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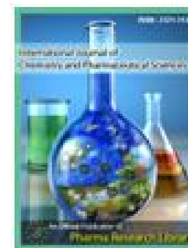

Review Article Open Access

Resealed Erythrocytes- A Review

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A B S T R A C T

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, nano erythrocytes, 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. Biopharmaceuticals, therapeutically significant peptides and proteins, nucleic acid-based biological, antigens, anticancer drug and vaccines, are among the recently focused pharmaceuticals for being delivered using carrier erythrocytes**.** Erythrocytes, also known as red blood cells, and have been extensively studied for their potential carrier capabilities for the delivery of drugs. The biocompatibility, nonpathogenicity, non-immunogenicity and biodegradability make them unique and useful carriers. 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. Current review highlights isolation, drug loading methods, Evaluation methods and applications of resealed erythrocytes for drug delivery

Keywords: Red blood cells, Heamoglobin, Resealed erythrocytes, Heamolysis

A R T I C L E I N F O

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1. Introduction

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. [1]

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 reticulo endothelial system (RES) as well non RES organs/sites. They have the capacity to carry large amounts of drug; and can behave as a slow-release long acting system. Potential clinical indications for "RES targeting" include iron over-storage diseases, parasitic diseases, hepatic tumors, cancer and lysosomal storage diseases carriers. Erythrocytes live only about 120 days because of wear and tear on their plasma membranes as they squeeze through the narrow blood capillaries. 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**. In this review, the potential applications of erythrocytes in drug delivery have been highlighted. [2]

Advantages of Resealed Erythrocytes [3, 4]:

a. They are the natural product of the body, which are biodegradable in nature. Isolation of erythrocytes is easy and larger amount of drug can be encapsulated in a small volume of cells.
b. The entrapment of drug does not require the chemical modification of the substance to be entrapped. This is in contrast with other systems which involve covalent coupling of the drug and carrier which may affect the inherent biological activity of the parent drug.
c. They are non-immunogenic in action and can be targeted to disease tissue/organ.
d. They prolong the systemic activity of drug while residing for a longer time in the body.
e. They protect the premature degradation, inactivation and excretion of proteins and enzyme and act as a carrier for number of drugs.
f. They can target the drug within reticuloendothelial system (RES).
g. They facilitate incorporation of proteins and nucleic acid in eukaryotic cells by cell infusion with RBC.

Disadvantages of Resealed Erythrocytes:

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

- 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.
- The rapid leakage of certain encapsulated substances from the loaded erythrocytes.
- Several molecules may alter the physiology of the erythrocyte.
- Possibility of clumping of cells and dose dumping may be there.

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 vernipuncture. 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 40^0C 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 haematocrit values as desired and are often stored in acid–citrate– dextrose buffer at 40^0C as long as 48 hours before use. [5, 6]

Table 1: Buffers used for isolation

S.NO	Species	Washing Buffer	Centrifugal Force (G)
1	Rabbit	10mmol KH2PO4/NaHPO4	500-1000
2	Dog	15mmol KH2PO4/NaHPO4	500-1000
3	Human	154mmol NaCl	<500
4	Mouse	10mmol KH2PO4/NaHPO4	100-500
5	Cow	10-15mmol KH2PO4/NaHPO4	1000
6	Horse	2mmol MgCl2, 10mmol glucose	1000
7	Sheep	10mmol KH2PO4/NaHPO4	500-1000
8	Pig	10mmol KH2PO4/NaHPO4	500-1000

2. Erythrocytes as Drug Carriers

The developing RBC has the capacity to synthesize haemoglobin. The adult RBC however loses their capacity

and serves only to carry haemoglobin. The use of cells as drug delivery system requires that the drug which are normally unable to permeate the membrane, should be made to traverse the membrane without causing any irreversible changes in membrane structure and permeability. Further the cells should be able to release the drug in controlled manner upon reaching the desired target. RBCs have solid content of about 35% (rest 65% being water). Apart from this the erythrocytes have phosphate content which is in organic nature. The osmotic pressure of the interior of erythrocytes is equal to that of plasma and termed as isotonic. [1,2] (Equal to the osmotic pressure of 0.9% NaCl). If medium is hypotonic water diffuses into the cells and they get swelled and eventually loose their hemoglobin content and may burst. If medium is hypertonic (osmotic pressure more than 0.9% NaCl) they will shrink and become irregular in shape.

Some of the hemoglobin is lost and other cellular constituents are retained in the cells. On resealing they lose some of properties of the normal erythrocytes and referred as "resealed erythrocytes". Such erythrocytes which contain no or little haemoglobin are called ghosts. 3 types of ghosts can be distinguished: type-1 ghosts which reseal immediately after haemolysis; type-2 ghosts which reseal after reversal of haemolysis by addition of alkali ions; type-3 ghosts which remain leaky under different experimental conditions. RBC's are biocompatible provided that compatible cells are used in patients; there is no possibility of triggered immunological response. [1-4]

Since resealed erythrocytes are being considered as novel carriers, it would be logical to review the properties of the novel drug carriers, which are

- It should be of appropriate size and shape to permit the passage through the capillaries.
- It should possess specific physico –chemical properties by which a desired target site could be recognized.
- Should be biocompatible and have minimum toxic side effects.
- Degradation products should be biocompatible.
- Minimum leaching\ leakage of drugs should takes place before target is reached.
- Possess ability to carry a broad spectrum of drugs with different properties. Physico- chemically compatible with drugs.
- The carrier system should have an appreciable stability during storage.

Erythrocytes can be used as carriers in two ways:-

- 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. [7]

3. Method 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 pharmacodynamic properties. [8-12]

Hypo-osmotic lysis method:

In this process, the intracellular and extracellular solutes of erythrocytes are exchanged by osmotic lysis and resealing. The drug present will be encapsulated within the erythrocytes membrane by this process. [13]

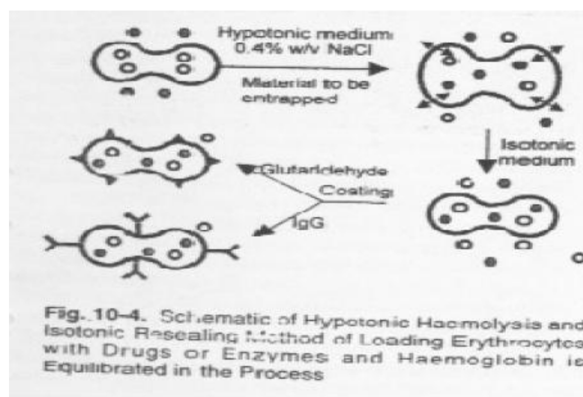

Figure 1: Schematic representation of hypo – osmotic lyses procedure

Hypotonic haemolysis:

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 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. [13]

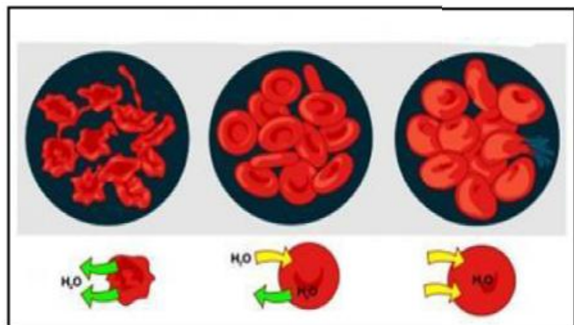

Figure 2: Hypotonic haemolysis

Hypotonic dilution:
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 haemoglobin 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 galactosidase and –glucosidase, asparginase, and arginase, as well as bronchodilators such as salbutamol.[14]

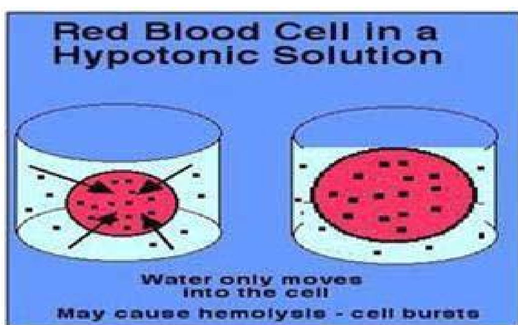

Figure 3: Hypotonic Haemolysis Method

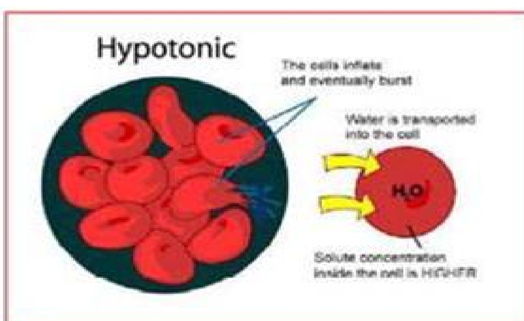

Figure 4: Hypotonic Dilution Methods

Use of red cell loader:
Novel method for entrapment of non-diffusible drugs into erythrocytes. 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 2h 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. [15, 16]

Figure 5: Use of Red Cell Loader

Hypotonic dialysis:
This method was first reported by Klibansky for loading enzymes and lipids. Several methods are based on the principle that semi permeable dialysis membrane maximizes the intracellular: extracellular volume ratio for macromolecules during lysis and resealing. In the process, an isotonic, buffered suspension of erythrocytes with a haematocrit 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 2hrs. 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. [12, 15]

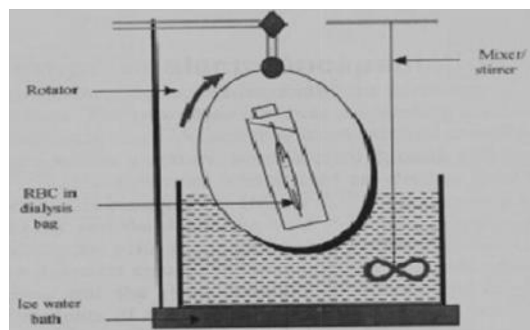

Figure 6: Hypotonic dialysis - Erythrocyte Dialyzer

Hypotonic pres well technique:
This method was investigated by Rechsteiner in 1975 and was modified by Jenner *et al*. For drug loading, this method is based on the principle of first swelling the erythrocytes without lysis by placing them in slightly hypotonic solution. The swollen cells are recovered by centrifugation at low speed. Then, relatively small volumes of aqueous drug solution are added to the point of lysis. The slow swelling of cells results in good retention of the cytoplasmic constituents and hence good survival in vivo. This method is simpler and faster than other methods, causing minimum

damage to cells. Drugs encapsulated in erythrocytes using this method include propranolol, asparginase, cyclopohphamide, cortisol-21-phosphate, 1-antitrypsin, methotrexate, insulin, metronidazole, levothyroxine, enalaprilat, and isoniazid.

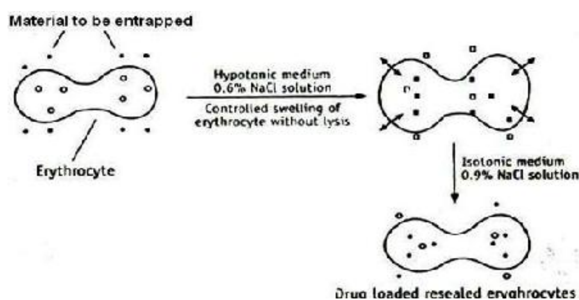

Figure 7: Drug Loaded In Erythrocyte by Hypotonic Preswelling Method

Isotonic osmotic lysis:

This method, also known as the osmotic pulse method, involves isotonic haemolysis. Erythrocytes are incubated in solutions of a substance with high membrane permeability; the solute will diffuse into the cells because of the concentration gradient. Chemicals such as urea solution, polyethylene glycol, and ammonium chloride have been used for isotonic haemolysis. In 1987, Franco *et al*. developed a method that involved suspending erythrocytes in an isotonic solution of dimethyl sulfoxide (DMSO). [1, 4, 13-16]

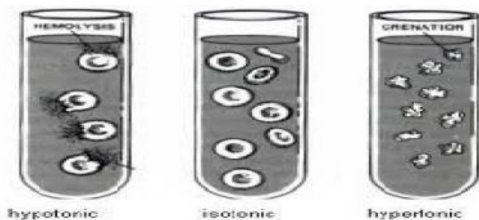

Figure 8: Isotonic Osmotic lysis

Membrane perturbation by chemical agent:

This method is based upon the increase in membrane permeability of erythrocytes when the cells are exposed to certain chemicals. In 1973, Deuticke *et al*. showed that the permeability of erythrocytic membrane increases upon exposure to polyene antibiotic such as amphotericin B. In 1980, this method was used successfully by Kitao and Hattori to entrap the antineoplastic drug daunomycin in human and mouse erythrocytes. Lin *et al*. Used halothane for the same purpose. However, these methods induce irreversible destructive changes in the cell membrane and hence are not very popular. [17]

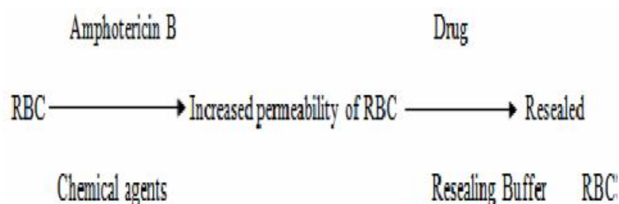

Figure 9: Resealing of RBC by chemical perturbation of the Membrane method

Electro-insertion or electro encapsulation:

This method is also known as electroporation, the method consist of creating electrically induced permeability changes at high membrane potential differences. In 1977, Tsong and Kinosita suggested the use of transient electrolysis to generate desirable membrane permeability for drug loading. Electrical breakdown is achieved by membrane polarization for microseconds using varied voltage of 2kv/cm is applied for 20 μsec.

The potential difference across the membrane is built up either directly by inter and intracellular electrodes or indirectly by applying internal electric field to the cells. The extent of pore formation depends upon the electric field strength, pulse duration and ionic strength of suspending medium. Once membrane is perforated, regardless of the size of pores, ions rapidly distribute between the extra and intracellular space to attain Donnan equilibrium, however the membrane still remains impermeable to its cytoplasmic macromolecules. The cell membrane eventually lyses because of the colloidal osmotic pressure of its macromolecular contents. In the case of red blood cells, the colloidal osmotic pressure of haemoglobin is about 30 m Osm. This pressure drives water and ion influx, as a result swelling of the cells occurs.

The membrane is ruptured when the cell volume reaches 155% of its original volume. Thus, cell lysis is a secondary effect of electric modification of the membrane. 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 tetrasaccharide stachyose or protein such as bovine serum albumin) and ribonucleases. This helps to counteract the colloidal osmotic swelling of electrically perforated erythrocytes. Under this osmatically balanced condition pores stay open at 4oC for few days. If drug molecules are added at this point, they permeate into red blood cells. A suitable procedure could be subsequently used to reseal these pores. 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. [18-20]

Entrapment by endocytosis:

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. This method was reported by Schrier *et al.* in 1975. The pores created by this method are resealed by using 154 mM of NaCl and incubation at 37 oC 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. [21-23]

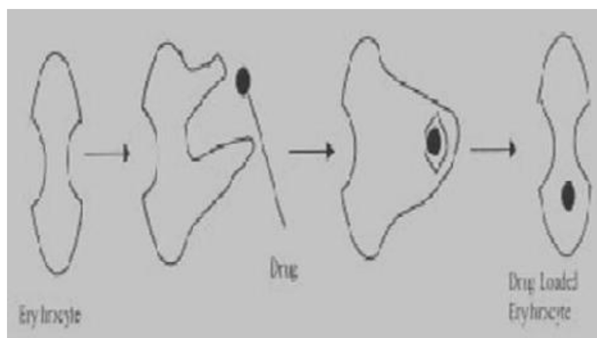

Figure 10: Entrapment by endocytosis

Loading by electric cell fusion:
This method involves the initial loading of drug molecules into erythrocyte ghosts followed by adhesion of these cells to target cells. The fusion is accentuated by the application of an electric pulse, which causes the release of an entrapped molecule. An example of this method is loading a cell-specific monoclonal antibody into an erythrocyte ghost. An antibody against a specific surface protein of target cells can be chemically cross-linked to drug-loaded cells that would direct these cells to desired cells. [21-23]

Lipid fusion technique:
In this method fused lipid vesicle containing bioactive molecule along with human erythrocytes leading to exchange of lipid entrapped drug molecule. This method provides very low encapsulation efficiency.[21-23]

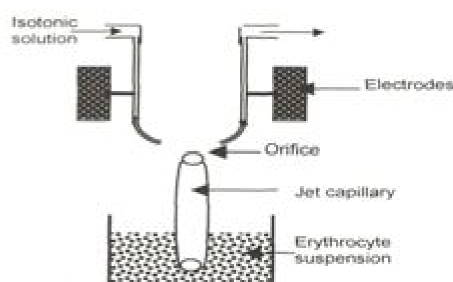

Figure 11: Loading by lipid fusion

Characterization of Resealed Erythrocytes: [24-26]
There are mainly three ways for a drug to efflux out from erythrocyte carrier's i.e. phagocytosis, diffusion through the membrane of the cell and use of specific transport system. The rate of diffusion depends upon the rate at which a particular molecule penetrates through a lipid bilayer it is greatest for molecule with high lipid solubility and gradually goes down with polarity or charged groups of the molecule. Prolongation of release could presumably be accomplished by entrapment of potent inhibitors of the appropriate transport protein along with the drug.

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% haematocrit in PBS) are stored at 4 0C in amber coloured glass containers. Periodically the clear supernatent are withdrawn using a hypodermic syringes equipped with 0.45. filter, deproteinized using methanol and were estimated for drug content. The supernatent of each sample after centrifugation is collected and assayed, % haemoglobin release may be calculated using the formula.

% haemoglobin release = A540 of sample - A540 of background/A540 of 100% haemoglobin

Mean corpuscular haemoglobin {MCH (pg)} = Haemoglobin (g/100ml) x10/Erythrocyte count (millions/cu mm) Where a A540 refers to absorbance at 540nm.

4. Physical Characterization

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

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

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

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

B] Cellular characterization:

1) 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.

2) Osmotic fragility and Osmotic shock study:
When red blood cells are exposed to solution of varying tonicities their shape change due to osmotic imbalance. To

study the effect of different tonicities, drug loaded erythrocytes are incubated separately in normal saline solution at 37= 2°C 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 haemoglobin release spectrophotometrically.

3) Turbulence shock:

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 haemoglobin in withdrawn sample is estimated. Drug loaded erythrocytes appear to be less resistant to turbulence, probably indicating destruction of cells upon shaking.

4) Entrapped magnetite study:

The hydrochloric acid is added to a fixed amount of magnetite bearing erythrocytes and contents are heated at 600c for 2 hr. Then 20% w/v trichloroacetic acid is added and suparnantent obtained after centrifugation is used to determine magnetite concentration using atomic absorption spectroscopy.

5) Erythrocyte sedimentation rate (ESR):

It is an estimate of suspensions stability of RBC in plasma& is related to number & size of red cells& to relative concentration of plasma proteins, especially fibrogen& the and globulins. The test performed by determining rate of sedimentation of blood cells in standard tube. Normal blood ESR is 0-15mm/hr.

6) The zeta sedimentation ratio:

Based on measure of closeness with RBC, will approach each other after standardised cycles of dispersion & compaction.

7. Shelf life and Stability and Crosslinking 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 and lyophillized at- 40 0C to 0.01 torr using a laboratory lyophilizer. The dried powder was filled in amber colour glass vials and stored at 4 0C for month. Improvement in shelf life of the carrier erythrocytes was achieved by storing the cells in powder from, ready for reconstitution at 4 ºC.

C] Biological characterization

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

5. Conclusion

The use of resealed erythrocytes extended promising for a safe and effective delivery of various bioactive molecules for effective 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. Until other carrier systems come of age, resealed erythrocytes technology will remain an active arena for the further research. In near future, erythrocytes based delivery system with their ability to provide controlled and site specific drug delivery will revolutionize in effective treatment of various disease. For the present, it is concluded that erythrocyte carriers are “nano device in field of nanotechnology” considering their tremendous potential.

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