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Review Article

Future Prospectus in Targetting Drug Delivery: A Review on Resealed Erytrocytes - A Promising Carrier

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Abstract: In this review we focus on the various features, drug loading technology and biomedical applications of resealed erythrocytes. Among the various carriers used for targeting drug delivery, erythrocytes constitute potential biocompatible carriers since they possess several properties which make them unique and useful carriers. They are biocompatible, biodegradable, possess long half lives, follow zero order release kinetics and can be loaded with a variety of biologically active compounds using various physical and chemical methods. Drug-loaded carrier erythrocytes are prepared by collecting blood sample from the organism of interest and separating erythrocytes from plasma. By using various methods (membrane perturbation, Electro-insertion, entrapment by endocytosis, Hypo-osmotic lysis) the cells are broken and the drug is entrapped into the erythrocytes, finally they are resealed and the resultant carriers are then called "resealed erythrocytes".
Keywords: Drug delivery system, Resealed erythrocytes, Carrier drug targeting, nano-RBCs

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. Various carriers has been used for the drug targeting among which cellular carrier offer a greater potential advantages related to its biodegradability, non-pathogenicity, non-immunogenicity, biocompatibility, self degradability along with high drug loading efficiency[1]. Leukocytes, platelets and erythrocytes have been proposed as cellular carrier systems [2].

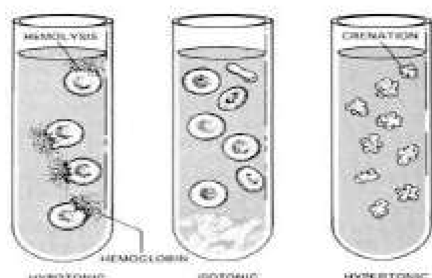

Fig.1: Effect of tonicity on erythrocytes

ERYTHROCYTES

Erythrocytes are natural products of the body, biodegradable in nature, isolation of these is easy and large amount of drug can proteins and enzymes, act as a carrier for number of drugs, target the drugs within the reticuloendothelial system (RES) as well non RES organs/sites. Potential clinical indications for "RES targeting" include iron over-storage diseases, parasitic diseases, hepatic tumors, cancer and lysosomal storage diseases carriers [3].

Fig.2: Morphology of Erythrocytes

Morphology and Physiology of Erythrocytes

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

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 [4]. 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 as long as 48 hours before use [5-6].

Advantages of erythrocytes as drug carriers

- Their biocompatibility, particularly when autologous cells are used, hence no possibility of triggered immune response
- Their biodegradability with no generation of toxic products
- The considerably uniform size and shape of the carrier
- Relatively inert intracellular environment
- Prevention of degradation of the loaded drug from inactivation by endogenous chemicals
- The wide variety of chemicals that can be entrapped,
- The modification of pharmacokinetic and pharmacodynamic parameters of drug
- Attainment of steady-state plasma concentration decreases fluctuations in concentration
-

Disadvantages [7-11]

- 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.
- The rapid leakage of certain encapsulated substances from the loaded erythrocytes.
- Several molecules may alter the physiology of the erythrocyte.

ERYTHROCYTES CAN BE USED AS CARRIERS IN TWO WAYS [12]

- 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.
- 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.

Methods of Drug Loading [13]

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).

a. Hypotonic hemolytic [14]

This method is based on the ability of erythrocytes to undergo reversible swelling in a hypotonic solution. 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 volume gain is ~25–50%. The cells can maintain their integrity up to a tonicity of ~150 m osm/kg, above which the membrane ruptures, releasing the cellular contents. At this point 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.

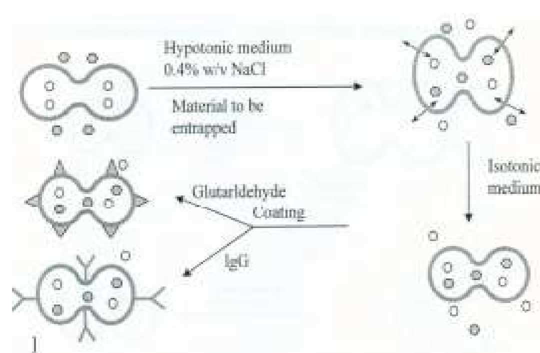

Fig.3:- Hypotonic haemolytic

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b. Use of red cell loader [15]

Red cell loader is a novel method for entrapment of non-diffusible drugs into erythrocytes, with as little as 50 ml of a blood sample; different biologically active compounds were entrapped into erythrocytes within a period of 2 hours at room temperature. The process is based on two sequential hypotonic dilutions of washed erythrocytes followed by concentration with a hemo filter and an isotonic resealing of the cells. There was ~30% drug loading with 35–50% cell recovery.

c. Hypotonic dilution [16]

Hypotonic dilution was the first method investigated for the encapsulation of chemicals into erythrocytes and is the simplest and fastest. In this method, a volume of packed erythrocytes is diluted with 2–20 volumes of aqueous solution of a drug.

The solution tonicity is then restored by adding a hypertonic buffer. The resultant mixture is then centrifuged, the supernatant is discarded, and the pellet is washed with isotonic buffer solution. The major drawbacks of this method include low entrapment efficiency and a considerable loss of hemoglobin and other cell components.

d. Hypotonic pre-swelling [17-18]

The technique is based upon initial controlled swelling in a hypotonic buffered solution. This mixture is centrifuged at low values. The supernatant is discarded and the cell fraction is brought to the lysis point by an aqueous solution of the drug to be encapsulated. Adding 100–120. 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.

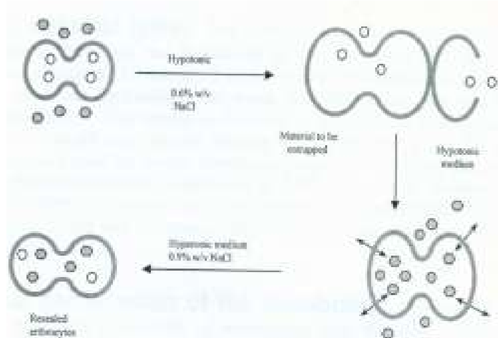

Fig.4: Hypotonic presswelling

e. Isotonic osmotic lysis [19]

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 isotonic. 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.

f. Hypotonic dialysis [20]

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.

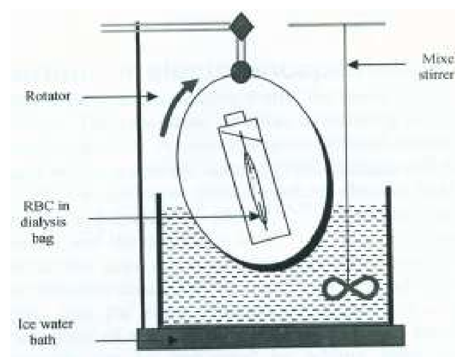

Fig.5: Hypotonic dialysis

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 that the permeability of erythrocyte membrane increases upon exposure to polyene antibiotic such as amphotericin B.

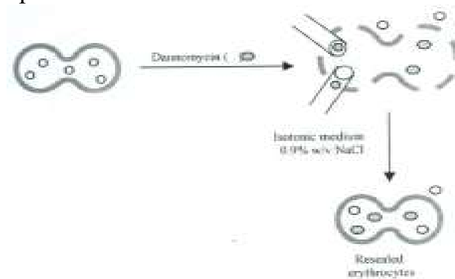

Fig.6: Chemical perturbation of the membrane

h. Electro-insertion or electro encapsulation [21]

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. The erythrocyte membrane is opened by a dielectric break down. Subsequently, the pores can be resealed by incubation at 37°C in an isotonic medium.

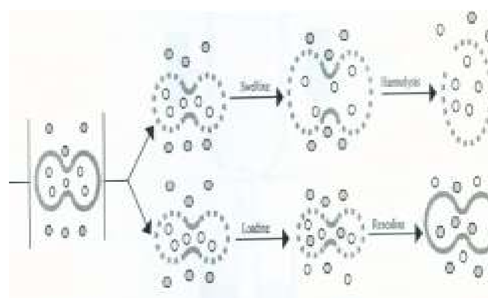

Fig.7: Electro-insertion or electro encapsulation

i. Entrapment by endocytosis [22]

Endocytosis involves the addition of one volume of washed 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°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.

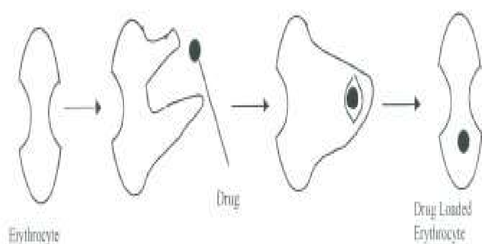

Fig.8: Entrapment by endocytosis

j. Loading by electric cell fusion [23]

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.

k. Loading by lipid fusion [24]

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%).

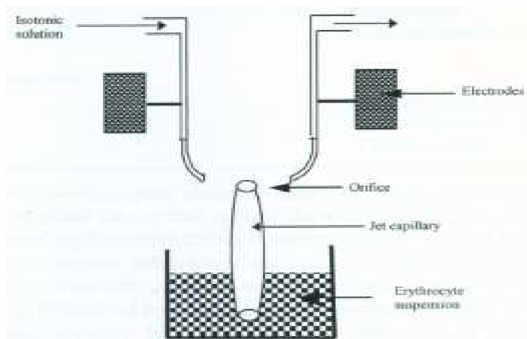

Fig.9: Loading by lipid fusion

CHARACTERIZATION OF RESEALED ERYTHROCYTES [25-30]

a. Drug content determination

Packed loaded cells are deprotenized with acetonitrile after centrifugation at 3000 rpm for a fixed time interval. The clear supernatant liquid is assayed for drug content.

b. In-vitro drug release and Hb content

The cell suspensions are stored at 4°C in ambered colored glass container. Periodically clear supernatant are drawn using a hypodermic syringe equipped with 0.45 are filter, deproteined using methanol and were estimated for drug content. The supernatant of each sample after centrifugation collected and assayed.

c. Percentage cell recovery

May be determined by counting the no. of intact cells per cubic mm of packed erythrocytes before and after loading the drug.

d. Morphology

Phase contrast or electron microscope may be used for normal and erythrocytes.

e. Osmotic shock /Osmotic fragility

For the study of osmotic shock loaded erythrocytes are incubated with saline solutions of different tonicities (0.9%w/v to 0.1w/v) at 37°C for 10 min, centrifuged and assayed for drug content and Hb content. For osmotic fragility a sudden exposure of drug loaded erythrocytes to an environment which is far from isotonic to evaluate the ability of resealed erythrocytes to withstand the stress and maintain their integrity as well as appearance.

f. Turbulence shock

It is the measure of simulating distribution of loaded cells during injection. In this drug loaded cells are passed through a 23 gauge hypodermic at a flow rate of 10 ml/min which is comparable to the flow rate of blood. It is followed by collecting of an aliquot and centrifugation sample is estimated. Drug loaded erythrocytes appears to be less resistant to turbulence, probably indicating destruction of cells upon shaking.

g. Determination of entrapped magnetite

Atomic absorption spectroscopic method is reported for determination of the concentration of a particular metal element in a sample. The HCl is added to a fixed amount of magnetite bearing erythrocytes and content are heated at 60°C for 2 hours.then 20%w/v trichloro acetic acid is added and supernatant obtained after centrifugation is used to determine magnetite concentration using atomic absorption spectroscopy.

h. **Erythrocyte sedimentation rate (ESR)**

It is an estimate of the suspension stability of RBC in plasma and is related to the number and size of the red cells and to relative concentration of plasma protein, especially fibrinogen and α, β globulins. This test is performed by determining the rate of sedimentation of blood cells in a standard tube. Normal blood ESR is 0 to 15 mm/hr, higher rate is indication of active but obscure disease processes.

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 4^0C. 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.

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. 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.

APPLICATIONS OF RESEALED ERYTHROCYTES

Resealed erythrocytes have several possible applications in various fields of human and veterinary medicine.

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.

Drug Targeting

Ideally, drug delivery should be site-specific and target-oriented to exhibit maximal therapeutic index with minimum adverse effects.

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
-

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.

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.

Treatment of Parasitic diseases

The ability of resealed erythrocytes to selectively accumulate within RES organs make them useful tool during the delivery of antiparasitics agents. Parasitic diseases that involve harboring parasites in the RES organs can be successfully controlled by this method.

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 [31]

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.

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 nucleotides or nucleoside analogs, their entrapment and exit through the membrane needs careful consideration. Nucleosides are rapidly transported across the membrane whereas nucleotides are not, and thus exhibiting prolonged release profiles. The release of nucleotides requires conversion of these moieties to purine or pyrimidine bases. Resealed erythrocytes have been used to deliver deoxycytidine derivatives, recombinant herpes simplex virus type 1 (HSV-1) glycoprotein B , azidothymidine derivatives, azathioprene.

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.

Improvement in oxygen delivery to tissues

Hemoglobin is the protein responsible for the oxygen-carrying capacity of erythrocytes. Under normal conditions, 95% of hemoglobin is saturated with oxygen in the lungs, whereas under physiologic conditions in peripheral blood stream only 25% of oxygenated hemoglobin becomes deoxygenated. Thus, the major fraction of oxygen bound to hemoglobin is recirculated with venous blood to the lungs. The use of this bound fraction has been suggested for the treatment of oxygen deficiency.

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.

ERYTHROCYTES CAN ALSO BE USED IN CANCER

Cancer is a term for used diseases in which abnormal cells divide without control and are able to invade other tissues.

Types of Cancer Classified by Body System [32]

- Blood cancer
- Bone cancer
- Brain cancer
- Breast cancer
- Gastrointestinal cancers
- Endocrine cancers
- Eye cancer
- Gynecologic cancers
- Genitourinary cancer
- Head and Neck cancer
- Respiratory cancer
- Skin cancer

NOVEL APPROACHES

Erythrosomes [33-35]

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 [36-37]

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.

FUTURE PERSPECTIVES

The concept of employing erythrocytes as drug or bio active carrier still needs further optimization a large amount of valuable work is needed so us to utilize the potential of erythrocyte in passive and as well as active targeting of drugs. Disease like cancer would surely find it cure. Genetic engineering aspects can be coupled to give a newer dimension to the existing cellular drug concept.

CONCLUSION

During the past decade, numerous applications have been proposed for the use of resealed erythrocytes as carrier for drugs, enzyme replacement therapy etc. 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. For the present, it is concluded that erythrocyte carriers are "golden eggs in novel drug delivery systems" considering their tremendous potential. Most of the studies in this area are in the in vitro phase and the ongoing projects worldwide remain to step into preclinical and then, clinical studies to prove the capabilities of this promising delivery system. It is also

very effective and safe delivery system for anti cancer drug with less or without toxicity.

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