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RESEALED ERYTHROCYTES: AN OVERVIEW

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ABSTRACT

Among the various carriers used for targeting drugs to various body tissues, the cellular carriers meet several criteria desirable in clinical applications, among the most important being biocompatibility of carrier and its degradation products. Leucocytes, platelets, erythrocytes, nanoerythrocytes, hepatocytes, and fibroblasts etc. have been proposed as cellular carrier systems. Among these, the erythrocytes have been the most investigated and have found to possess greater potential in drug delivery. Resealed Erythrocytes are biocompatible, biodegradable, possess long circulation half-life and can be loaded with variety of active substances. Carrier erythrocytes are prepared by collecting blood sample from the organism of interest and separating erythrocytes from plasma. By using various methods 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". So many drugs like aspirin, steroid, cancer drug which having many side effects are reduce by resealed erythrocyte.

INTRODUCTION

To achieve a required the rapeutic concentration the drug has to be administered in large quantities, the major part of which is just wasted in normal tissues. Ideally, a "perfect" drug should exert its pharmacological activity only at the target site, using the lowest concentration possible and without negative effects on non-target compartments. The delivery systems currently available enlistcarriers that are either simple, soluble macromolecules (such as monoclonal antibodies, soluble synthetic polymers, polysaccharides and particulate biodegradable polymers) or more complex multicomponent structures (microcapsules, Microparticles, cells, cell ghosts, lipoproteins, liposomes, erythrocytes).[1]

Erythrocytes

Red blood cells (also referred to as erythrocytes) are the most common type of blood cells and the vertebrate organism's principal means of delivering oxygen (O_2) to the body tissues via the blood flow through the circulatory system. The cells develop in the bone marrow and circulate for about 100–120 days in the body before their components are recycled by macrophages. Each circulationtakes about 20 seconds. Approximately a quarter of the cells in the human body are red blood cells.[2]

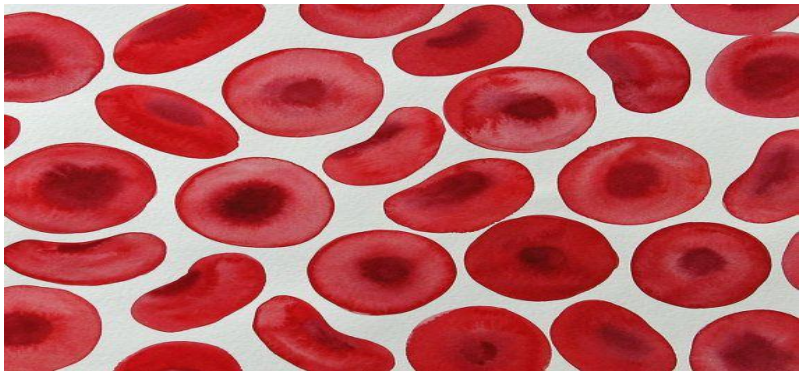


Fig 1: Erythrocytes

Composition of Erythrocytes

Blood contains 55% of plasma and other 45% made of corpuscles. Erythrocytes have diameter ranging from 6-9 μ and the thickness is nearly 1-2 μ. Erythrocytes have a solid content of about 35% most of which is Hb and rest 65% being water. Lipidcontent of erythrocytes includes cholesterol, lecithin and cephalexins. The concentration of K+ is more in erythrocytes and Na+ in plasma. The osmotic pressure of the interior of the erythrocytes is equal to that of the plasma and termed as isotonic (0.9% NaCl or normal physiological saline) Changes in the osmotic pressure of the medium surrounding the red blood cells changes the morphology of the cells. If blood is

placedinto a tube and centrifuged, the cells and the plasma will separate. The erythrocytes, which are heavy, will settle down to the bottom of the tube, while the plasma rises up to the top and the leukocytes and platelets will form a thin layer (buffy coat) between the erythrocytes and the plasma. The hematocrit[3] is defined as the percentage of whole blood made up of erythrocytes

Resealed Erythrocytes

Such drug-loaded carrier erythrocytes are prepared simply by collecting blood samples from the organism of interest, separating erythrocytes from plasma, entrapping drug in the erythrocytes, and resealing the resultant.[4]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 reticuloendothelial system (RES).[5]

Properties of resealed erythrocyte of novel drug delivery carriers[6, 7, 8, 9, 10]

1. The drug should be released at target site in a controlled manner.
2. It should be appropriate size, shape and should permit the passage through capillaries and minimum leakage of drug should take place.
3. It should be biocompatible and should have minimum toxic effect.
4. It should possess the ability to carry a broad spectrum of drug.
5. It should possess specific physicochemical properties by which desired target size could be recognized.
6. The degradation product of the carriers system release of the drug at the selected site should be biocompatible. It should be physicochemical compatible with drug.
7. The carrier system should have an appreciable stability during storage.

Advantages[11, 12, 13]

1. They are natural part of body, so they are biodegradable in nature.
2. The entrapment of drug does not require the chemical modification of drugs
3. The entrapment of drug also does not require the chemical modification of the substance to be entrapped.
4. They are non-immunogenic in action and can be targeted to disease tissue/organ.
5. They prolong the systemic activity of drug.
6. Isolation of erythrocyte is easy and larger amount of drug can be encapsulated in small volume of cells
7. They facilitate incorporation of protein and nucleic acid in eukaryotic cells by cell infusion with RBC.

8. Entrapment of drug can be possible without chemical modification of the substance to be entrapped.
9. Possible to maintain steady-state plasma concentration, decrease fluctuation in concentration.
10. Protection of the organism against toxic effect of drug.
11. Targeting to the organ of the RES.
12. Ideal zero-order drug release kinetic.
13. Prolong the systemic activity of drug byresiding for a longer time in the body.

Disadvantages[14]

1. They have a limited potential as carrier tonon-phagocyte target tissue.
2. Possibility of clumping of cells and dose dumping may be there.

Erythrocytes can be used as carriers in two ways[15]

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.

ISOLATION OF ERYTHROCYTES: [16]

- Blood is collected into heparin zed tubes by venipuncture.
- Blood is withdrawn from cardiac/splenic puncture (in small animal) and through veins (in large animals) in a syringe containing a drop of anti-coagulant.
- The whole blood is centrifuged at 2500 rpm for 5 min. at 4 ±1 0 C in a refrigerated centrifuge.
- The serum and Buffycoats 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 40C 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.

Table 1. Various condition and centrifugal force used for isolation of erythrocytes[17]

Sr.no.	Species	Washing Buffer	Centrifugal force(g)
1	Rabbit	10mmol KH_2PO_4/$NAHPO_4$	500-1000
2	Dog	15mmol KH_2PO_4/$NAHPO_4$	500-1000
3	Human	154mmol NACL	<500
4	Mouse	10mmol KH_2PO_4/$NAHPO_4$	100-500
5	Cow	10-15mmol KH_2PO_4/$NAHPO_4$	1000
6	Horse	2mmol $MGCL_2$ /10mmol Glucose	1000
7	Sheep	10mmol KH_2PO_4/$NAHPO_4$	500-100

Methods of Drug Loading in Resealed Erythrocytes:

Several methods can be used to load drugs or other bioactive compounds in erythrocytes, including physical (e.g., electrical pulse method) osmosis-based systems, and chemical methods (e.g., chemical perturbation of the erythrocytes membrane). Irrespective of the method used, the optimal characteristics for the successful entrapment of the compound requires the drug to have a considerable degree of water solubility, resistance against degradation within erythrocytes, lack of physical or chemical interaction with erythrocyte membrane, and well-defined pharmacokinetic and pharmacodynamics properties 30.

The several methods are giving in follows:-

- Hypotonic hemolysis method
- Use of red cell loader method
- Hypotonic dilution method
- Hypotonic preswelling
- Isotonic osmotic lysis
- Chemical perturbation of membrane
- Electro- insertion or electro encapsulation
- Entrapment by endocytosis

Hypotonic Hemolysis[18, 19]

This method is based on the ability of erythrocytes to undergo reversible swelling in a hypotonic solution. Erythrocytes have an exceptional capability for reversible shape changes with or without accompanying volume change and for reversible deformation under stress. An increase in volume leads to an initial change in the shape from biconcave to spherical. This change is attributable to the absence of superfluous membrane; hence, the surface area of the cell is fixed.

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

Use of Red Cell Loader[20, 21, 22]

This is a novel method and it was developed by Magnani and coworkers in 1998 for the entrapment of non-diffusible drugs in human erythrocytes.They developed a piece of equipment called a "red cell loader" 33.The method requires as little as 50ml of blood. 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 cells.

Hypotonic dilution or Dilutional method[23, 24]

Hypotonic dilution was the first method investigated for the encapsulation of chemicals into erythrocytes 14 and is the simplest and fastest 34.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 methodinclude a low entrapment efficiency and a considerable loss of hemoglobin and other cell components. This reduces the circulation half-life of the loaded cells. These cells are readily phagocytosed by RES macrophages and hence can be used for targeting RES organs.

Hypotonic dilution is used for loading enzymes such as Bgalactosidase and B-glucosidase, asparginase, and arginase, as well as bronchodilators such as salbutamol.

Hypotonic preswelling[25-26]

This method was developed in 1975 by Rechsteiner 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. The supernatant is discarded and the cell fraction is brought to the lysis point by adding 100-120 liters portions of an aqueous solution ofthe 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.Drugs encapsulated in erythrocytes using this method include propranolol, asparginase, cyclopohphamide, cortisol-21-phosphate, w1- antitrypsin, methotrexate, insulin, metronidazole, levothyroxine, enalaprilate and isoniazid.

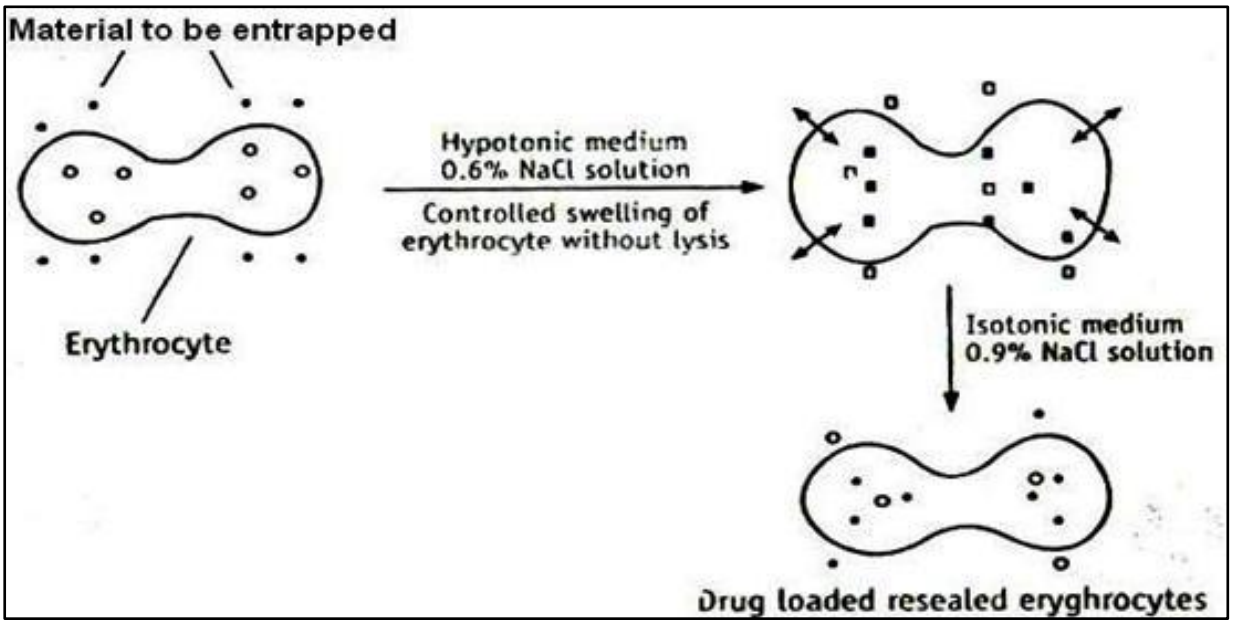


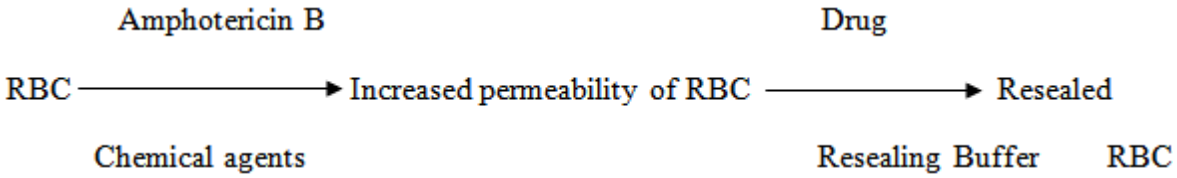
Fig. 3: Drug Loaded In Erythrocyte by Hypotonic Preswelling

Isotonic osmotic lysis[27]

This method is 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. However, this method also is not immune to changes in membrane structure composition.

Chemical perturbation of the membrane[28, 29]

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



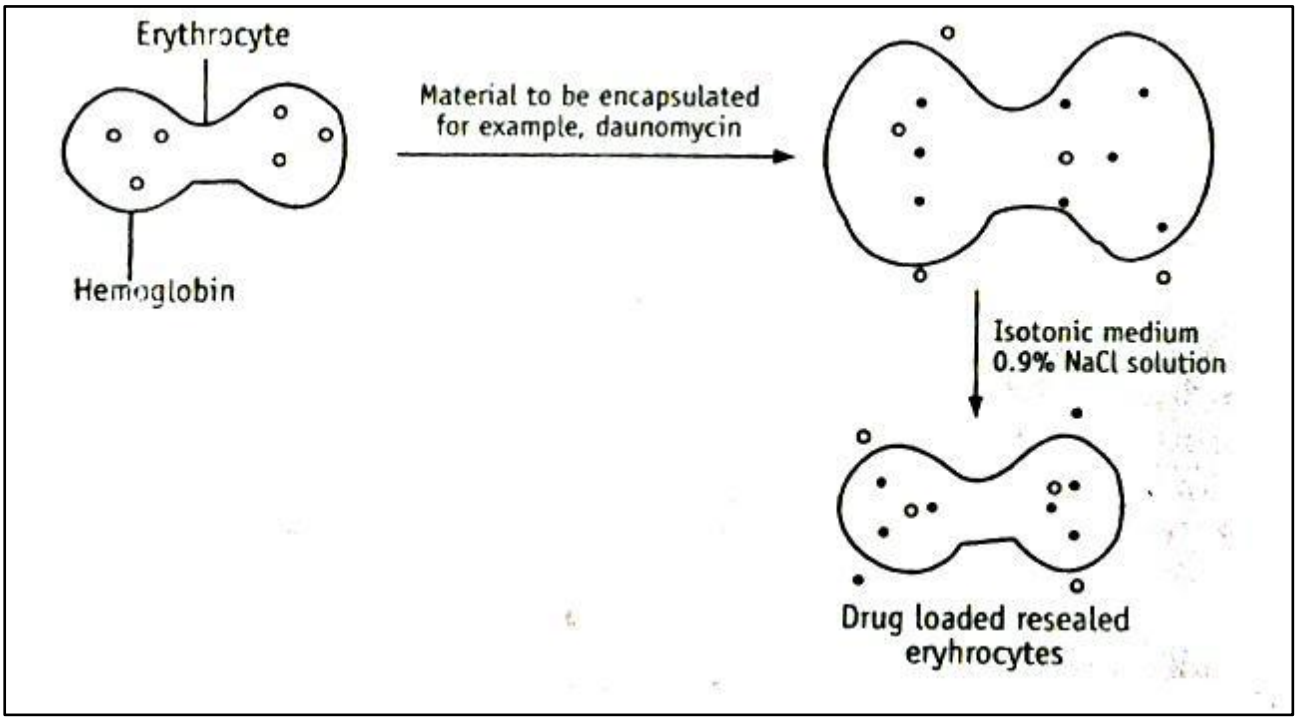


Fig. 4: Resealing of RBC by Chemical Perturbation of the Membrane Method

Electro-insertion or electroencapsulation[30, 31]

In 1973, Zimmermann tried an electrical pulse method to encapsulate bioactive molecules. This method is also known as electroporation, the method consist of reating electrically induced permeability changes at high membrane potential differences.In 1977, Tsong and Kinosita suggested the useof transient electrolysis to generate desirable membrane permeability for drug loading. Electrical breakdown is achieved by membrane polarization for microseconds using varied voltage of2kv/cm is applied for 20 µsec. Once membrane is perforated, regardless of the size of pores, ionsrapidly distribute between the extra and intracellular space to attain Donnan equilibrium, however the membrane still remains impermeable to its cytoplasmic macromolecules. In red blood cells,the colloidal osmotic pressure of hemoglobin is about 30 mOsm. This pressure drives water and ion influx, as a result swelling of the cells occurs. The membrane is ruptured when the cell volumereaches 155% of its original volume. Since the cell lysis is due to colloidal osmotic swelling, the rational to prevent lysis is to balance the colloidal osmotic pressure of cellular macromolecules. This can be affected by addition of large molecules

(like tetrasaccharidemmstachyose or protein such as bovine serum albumin) and ribonucleases. This helps to counteract the colloidal osmoticswelling of electrically perforated erythrocytes. Under this osmotically balanced condition pores stay open at 4oC for few days. If drug molecules are added at this point, they permeate into red blood cells. 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.

Entrapment by endocytosis[32,33]

Endocytosis involves the addition of one volume of washed packed erythrocytes to nine volumes of buffer containing 2.5 mM ATP, 2. 5 mM MgCl2, and 1mM CaCl2, followed by incubation for 2 min at room temperature. The pores created by this method are resealed by using 154 mm of NaCl and incubation at 37oC for 2 min. The entrapment of material occurs by endocytosis.This method was discovered by Schrier et al. in 1975.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.

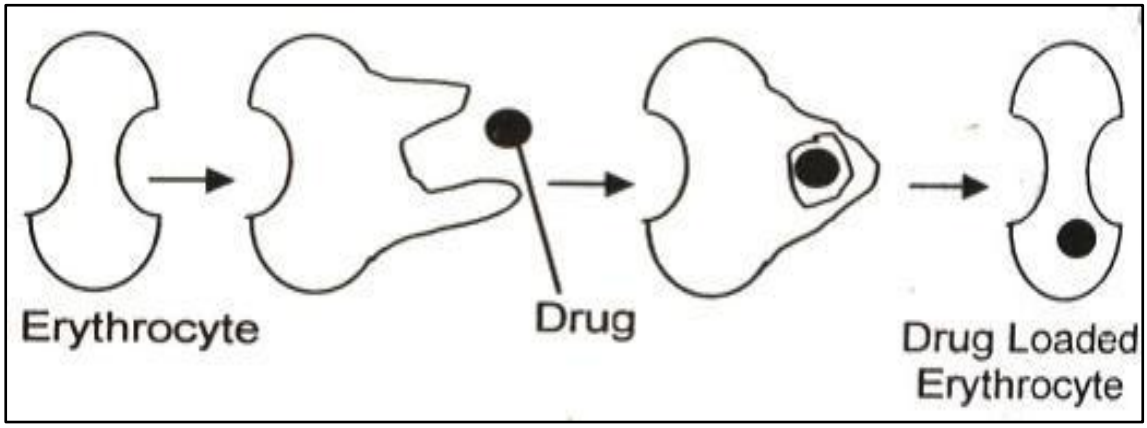


Fig. 5: Drug Entrapment by Endocytosis Method

Characterization of Resealed Erythrocytes[34, 35, 36]

Drug content quantification

To determine the drug content, packed loaded cells are deproteinized with acetronitrile after centrifugation at 3000 rpm for a fixed time interval. The clear supernatant liquid is analysed spectrophotometrically.

In-vitro drug release and haemoglobin content study

In-vitro release of drug(s) and haemoglobin are monitored periodically form drug loaded cells. The cells suspension (5% hematocrit in PBS) is stored at 40C in amber coloured glass containers.

Periodically the clear supernatant are withdrawn using a hypodermic syringes equipped with 0. 45 m filter, deproteinized using methanol and were estimated for drug content. The supernatant of each sample after centrifugation is collected and assayed, % haemoglobin release may be calculated using the formula.

$$\% \text{ hemoglobin release} = \frac{A_{540} \text{ of sample} - A_{540} \text{ of background}}{A_{540} \text{ of 100\% hemoglobin}}$$

Or

$$\text{Mean corpuscular hemoglobin \{MCH (pg)\}} = \frac{\text{Hemoglobin (g/100ml) x 10}}{\text{Erythrocyte count (millions/cu mm)}}$$

Erythrocyte count (millions/cu mm)

Where a A540 refers to absorbance at 540nm.

Percent cell recovery and Morphological study

Percent cell recovery may be determined by counting the no. of intact cells per cubic mm of packed erythrocytes before and after loading the drug. Phase contrast or electron microscope may be used for normal and drug loaded erythrocytes.

Fragility and Osmotic shock study

To study the effect of different tonicities, drug loaded erythrocytes are incubated separately in normal saline solution at 37 ± 2oC for 10 minutes, followed by centrifugation at 2000 rpm for 10 min. For osmotic shock study, dispersing the resealed erythrocyte suspension in distilled water and centrifuged at 300 rpm for 15 min. The supernatant was estimated for percent hemoglobin release spectrophotometrically.

Turbulence shock study

It is the measure of simulating destruction of loaded cells during injection. Normal and drug loaded cells are passed through a 23 gauge hypodermic needle at a flow rate of 10 ml/min which is comparable to the flow rate of blood. It is followed by collection of an aliquot and centrifugation at 2000 rpm for 10 minutes. The hemoglobin in withdrawn sample is estimated. Drug loaded erythrocytes appear to be less resistant to turbulence, probably indicating destruction of cells upon shaking.

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. Test is performed by determining 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 disease processes.

Self-life and Stability and Cross linking of Released Erythrocytes

Glutaraldehyde (0. 2%) treated erythrocytes in a sintered glass funnel (G-4) by filtration and dried in vacuum (200mm Hg) for 10 hr. alternatively the erythrocyte suspension was filled into vials andlyophilized at- 400C to 0. 01 torr using a laboratory lyophilizer. The dried powder was filled in amber colour glass vials and stored at 40C for month. Improvement in shelf life of the carrier erythrocytes was achieved by storing the cells in powder from, ready for reconstitution at 40C.

Mechanism of Drug Release from Resealed Erythrocyte

There are mainly three ways for a drug to efflux out from erythrocyte carriers.

1. Phagocytosis
2. Diffusion through the membrane of the cell and
3. Using a specific transport system.

RBCs are normally removed from circulation by the process of phagocytosis. The degree of cross-linking determines whether liver or spleen will preferentially remove the cells Carrier erythrocytesfollowing heat treatment or antibody cross-linking are quickly removed from the circulation by phagocytic cells located mainly in liver and spleen. The rate of diffusion depends upon the rate atwhich a particular molecule penetrates through a lipid bilayer. It is greatest for a molecule with high lipid solubility. Many substances enter cells by a specific membrane protein system because thecarriers are proteins with many properties analogous to that of enzymes, including specificity.

Route of administration[37]

Intra peritoneal injection reported that survival of cells in circulation was equivalent to the cells administered by IV injection. They reported that25% of resealed cell remained in circulation for 14 days they also proposed this method of injection as a method for extra vascular targeting of RBCs to peritoneal macrophages. Subcutaneous route for slow release of entrappedagents. They reported that the loaded cell released encapsulated molecules at the injection site.

Application[38, 39, 40]

(1) In-Vitro Application:

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 biochemical defects such as the glucose- 6- phosphate dehydrogenase (G6PD) deficiency can be useful tool for discerning the mechanism that eventually causes theseeffects. The most frequent in vitro application of RBC is that of

microinjection. A protein or nucleic acid was injected into eukaryotic cells by fusion process. Similarly, when antibody molecules areintroduced using erythrocytic carrier system, they immediately diffuse throughout the cytoplasm. Antibody RBC auto-injected into living cells has been used to confirm the site of action of fragment of diphtheria toxin. Antibodies introduced using RBC mediated microinjection is recorded not to enter the nucleus, thus limiting the studies to the cytoplasmic level.

(2) In – Vivo Application

i) Targeting of bioactive agents to RE System

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, gluteraldehyde, carbohydrates such as sialic acid and sulphydryl.

ii) Targeting to sites as other than RES Organs

Resealed erythrocytes have the ability to deliver a drug or enzyme to the macrophage-rich organs. Organ targeting other than RES have been tried recently with resealed erythrocytes. Some of the representative approaches are discussed in brief.

iii) Erythrocytes as Circulating Bioreactors

Erythrocytes have been realized as carriers for enzymes to serve As circulating bioreactors. Sometimes it is desirable to decrease the level of circulating metabolites that can enter erythrocytes.

Erythrocytes have also been used as circulating bioreactors for the controlled delivery of antiviral drugs.

iv)Erythrocytes as Carriers for Drugs

Various bioactive agents encapsulated in erythrocytes are developed for the slow and sustained release in circulation to allow effective treatment of parasitic disease. Resealed erythrocytes serve as an ideal carrier for antineoplastic agents, antimicrobial drug and vitamins and steroids.

v) Erythrocytes as Carriers for Enzymes

Enzymes can be injected into the blood stream to replace a missing or deficient enzyme in metabolic disorders or to degrade toxic compounds accumulated in the blood due to a disease

Likewise, environmental, lysosomal storage disorders such as Gaucher's disease, hyperarginiaemia, hyperuricaemia, hyperphenyl- alaninaemia and kidney failure are only few examples of metabolic disorders that can be treated by administration of enzymes.

Recent developments

Nanoerythrosomes

Nanoerythrosomes are vesicles prepared by the extrusion of RBC ghosts, the average diameter of these vesicles being 100nm. The process gave small vesicles with the size of a liposomes. Thesespheroid particles were named 'nanoerythrosomes' and appear to be stable and maintain both the cytotoxic and antineoplastic activity of daunorubicin against mice leukaemia P338D cells.. Significant advances have been made with the use of erythrocyte for specific targeting to cells of the immune system antiviral drugs can be pretreated to deliver drug directly to macrophages.

Several laboratory techniques have developed for the encapsulation of allosteric effector of haemoglobin, inositol hexaphosphate, which are effective at oxygen delivery, much more effective than normal erythrocytes.

CONCLUSION

The use of resealed erythrocytes looks promising for a safe and effective 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. It is very effective and safe delivery system for anti-cancer drug with or without less toxicity.For the present, it is concluded that erythrocyte carriers are most effective in novel drug delivery systems considering their tremendous potential. The future studies would concentrate on manipulation of the autologous properties of erythrocytes, improved understanding biology of the red cells and its membrane, development of pulsatile and feedback control system, selective drug delivery to CNS and delivery of peptide and protein drugs. Main suggestion for future study is that by carrier through we can transplant steroids and hormones to the targeting site. So we can decrease many side effects. In these field no one can deep think but by resealed erythrocyte we can improvise drug targeting area and reduces so many side effect.

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