC ghost, the average diameter of these vesicles being 100 nm. The process gave small vesicles with the size of liposomes. The drugs can be conjugated to the Nanoerythrocytes using certain cross linking agents such as glutaraldehyde have higher activity than when compared to free drug and they offer higher stability. The Nanoerythrocytes are characterized for various parameters like surface morphology, percentage of drug conjugation, centrifugal stress, in vitro release etc. Thus nEs are very versatile bioactive drug carriers or drug delivery system (DDS). These nEs are stable and maintain both the cytotoxic & antineoplastic activity of daunorubicin against mice leukemia p338D cells. The Nanoerythrocytes compositions further leads to the formation of bioassays. In case of Gauchers disease glucocortisone was encapsulated in erythrocytes and heparin was encapsulated in erythrocytes to prevent thromboembolism.

Preparation of (nEs) and drug loading

There are three methods for loading of drugs in NES.

1. Extrusion

The erythrocyte ghost suspension (50% haematocrit) is extruded through the 25mm polycarbonate membrane filter (0.4µm) pore obtained by 8-10 consecutive extrusions under nitrogen pressure. The ghosts obtained are stained with uranyl acetate and they are observed under microscope. The extrusions are performed in a thermostatically controlled extrusion device at 37°C and the final preparation is stored in a refrigerator at 40°C. The extrusions are performed at 37°C in thermostatically controlled extrusion device.

2. Sonication

Erythrocyte ghosts are converted into small vesicles using a dismembrator.

3. Electrical breakdown method

It is used to convert ghosts into small vesicles under the influence of electric potential

Advantages

1. Non immunogenic in action and can be targeted to disease tissue /organ.
2. Prolong the systemic activity of drug while residing for a longer time in the body.
3. Protect the premature degradation, inactivation and excretion of proteins and enzymes.
4. Target the drugs within RES as well as non RES organs/sites.
5. No possibility of triggered immunological response.
6. Drug is chemically bonded with the protein of the erythrocyte membrane.
7. Less prone to aggregation and fusion.
8. Flexibility of membrane allows them to escape RES for longer periods.

Applications

1. nEs are also used for delivery of mitomycin-c, hydroxyurea & 6-mercaptopurine. Cytotoxicity is more in the form of nE combinations than the free drug. They are also used for the delivery of insulin.
2. Doxorubicin-E conjugates have higher antineoplastic activity than the free drug on CDF1 leukemia tumors.
3. Nanoerythrocytes (nEs) were designed in such a way such that with low doses, they maintain the optimum concentration of the drug for prolonged periods of time & they provide sustained action.

DISCUSSION

The preparation of resealed erythrocytes is very easy. There are several techniques identified now days by which we can easily entrap the drug into erythrocytes. It is having both types of application *in vitro* as well as *in vivo*. The traditional drug delivery system had many disadvantages, such as high toxicity effect and high minimum effective dose. Among the various carriers, the most important are the one that at least mimic body's intrinsic components and avoid provoking an immune response on IV administration. Many drug therapies have disadvantages of patient non-compliance due to more dosing frequency.

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, Proteins, steroids. Resealed erythrocytes are used for targeting drug delivery system



depending on sustain release insulin, antiviral agent, Oxygen deficiency therapy etc. was found to be very beneficial to use as a novel drug delivery system.

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