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RESEALED ERYTHROCYTES – A REVIEW

Abhilash M*, Sandeep Kumar MS, Sathya Prasad S, SK Senthil kumar, S Parthiban

Department of Pharmaceutics, Bharathi college of Pharmacy, Bharathi Nagara, Mandya, Karnataka – 571 422, India.

ABSTRACT

Now days the research work in the drug development is mainly focusing on targeted drug delivery for better therapeutic effect. Various carriers for delivering the drug have been reported, among these erythrocytes (RBC) constitute potential biocompatible carriers since they possess several properties which make them unique and useful carriers. Erythrocytes are biocompatible, biodegradable, possess long circulation half-lives, and can be loaded with a variety of biologically active compounds using various chemical and physical methods.

KEY WORDS: Erythrocytes, Advantages, *Invitro* characterization.

INTRODUCTION

Erythrocytes, also known as red blood cells, 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 [1].

Erythrocytes, the most abundant cells in the human body, have potential carrier capabilities for the delivery of drugs. Erythrocytes are biocompatible, biodegradable, possess very long circulation half-lives and can be loaded with a variety of chemically and biologically active compounds using various chemical and physical methods. Application of erythrocytes as promising slow drug release or site-targeted delivery systems for a variety of bioactive agents from different fields of therapy has gained a remarkable degree of interest in recent years. Biopharmaceuticals are among the most widely exploited candidates for being delivered to the host body using these cellular carriers. In this review, the potential applications of erythrocytes in drug delivery have been highlighted [2].

MORPHOLOGY AND PHYSIOLOGY OF ERYTHROCYTES

- Erythrocytes are the most abundant cells in the human body.
- Erythrocytes are biconcave discs with an average diameter of 7.8µm, a thickness of 2.5 m in periphery.
- The flexible, biconcave shape enables erythrocytes to squeeze through narrow capillaries, which may be only 3µm 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.
- Erythrocytes are highly specialized O2 carrier system in the body. Because a nucleus is absent, all the intracellular space is available for O2 transport.
- Also, because mitochondria are absent and because energy is generated aerobically 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.

Corresponding Author:- **Abhilash M** Email:-: abhilash9164709809@gmail.com

- 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

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 hrs 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 weight10–250kDa).In1973, 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 [2].

Factors which considering resealed erythrocytes as carrier

- Its shape and size to permit the passage through the capillaries.
- Its specific physico-chemical properties by which a prerequisite site can be recognized.
- Its biocompatible and minimum toxicity character.
- Its degradation product, after release of the drug at the target site, should be biocompatible.
- Low leaching/leakage of drug should take place before target site is reached.
- Its drug released pattern in a controlled manner.
- High drug loading efficiency for broad spectrum of drugs with different properties.
- Physico-chemical compatibility with the drug.
- The carrier system should have an appreciable stability during storage [5].

ADVANTAGES OF ERYTHROCYTES AS DRUG CARRIERS

Advantages include

- The considerably uniform size and shape of the carrier
- Their biodegradability with no generation of toxic products.
- 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 kinetics.
- The lack of occurrence of undesired immune response against encapsulated drug.
- The large quantity of drug that can been capsulated within a small volume of cells ensures dose sufficiency.
- A longer life span in circulation as compared with other synthetic carrier 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 [3].

DRAWBACKS

The use of erythrocytes as carrier systems also presents some disadvantages which can be summarized as

1. The major problem encountered in the use of biodegradable materials or natural cells as drug carriers is that they are removed in vivo by the RES as result of modification that occurred during loading procedure in cells. This, although expands the capability to drug targeting to RES, seriously limits their life-span as long-circulating drug carriers in circulation and, in some cases, may pose toxicological problems.
2. The rapid leakage of certain encapsulated substances from the loaded erythrocytes.
3. Several molecules may alter the physiology of the erythrocyte.
4. Given that they are carriers of biological origin, encapsulated erythrocytes may present some inherent variations in their loading and characteristics compared to other carrier systems.
5. The storage of the loaded erythrocytes is a further problem provided that there are viable cells and need to survive in circulation for a long time upon re-entry to

the host body. Conditioning carrier cells in isotonic buffers containing all essential nutrients, as well as in low temperatures, the addition of nucleosides or chelators, lyophilization with glycerol or gel immobilization have all been exploited to overcome this problem.

6. Possible contamination due to the origin of the blood, the equipment used and the loading environment [4].

METHODS OF DRUG LOADING

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 .

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 shaper 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 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. 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 [3].

USE OF RED CELL LOADER

Magnaniet al developed a novel method for entrapment of non-diffusible 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 [3].

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 low entrapment efficiency and a considerable loss of hemoglobin and other cell component. 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, asparginase and arginase as well as bronchodilators such as salbutamol [3].

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 100–120µL 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 renewal there sealed 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 21phosphate, 1antitrypsin, methotrexate, insulin, metronidazole, levothyroxine, enalapril, and isoniazid [6].

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 semipermeable dialysis membrane maximizes the intracellular and 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 2h. 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 [5].

The drug to be loaded can be added by either dissolving the drug in isotonic cell suspending buffer inside a dialysis bagat 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 galactosidase, glucose rebrosidase, asparginase, inositol hexaphosphatase, well as drugs such as gentamicin, adriamycin, pentamidine and furamycin, interlukin-2, desferroxamine, and human recombinant erythropoietin [6].

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 [7].

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 mouseerythrocytes.Linetal used halothane for the same purpose. However, these methods induce irreversible destructive changes in the cell membrane and hence are not very popular [7].

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 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 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 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., tetra saccharide 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 0.2µm 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 [7].

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 2.5mM 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 37C 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 [7].

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 directly these cells to desired cells [4].

LOADING BY LIPID FUSION

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

In vitro characterization

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 [4,8].

IN VITRO CHARACTERIZATION

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. 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 visco elasticity of the cell membrane, viscosity of the cell contents, and the cellular surface-to-volume ratio.

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 [10].

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 haemolysis.

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, and enalaprilat, and enzymes such as asparginase peptides, including progesterone 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 [11].

IN VITRO STORAGE

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 4C. 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 [12].

IN VIVO LIFE SPAN

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 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 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 [13].

APPLICATIONS OF RESEALED ERYTHROCYTES

Slow drug release

Erythrocytes have been used as circulating depots for the sustained delivery of antineoplastics, antiparasitics, veterinary antiamoebic, 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.

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.

Targeting 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

- surface modification with antibodies
- surface modification with gluteraldehyde
- surface modification with carbohydrates such as sialic acid
- surface modification with sulphydryl
- surface chemical cross-linking e.g. delivery of 125I-labeled carbonic anhydrase loaded in erythrocytes cross-linked with *bis* (sulfosuccinimidyl) suberate and 3,3-dithio (sulfosuccinmidyl propionate) [14].

Targeting the liver Enzyme deficiency/replacement therapy

Many metabolic disorders related to deficient or missing enzymes can be treated by injecting these enzymes. However, the problems of exogenous enzyme therapy include a shorter circulation half-life of enzymes, allergic reactions, and toxic manifestations. These problems can be successfully overcome by administering the enzymes as resealed erythrocytes. The enzymes used include glucosidase, glucoronidase, galactosidase. The disease caused by an accumulation of glucocerebrosides in the liver and spleen can be treated by glucocerebrosidase-loaded erythrocytes.

Treatment of hepatic tumors

Hepatic tumors are one of the most prevalent types of cancer. Antineoplastic drugs such as methotrexate, bleomycin, asparginase, and adriamycin have been successfully delivered by erythrocytes. Agents such as daunorubicin diffuse rapidly from the cells upon loading and hence pose a problem. This problem can be overcome by covalently linking daunorubicin to the erythrocytic membrane using gluteraldehyde or cisaconitic acid as a spacer. The resealed erythrocytes loaded with carboplatin show localization in liver.

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.

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.

Removal of toxic agents

Cannon et al. reported inhibition of cyanide intoxication with murine carrier erythrocytes containing bovine rhodanase and sodium thiosulfate. Antagonization of organophosphorus intoxication by resealed erythrocytes containing a recombinant phosphodiestrase also has been reported [15].

Targeting organs other than those of RES

Recently, resealed erythrocytes have been used to target organs outside the RES.

The various approaches include

- Entrapment of paramagnetic particles along with the drug
- Entrapment of photosensitive material
- The use of ultrasound waves
- Antibody attachment to erythrocyte membrane to get specificity of action

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 either nucleotides or nucleoside analogs, their entrapment and exit through the membrane needs careful consideration.

Enzyme 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, organophosphorus),and as drugs [16].

Improvement in oxygen delivery to tissues

Hemoglobin is the protein responsible for the oxygen-carrying capacity of erythrocytes. Under normal conditions, % of hemoglobin is saturated with oxygen in the lungs, whereas under physiologic conditions in peripheral blood stream only _25% of oxygenated hemoglobin becomes deoxygenated. Thus, the major fraction of oxygen bound to hemoglobin is recirculated with venous blood to the lungs. The use of this bound fraction has been suggested for the treatment of oxygen deficiency. An application of IHP-loaded erythrocytes for improved oxygen supply is beneficial under the following conditions:

- High altitude conditions where the partial pressure of oxygen is low.
- Reduction in the number of alveoli, where exchange surface of the lungs is decreased.
- Increased resistance to oxygen diffusion in the lungs.
- Reduction in oxygen transport capacity.
- Mutation or chemical modification, which involves a decrease in oxygen affinity for hemoglobin.
- Increased radiosensitivity of radiation-sensitive tumors.
- Restoration of oxygen-delivery capacity of stored blood.
- Ischemia of myocardium, brain, or other tissues [17.

Microinjection of macromolecules

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). A relatively simple structure and a lack of complex cellular components (e.g., nucleus) in erythrocytes make them good candidates for the entrapment of macromolecules. In microinjection, erythrocytes are used as microsyringes for injection to the host cells [18].

NOVEL APPROACHES

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.

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 [19].

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 reseal 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 [4].

FUTURE PERSPECTIVES

- The concept of employing erythrocytes as drug or bioactive carrier still needs further optimization.
- A large amount of valuable work is needed so as to

utilize the potentials of erythrocytes in passive as well as active targeting of drugs.

- The resealed erythrocytes can be utilized for in humans as carriers for drugs.
- Scientists have demonstrated that such engineered red blood cells are sutable for blood transfusion.

Future studies would concentrate on the following:

1. Manipulation of autologous properties of erythrocytes, improved understanding of the biology of the red cells and its membrane development of pulsatile and feedback control system, selective drug delivery to CNS and delivery peptide and protein drugs.
2. Technical improvement in the procedure for preparing resealed erythrocytes , routes of administration, stability, crosslinking of resealed erythrocytes, aseptic and sterile processing, optimization techniques , pilot- plant scale up studies and innovative ideas for the application of resealed erythrocytes. Either as carriers or as cellular bioreactors would pave the way for automation and commercialization of this novel drug delivery system.
3. With the availability of technology to clone human DNA prokaryotes and the potential to produce large quantity of human enzymes, the possibility of enzyme replacement therapy targeting and the use of RBC carrier reservoirs should become more of realties.
4. In future greatest interest seems to be related to the targeting of immune-modulators on the phagocytic system anticancer drugs.
5. Drug loaded magnetite bearing cells serve as a promising carrier for delivering the drug to specific site [20].

Table 1. Parameter Method/instrument used for determination of characterization

Parameter	**Method/instrument used**
I. Physical characterization	
Shape and surface morphology	Transmission electron microscopy, scanning electron microscopy, phase contrast microscopy, optical microscopy.
Vesicle size and size distribution	Transmission electron microscopy, optical microscopy.
Drug release	Diffusion cell, dialysis
Drug content	Deproteinization of cell membrane followed by assay of resealed drug, radio labeling
Surface electrical potential	Zeta potential measurement
Surface pH	pH-sensitive probes
Deformability	Capillary method.
II. Cellular characterization	
% Hb content	Deproteinization of cell membrane followed by hemoglobin assay
Cell volume	Laser light scattering
% Cell recovery	Neubaur's chamber, hematological analyzer
Osmotic fragility	Stepwise incubation with isotonic to hypotonic saline solutions and determination of drug and hemoglobin assay
Osmotic shock	Dilution with distilled water and estimation of drug and hemoglobin
Turbulent shock	Passage of cell suspension through 30- gauge hypodermic needle at 10 mL/min flow rate and estimation of residual drug and hemoglobin, vigorous shaking followed by hemoglobin estimation
III. Biological characterization sterility Pyrogenicity Animal toxicity	 Sterility test Rabbit method, LAL test, toxicity tests [9]

Fig 1. Morphology of Erythrocytes

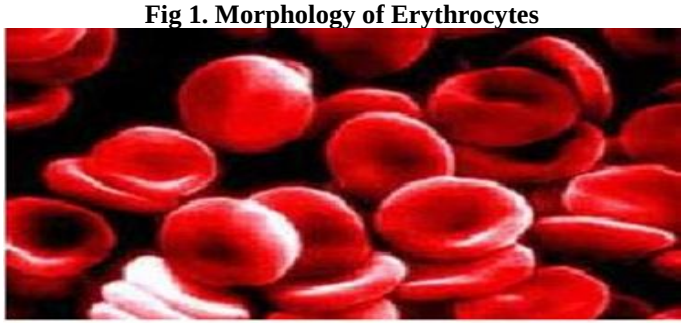


Fig 2. Hypotonic Dialysis

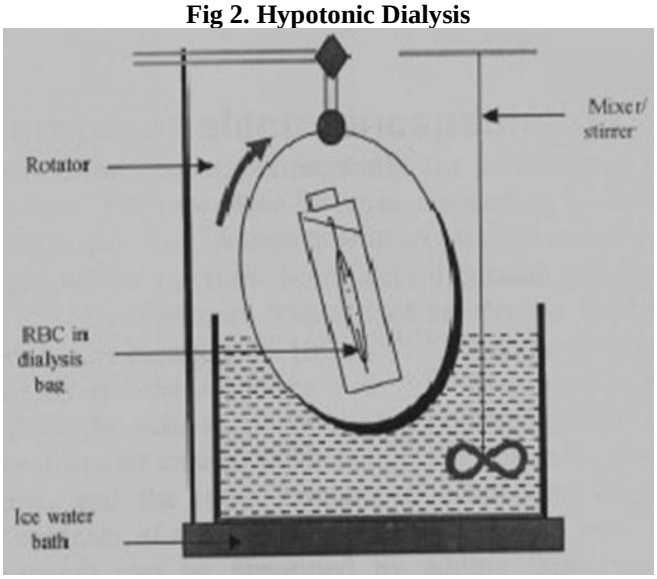
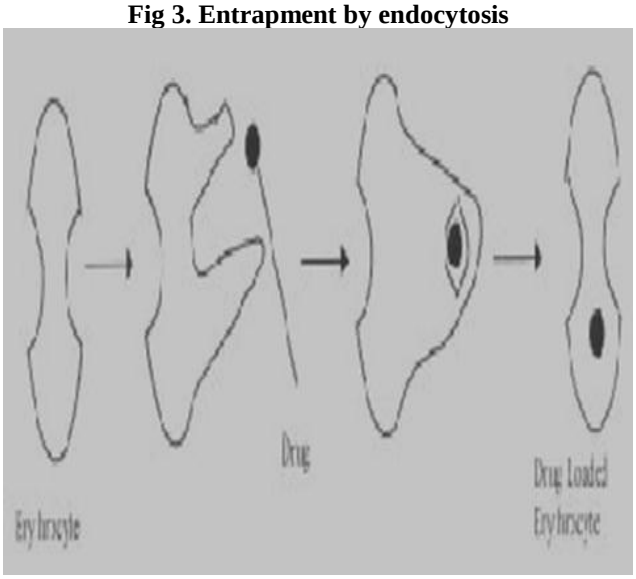


Fig 3. Entrapment by endocytosis



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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