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RESEALED ERYTHROCYTES AS A POTENTIAL DRUG CARRIER SYSTEM

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ABSTRACT

Novel drug delivery system has been introduced to overwhelm the drawback of fluctuating drug levels associated with conventional dosage forms. Out of various cellular carrier systems, erythrocytes (red blood cells, RBC) have been the most widely investigated and found to possess great potential for the delivery of drugs and drug-loaded microspheres. These cellular carriers, having remarkable biocompatibility, biodegradability, and life-span in circulation, can be loaded by a wide spectrum of compounds of therapeutic value using different chemically, as well as physically, based methods. Most of the characteristics of the erythrocytes, including shape, membrane fragility, deformability, and hematologic indices undergo some degree of irreversible changes during the loading procedure. Carrier erythrocytes are prepared simply by collecting blood sample from the organism of interest, separating erythrocytes from plasma, then by using various physical and chemical methods the cells are broken. The drug is entrapped into the erythrocytes and finally resealing the resultant cellular carriers. Hence these carriers are suitably known as Resealed erythrocytes. Surface modification with glutaraldehyde, antibodies, carbohydrates like sialic acid and biotinylation of loaded erythrocytes (biotinylated erythrocytes) is possible to achieve various levels of targeting and increased circulation half-life. Upon reinjection the drug loaded erythrocytes serve as slow circulation depots, targets the drug to the reticuloendothelial system (RES), prevents degradation of loaded drug by endogenous chemicals, attain steady state concentration of drug and decrease the toxic-effects of loaded drug.

INTRODUCTION: The Drug carrier system including liposomes, nanoparticles, niosomes, resealed erythrocytes etc act on specific target, promote therapeutic effect of the drug, decrease toxic effect (by increasing drug level and persistence in vicinity of target cells, hence decreasing the drug exposure to non-target cells) and finally increases the dose effectiveness also [1].

Characteristics of Erythrocytes: The erythrocytes have flexible, elastic, biconcave and nucleated structure with mean diameter of 7.3µm and thickness of 2.2µm. The chemical constituents include water (63%), Haemoglobin (33.67%), methe-haemoglobin (0.5%), glucose (0.8%), minerals (0.7%), non- haemoglobin protein (0.9%) and lipids (0.5%). The prime function of these RBC's is to transport gases for respiratory processes. The production rate of RBC is 2.5 million per second and life span of 100-120 days [2, 3].

The mature RBC has no nucleus and no mitochondria therefore generate ATP anaerobically, for this they are more preferred for resealing. At 0.9% NaCl solution, the erythrocytes are biconcave in shape which swell in hypotonic solution and shrinked in hypertonic solution.

Desirable properties essential for suitability of RBC as Drug Carrier [4, 5]:

1. Biodegradability : Resealed erythrocytes release the drug loaded in it on biodegradation , therefore the biodegraded product is of utter importance (90% RBC's are degraded by macrophages present in Reticuloendothelial system (RES) and remaining 10% by haemolysis in circulation)
2. Circulate throughout the circulatory system (On addition of calcium chelating agents or purine nucleosides, the circulation is further enhanced)
3. Large quantity of materials or bioactive agents can be encapsulated within small volumes of cells.
4. Can be utilized for organ targeting within RES.
5. Erythrocytes are biocompatible provided that compatible cells are used in patients.
6. There should be no possibility of triggered immunological reaction.
7. The flexibility of red blood cells to retain its shape and morphology when placed in isotonic saline also make them suitable carriers for drugs and enzymes.
8. It should have an appreciable stability during storage.
9. It should have specific physicochemical properties by which a desired target site could be recognized.
10. Minimum leakage of drug should take place before target site is reached.

Objective / Purpose of Resealing Erythrocytes:

1. To improve existing dosage forms and development of more sophisticated dosage forms.
2. For Avoiding immune response or reaction on IV administration.
3. For Achieving maximum therapeutic benefit for safe and effective management of diseases.
4. To have accurate dose on targeting sites and hence reducing the side effects.
5. To improve Patient compliance.

Advantages of Resealed Erythrocytes:

1. No chance of triggered immune response.
2. Biodegradability with no generation of toxic or harmful products.
3. The considerable uniform size and shape of the carrier.
4. Relatively inert intracellular environment.
5. Prevention of degradation of the loaded drug from inactivation by endogenous chemicals.
6. The wide variety of chemicals and enzymes can be entrapped.
7. The modification of pharmacokinetic and pharmacodynamic parameters of drug can be done.
8. Attainment of steady-state plasma concentration which decreases fluctuations in concentration of drug.
9. Protection of the organism against toxic effects of drugs (e.g., antineoplastics)
10. Ease of circulation and ability to target RES organ
11. Prolong systemic activity of the drug while residing for a longer time in the body.

12. Possibility of decreasing the side effects of the drug.

13. The entrapment of drugs does not require the chemical modification of the substance to be entrapped.

14. No chance of triggered immune response.

15. Biodegradability with no generation of toxic or harmful products.

16. The considerable uniform size and shape of the carrier.

Limitations encountered:

1. The major problem encountered in the use of resealed erythrocytes is that they are removed in vivo by the RES as a result of modification that occurred during loading procedure in cells.

2. There is lack of reliable and practical storage methods.

3. They have limited potential as carrier to non phagocytic target tissue.

4. There can be cell clumping and dose dumping problem.

5. Leakage of certain encapsulated substances from the loaded erythrocytes may be there.

6. There is possibility of contamination due to the origin of the blood, the equipment used and the loading environment.

Source of Erythrocytes [6]: Different mammalian erythrocytes have been exploited for drug loading, resealing and subsequent use in delivery of drug, enzyme and other bioactive agents' delivery. Majority of them is constructed from red blood cells of mice, cattle, pigs, dogs, sheep, goats, monkeys, chicken, rats and rabbits. But to avoid antigen-antibody reaction, blood group and compatibility parameters should be evaluated.

Isolation of Erythrocytes: (table 1)

1. Fresh whole blood should be used for the isolation of erythrocytes for drug delivery purpose.

2. Blood is withdrawn from cardiac / splenic puncture in small animals and through veins in large animals in a syringe containing a drop of anticoagulant.

3. The blood is collected into heparinized tubes.

4. The whole blood is centrifuged at 2500 rpm for 5 minutes at 4±1ºC in a refrigerated centrifuge.

5. After centrifugation, the serum and buffy coats are carefully removed and packed cells are washed three times with phosphate buffer saline of 7.4 pH.

6. The washed erythrocytes are diluted with phosphate buffer saline.

7. The washed erythrocytes are often stored in acid-citrate-dextrose buffer at 4^{o}C up to 48 hr prior to use.

TABLE 1: VARIOUS CONDITIONS AND CENTRIFUGAL FORCE USED FOR THE ISOLATION OF ERYTHROCYTES

Species	Washing Buffer	Centrifugal Force (g)
Mouse	10 mmol KH_2PO_4 /Na_2HPO_2, pH 7.0 ; 5mmol adenosine ; 5mmol $MgCl_2$; 10 mmol glucose	100-500
Human	154 mmol NaCl or 10mmol KH_2PO_4 / Na_2HPO_4,pH 7.0 ; 2mmol $MgCl_2$; 10 mmol glucose	<500
Rabbit	10 mmol KH_2PO_4 / Na_2HPO_4 pH 7.0	500-1000
Dog	15 mmol KH_2PO_4 /Na_2HPO_4, pH 7.0 ; 5 mmol $MgCl_2$; 10 mmol glucose	500-1000
Cow	10-15 mmol KH_2PO_4/ Na_2HPO_4 ,pH 7.0 ; 2 mmol $MgCl_2$; 10 mmol glucose	1000
Goat	10 mmol KH_2PO_4 / Na_2HPO_4, pH 7.0	500-1000
Horse	10 mmol KH_2PO_4 / Na_2HPO_4, pH 7.0 ; 2mmol $MgCl_2$; 10 mmol glucose	1000
Pig	10 mmol KH_2PO_4 / Na_2HPO_4,pH 7.0	500-1000
Sheep	10 mmol KH_2PO_4 / Na_2HPO_4, pH 7.0 ; 5 mmol $MgCl_2$	500-1000

Requirements for Encapsulation: Variety of bioactive agents (5000-600,000 daltons in size) can be entrapped in erythrocytes. Once encapsulated, charged molecules are retained longer than uncharged molecules. Both polar and non polar molecules have been successfully entrapped. Sucrose is used as a marker for encapsulation studies. Hydrophobic molecules may be entrapped by absorbing over other molecules while non polar molecules may be entrapped in their respective salts. Moreover, if the molecules interact with membrane and cause deleterious effect on membrane structure then that molecule should not be considered for encapsulation in erythrocytes (**fig. 1**).

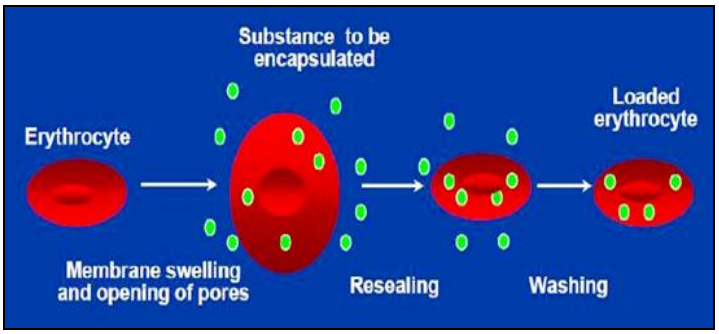

FIG. 1: GENERAL MECHANISM OF RESEALING OF ERYTHROCYTE

Methods of Drug Loading: In general, the potential use of erythrocytes depends on their ability to encapsulate exogenous enzymes or other bioactive molecules into erythrocytes. Several methods can be used to load drugs, enzymes or other bioactive compounds in erythrocytes. 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, with well-defined pharmacokinetic and pharmacodynamic properties [7, 8].

The various methods used are listed below:

A. **Hypo-Osmotic Lysis Method:** This method is based upon hypotonic lysis of erythrocytic cells in a solution containing the drug or enzyme to be entrapped followed by restoration of tonicity in order to reseal them (erythrocytes are resealed on addition of sufficient 1.54M KCl, which restores isotonicity). Hence, a heterogeneous population of ghosts cells is obtained.

Ghosts' cells: These are red blood cells which have limited or no haemaglobin. They are of following types:

- Type-1 ghost cells: which reseal immediately after haemolysis
- Type-2 ghost cells: which reseal after reversal of haemolysis by addition of alkali ions.
- Type-3 ghost cells: which remain leaky under different experimental conditions.

The ratio of above three fractions depends upon:

- Temperature at which haemolysis is affected.
- Time interval between haemolysis and restoration of tonicity.

Types of Hypo-Osmotic Lysis Method:

a) **Isotonic Osmotic Lysis:** This method, also known as the osmotic pulse method, involves isotonic haemolysis 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 transerythrocytic 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.

Various methods based on this mechanism include:

- Conventional haemolysis in isotonic urea solutions
- Polyethylene induced haemolysis
- Ammonium chloride induced haemolysis

Advantage: Better *in-vivo* surveillance of erythrocytes.

Limitations of this method:

- The process is time consuming.

- Generally impossible to rule out changes in membrane structure and composition due to presence of the membrane lytic substances.

- The ghosts cell obtained have limited application as carriers.

b) **Hypotonic Pre-swelling Method:** 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 without lysis in a slightly hypotonic buffered solution. This mixture (i.e., 1 volume washed cells suspended in 5 volume of 0.6% w/v NaCl solution) is centrifuged at low *g* values for 5 minutes at 0^{o}C.The cells are recovered and swelling procedure is further extended by addition of hypotonic buffer equal to half the volume of the swollen cells to effect lysis of the cells.

This hypotonic buffer medium also contains the material to be loaded. Then the cells are allowed to remain lysed for 10 minutes at 0^{o}C.This is further followed by restoration of tonicity and resealing of membrane. 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.

Advantages of this method include;

- Good retention of cytoplasm constituents with good in vivo survival because of gentle swelling of cells.

- The % of loading is in between 20-70%

c) **Dialysis Method:** 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 85-95% is prepared and placed in a conventional dialysis tube immersed in 10-20 volumes of a hypotonic buffer (Phosphate buffer saline, pH7.4).The dialysis bag is inflated with an air bubble of nearly 25% of the internal volume. Air bubble is critical for the procedure, in that during lysis and resealing, it travels through the length of dialysis tubing and hence serves to blend the contents of the dialysis bag in a uniform mixture. Now sealed the dialysis bag in such a way that erythrocyte suspension occupies no more than 75% of the internal volume. Both ends of dialysis bag are tied with thread.

The sealed bag is then placed in a bottle containing atleast 200 ml of lysis buffer (0.1% w/v NaCl, 0^{o}C) and placed on a mechanical agitator for 2 hr at 4^{o}C. After the initial lysis at 0^{o}C, the dialysis bag is transferred to a bottle containing at least 200 ml of resealing buffer (isotonic buffer) at room temperature for 30 minutes. The loaded erythrocytes thus, obtained are then washed with cold Phosphate buffer saline at 4^{o}C.The cells are finally resuspended in Phosphate buffer saline.

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. The use of standard haemodialysis 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 were 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 [9, 10].

Haematocrit value: It refers to the fraction of the erythrocytes’ portion to total blood. It is also expressed as Volume ratio of packed red cell to total volume of the blood. This volume indicates both size and number of erythrocytes. Volume of packed red cell (VPRC) is expressed in International units, the normal VPRC for males is 0.45 litres per litre; and for females, and it is about 0.41 litres per litre.

Advantages of the method:

- The loaded cells exhibit the same circulation half life as that of normal cells.

- Also, this method allows higher % of encapsulations of extracellular material, when a high haematocrit is used; at low haematocrit, however, it entraps substances of large molecular weight or dimension more effectively than dilution method.

- The process is amenable to automation with control of process variables.

- This method is also preferred in case for obtaining better in vivo survival of erythrocytes, as it imparts structural integrity to membrane due to lesser ionic load and related in process stresses.

- The method was successfully utilized for encapsulation of ^{125}I albumin.

- This method has also been used for loading enzymes such as- galactosidase, glucoserebrosidase, asparginase, inositol hexaphosphatase, as well as drugs such as gentamicin, Adriamycin, interlukin-2, desferroxamine.

Disadvantages:

- The drawbacks include a long processing time and the need for special equipment.

- In most cases the size distribution of loaded ghosts is found to be homogeneous. Thus the drug concentration in individual ghosts may vary considerably.

d) **Hypotonic dilution / Dilutional Haemolysis:** Hypotonic dilution was the first method investigated for the encapsulation of chemicals into erythrocytes. In this method 1 volume of packed erythrocytes is diluted with 2-20 volumes of aqueous solution of a drug to be loaded. The mixture is then placed in hypotonic medium (0.4% NaCl) at 0^{o}C for 5 minutes. The membrane of the erythrocyte gets ruptured and then the erythrocytes are resealed after incubation at 25^{o}C in isotonic medium [11,12].

Advantages:

- Fastest and simplest method especially for low molecular weight drugs.

Disadvantages:

- The major drawbacks of this method include low entrapment efficiency (1-8%) and a considerable loss of hemoglobin and other cell components which reduces the circulation half life of the loaded cells.

A. **Endocytosis Method of drug loading:** This method was reported by Schrier *et al*., in 1975. Endocytosis involves the three steps:

a. Addition of one volume of washed packed erythrocytes to nine volumes of buffer containing 2.5 mM ATP, 2.5 mM $MgCl_2$, and 1mM $CaCl_2$, followed by incubation for 2 min at room temperature.

b. The pores created by this method are resealed by using 154 mM of NaCl and incubation at 37^{o}C for 2 min.

c. The entrapment of material occurs by endocytosis, following incubation of washed resealed cells with buffer containing the material to be entrapped for 30 minutes at 37^{o}C.

Advantages of the method:

- The vesicle membrane separates the endocytosed material from cytoplasm thus protecting it from the erythrocytes and vice-versa.

- This method also provides an alternative means for loading erythrocytes and allow for successful encapsulation of DNA or particles as large as bacteria which are difficult to be entrapped using hypotonic haemolysis methods.

- 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.

B. **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 attenuated by the application of an electric pulse, which causes the subsequent 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 the target point for adhesion.

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

D. **Normal Transport mechanism:** This method involves the loading of erythrocytes with drug without disrupting the erythrocyte membrane in any way by incubating the drug and erythrocytes for varying period of time. The drug will enter by following the kinetics order and will exit in the circulation in the same kinetics order.

E. **Chemical perturbation of the membrane:** This method is based on the increase in membrane permeability of erythrocytes when the cells are exposed to certain chemical agent. This allows the low molecular weight substances to get entrapped. 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.

Disadvantages:

- These methods induce irreversible destructive changes in the cell membrane and hence are not very popular.
- Moreover, the *in-vivo* survival of erythrocyte is poor.

F. **Electro-insertion / Electro- encapsulation / Electric Breakdown Method:** 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^{o}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 µsec. 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 strength 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., tetrasaccharide stachyose and bovine serum albumin) and ribonuclease [13].

Advantages of the method:

- One advantage of this method is a more uniform distribution of loaded cells in comparison with osmotic methods.
- Excellent *in vivo* performance has been reported and normal haemoglobin properties were retained.

- The technique can also be used to insert proteins into erythrocyte membrane.

- Since the method causes no to negligible memranolytic or deleterious effects, the erythrocytes are not intercepted and sequestrated by Reticulo-endothelial system. Thus, they are allowed to remain in circulation and during the course control the release of contents.

- The drug loaded erythrocyte prepared by this method can give a sustained release of the entrapped drug.

- 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 0.2 µm can be entrapped within erythrocytes by this method

Disadvantages:

- The need for special instrumentation for the method

- The sophistication of the process.

- Entrapment efficiency of this method is 35%.

- It is a time consuming and expensive technique

G. **Loading by "Red Cell Loader":** Magnani *et al*., 1998 developed a novel method for entrapment of non-diffusible drugs into erythrocytes. They developed equipment called as "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 hour at room temperature under blood banking conditions. The process is based on two sequential and controlled hypotonic dilutions of washed erythrocytes followed by concentration with a haemofilter and an isotonic resealing of the cells. The modifications are largely incorporated to increase their recognition by tissue macrophages to perform as drug targeting system. There was 30% drug loading with 35-50% cell recovery.

Advantages:

- The processed erythrocytes had normal survival in vivo.

- The same cells could be used for targeting by improving their recognition by tissue macrophages.

- Entrapment of a variety of biological compounds in erythrocyte in as little time as 2 hour at room temperature under blood banking conditions is possible.

Various factors affecting the loading of drugs in Erythrocytes include:

- Drug concentration

- Haematocrit value

- Lysing time

- Resealing time

- Mixing

- Magnetite concentration (Magnetites, for example ferro-fluids, upon entrapment in erythrocyte ghosts can be directed to deliver the entrapped drug under the influence of externally applied magnetic fields)

- Temperature (low temperature for the lysis in order to allow high entrapment of exogenous molecules; high temperature, 37^{o}C, for optimal resealing in order to obtain discocyte cells that are capable to survive in circulation)

Release Characteristics of Loaded Drugs [14, 15]: These are important to study so as to control efflux rate before constant release rate over prolonged period can be achieved. There are mainly three ways for a drug to efflux out from erythrocyte carriers.

I. **Phagocytosis:** RBC's are normally removed from circulation by process of phagocytosis following

heat treatment or antibody cross linking. The drug could be released from macrophages after phagocytosis if linkage is susceptible to lysosomal enzymes.

II. **Diffusion through the cell membrane:** The rate of diffusion depends upon the rate at which a particular molecule penetrates through a lipid bilayer. It is greatest for a molecule with high lipid solubility. Hence considerable control over the rate of drug release is possible by introducing or eliminating polar or charged substituent.

III. **Using a specific transport system:** Many substances enter cells by specific membrane protein system because the carriers are proteins and specific as that of enzymes. Moderate modification in compound can often alter the rate of exit.

- The release of drugs from erythrocytes rapidly follows sustained release profile and rate of exit is proportional to instantaneous intracellular drug concentration i.e. first order kinetics. However, erythrocytes' carriers have the potential of releasing encapsulated substance following zero order kinetics. By incorporating polymers, release pattern may be modified. If the drug is encapsulated in a random population of erythrocytes, then constant fraction of cells will be removed each day and constant amount of drug will be made available each day.

Routes of Administration: It had been proposed that the survival of erythrocytes in circulation by intraperitoneal injection was equivalent to their administration by i.v. injection. Subcutaneous route was also evaluated for slow release of entrapped agents. Moreover erythrocytes based nasal delivery of propanolol had also been proposed.

Storage of Erythrocytes: The storage of resealed erythrocytes places a major challenge in their practical utility as drug delivery system and is important pre-requisite for the erythrocytes as a drug carrier. The Encapsulated preparation should be stored in such a way that there should be no loss of integrity. There are following methods for the storage of loaded erythrocytes:

I. Suspending in Hank's balanced salt solution at $4^{o}C$ for two weeks.

II. After encapsulation suspending the cells in oxygenated Hank's balanced salt solution containing 1% soft bloom gelatin. The cells can be recovered by liquefying the gel by placing the tubes in water bath at $37^{o}C$ and centrifugation under clinical conditions.

III. Or Cryopreservation of erythrocytes at liquid nitrogen temperature.

Stability and cross linking of Resealed Erythrocytes: The cells treated with dimethyl sulfoxide,dimethyl-3,3 dithiobis propionamide, toluene 2,4-diisocynate and glutaraldehyde are even resistant to sonication, freezing and thawing. Osmotic fragility tests demonstrate that the RBC which survives the cross linking process display a very similar hemolysis curve as produced by normal RBC.

Talwar and jain collected glutaraldehyde (0.2%) treated erythrocytes in a sintered glass funnel by filtration and dried in vacuum (200 mm Hg) for 10 hour. Alternatively the erythrocyte suspension was filled into vials and lyophilized at $-40^{o}C$ to 0.01 torr using a laboratory lyophilizer. The dried powder was filled in amber color glass vials and stored at $4^{o}C$ for a month. Improvement in shelf life of the carrier erythrocytes was achieved by storing the cells in powder form, ready for reconstitution at $4^{o}C$.

This is important in the large scale manufacturing of drug loaded resealed erythrocytes. *In-vitro* stability, band-3-structural changes, the extent of cross linking and of membrane modification must be considered in order to study the *in-vivo* stability of carrier erythrocytes and their survival in circulation [16].

Visualization of Individual Cells: Fluorescent compounds such as FITC-BSA can be readily entrapped in erythrocytes by hypo-osmotic lysis or by endocytosis procedure. When viewed by fluorescence microscopy, erythrocytes made by the hypo-osmotic procedure appear uniformly fluorescent and erythrocytes loaded by endocytosis have a more irregular fluorescent pattern corresponding to the endocytic vacuoles.

This system may have considerable potential for certain type of experiment since it permits individual cells to be located among the normal endogeneous erythrocytes, for example, the flow properties of erythrocytes containing inclusions or intracellular parasites could be monitored. The testing of prototype antisickling compounds which do not enter erythrocytes readily could be accomplished by high osmotic entrapment along with the fluorescent compounds and subsequent recording of the circulatory properties in the micro vasculature (**TABLE 2**).

TABLE 2: ERYTHROCYTE CHARACTERIZATION WITH THEIR QUALITY CONTROL ASSAYS:

CHARACTERIZATION PARAMETERS	ANALYTICAL METHODS / INSTRUMENTATION
1. PHYSICAL CHARACTERIZATION:	
• Shape and surface morphology	• Transmission electron microscopy (TEM), Scanning electron microscopy, Phase-contrast optical microscopy
• Vesicle size and size distribution	• TEM, Optical microscopy
• Drug release	• Diffusion cell / dialysis
• %Encapsulation	• Deproteinization (using methanol or acetonitrile) of cell membrane and assay for released drug or radio-labeled markers
• Electrical surface potential and surface pH	• Zeta potential measurements and pH sensitive probes.
2. CELL RELATED CHARACTERIZATION:	
• % Haemoglobin content / volume	• Deproteinization (using methanol or acetonitrile) of cell membrane and assay for Hb; Laser light scattering for cell volume.
• Mean corpuscular haemoglobin	• Laser light scattering
• % Cell recovery	• Haematological analyzer; Neubeur's chamber
• Osmotic fragility	• Stepwise incubation with isotonic to hypotonic saline solutions and estimation of drug and Hb.
• Osmotic shock	• Dilution with distilled water and estimation of drug and Hb.
• Turbulent shock	• Passing cell suspension through a 23 gauge needle, hypodermic needle (10 ml / min) and estimation of residual drug and Hb.
• Erythrocyte sedimentation rate	• ESR apparatus
3. BIOLOGICAL CHARACTERIZATION:	
	• Aerobic or anaerobic cultures
• Sterility	• Rabbit fever response test of LAL test
• Pyrogenicity	• Toxicity tests
• Animal toxicity	

***In-vitro* characterization of Resealed Erythrocytes:** These characterizations are important to ensure their in-vivo performance and therapeutic benefits [17].

I. **Drug Content Determination:**

Method: 0.5ml packed loaded erythrocytes are deproteinized with acetonitrile (2 ml) and then centrifuged at 2500 rpm for 10 minutes. Now the clear supernatant liquid is analyzed for drug content.

II. ***In-vitro* drug release and Hb content:** Both these properties are monitored periodically from drug-loaded cells.

Method: The cell suspension (5% Haematocrit in Phosphate buffer saline) is stored at $4^{o}C$ in amber colored glass containers. Periodically the clear supernatant are withdrawn using a hypodermic syringe equipped with 0.45µ filter, deproteinized with methanol and then estimated for drug content. The supernatant of each sample after centrifugation is collected and assayed. Hence, % Hb (Haemoglobin) release is calculated.

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

A_{540}=Absorbance at 540 nm, A_{540} of 100% Hb

$$\text{Mean Corpuscular Hb} = \frac{\text{Hb (g/100 ml)} \times 10}{\text{Erythrocyte count (millions/cu mm)}}$$

III. **Percent cell recovery:** It is determined by counting the number of intact cells per cubic mm of packed erythrocytes before and after loading the drug.

IV. **Morphology :** Following types of microscopy are used for the morphological study of normal and drug loaded erythrocytes:

- Phase contrast microscopy
- Electron microscopy

a. Scanning electron microscopy

b. Transmission electron microscopy

V. **Osmotic fragility:** This method is based on resistance of cells to haemolysis in decreasing concentration of hypotonic saline.

It is a reliable parameter for:

- *In-vitro* evaluation of carrier erythrocytes with respect to shelf life
- *In-vivo* survival of erythrocytes
- Study of effect of the encapsulated substances
- For stimulating and mimicking the bio-environmental conditions that are encountered on in-vivo administration.

Method: Normal and drug - loaded erythrocytes are incubated separately in stepwise decreasing % of NaCl solution (0.9%) at $37^{o}C \pm 2^{o}C$ for 10 minutes , followed by centrifugation at 2000 rpm for 10 minutes. Then the supernatant liquid is examined for drug and haemoglobin content.

VI. **Osmotic shock:** This is used to describe a sudden exposure of drug loaded erythrocytes to an environment, which is far from isotonic so as to evaluate the ability of resealed erythrocytes to withstand the stress and maintain their integrity as well as appearance.

Method: Erythrocyte suspension (10% haematocrit,1 ml) was diluted with distilled water(5 ml) and centrifuged at 300 rpm for 15 minutes. Supernatant was estimated for % Hb release spectrophotometrically .

VII. **Turbulence shock:** This parameter indicates the effects of shear and pressure, by which resealed erythrocyte formulations are injected, on the integrity of the loaded cells. Drug loaded erythrocytes appear to be less resistant to turbulence because resealing of erythrocytes make them sensitive towards turbulence/ Mechanical agitation and hence estimation of turbulence shock study provides their expected performance in-vivo.

Method: Loaded erythrocytes(10% haematocrit,5 ml) are passed through 23-gauge hypodermic needle at a flow rate of 10 ml/minute(which is comparable to the flow rate of blood).After every pass, aliquot of suspension is withdrawn and then centrifuge at 2000 rpm for 10-15 minutes. Now the Hb content is estimated spectrophotometrically.

VIII. **Determination of entrapped Magnetite:** Resealed erythrocytes are entrapped with magnetite to make them Magnoresponsive.

Method: Magnetite bearing erythrocytes and Hydrochloric acid are heated at 60ºC for 2 hour. Now 20% w/v trichloroacetic acid is added. Centrifugation is done and supernatant is examined for Magnetite concentration using atomic absorption spectroscopy.

IX. **Erythrocyte Sedimentation Rate (ESR):** ESR is the estimation of suspension stability of RBC in plasma and is related to :

- Number and size of red cells.
- The relative concentration of plasma proteins(especially fibrogen ,alpha and beta globulins)

This test is performed by determining the ESR of blood cells in a standard tube of ESR apparatus. Higher rate of ESR is indication of active but obscure disease processes. The normal blood ESR is found to be 0 to 15 mm/hour.

X. **The Zeta Sedimentation Ratio:** It is based on a measure of the closeness with which RBC's will approach each other after standardized cycles of dispersion and compaction.

XI. **Miscellaneous:** Lipid composition, Membrane fluidity, rheological properties, density gradient separation, energy metabolism, Biological characterization(sterility test using aerobic and anaerobic cultures, Pyrogenicity using rabbit fever response or LAL test, animal toxicity study)

Applications of Resealed Erythrocytes [18]: Resealed erythrocytes have several possible applications in various fields of human and veterinary medicine. Such cells could be used as circulating carriers to disseminate a drug within a prolonged period of time

in circulation or in target-specific organs, including the liver, spleen, and lymph nodes. A majority of the drug delivery studies using drug-loaded erythrocytes are in the preclinical phase. In a few clinical studies, successful results were obtained.

A. *In-vivo* applications [19]:

a. **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.

b. **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. Surface-modified erythrocytes are used to target organs of mononuclear phagocytic system/ reticuloendothelial system because the changes in the membrane are recognized by macrophages. However; resealed erythrocytes also can be used to target organs other than those of RES.

c. **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).

d. **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 glucocerebrosidase in the liver and spleen can be treated by glucocerebrosidase- loaded erythrocytes.

e. **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 glutaraldehyde or cis-aconitic acid as a spacer. The resealed erythrocytes loaded with carboplatin show localization in liver.

f. **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.

g. **Removal of RES iron overload:** Desferrioxamine (iron-chelating agent) 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.

h. **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.

i. **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

j. **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,

k. **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. The problems involved in the direct injection of enzymes into the body have been cited. One method to overcome these problems is the use of enzyme-loaded erythrocytes. These cells then release enzymes into circulation upon hemolysis and act as a "circulating bioreactors" in which substrates enter into the cell, interact with enzymes, and generate products or accumulate enzymes in RES upon hemolysis for future catalysis.

The first report of successful clinical trials of the resealed erythrocytes loaded with enzymes for replacement therapy is that of –glucosere brosidase for the treatment of Gaucher's disease. The disease is characterized by inborn deficiency of lysosomal- glucosere brosidase in cells of RES thereby leading to accumulation of glucosere brosides in macrophages of the RES. To treat lead poisoning, the concentration of aminolevulinate dehydrogenase (ALA-D) in erythrocytes decreases. This leads to an accumulation of aminolevulinic acid in tissues, blood, and urine. This state leads to acute porphyria and CNS related problems.

l. **Improvement in oxygen delivery to tissues:** Haemoglobin is the protein responsible for the oxygen-carrying capacity of erythrocytes. Under normal conditions, 95% of haemoglobin is saturated with oxygen in the lungs, whereas under physiological conditions, in peripheral blood stream, only 25% of oxygenated haemoglobin becomes deoxygenated. Thus, the major fraction of oxygen bound to haemoglobin is recirculated with venous blood to the lungs.

The use of this bound fraction has been suggested for the treatment of oxygen deficiency. 2, 3 Diphosphoglycerate (2, 3-DPG) is a natural effector of hemoglobin. The binding affinity of haemoglobin for oxygen changes reversibly with changes in intracellular concentration of 2, 3-DPG. This compensates for changes in the oxygen pressure outside the body, as the affinity of 2, 3-DPG to oxygen is much higher than that of haemoglobin. Other organic polyphosphates can serve as allosteric effectors of haemoglobin with binding affinities higher than those of 2, 3-DPG and can compete with 2, 3-DPG for binding to haemoglobin. Inositol hexophosphate (IHP) is one of the strongest

effectors of this type. However, because of its ionization at physiological pH, it cannot enter erythrocytes.

Hence, it is entrapped by the electroporation process. Upon encapsulation, IHP irreversibly binds to haemoglobin, thereby decreasing the oxygen affinity to haemoglobin and subsequent shift of oxygen binding isotherm to the right. As a result, the oxygen pressure corresponding to 50% of the total binding capacity of hemoglobin to oxygen (P50 value) increases from 26–27 mm Hg to 50 mm Hg.

In the presence of IHP encapsulated in erythrocytes, the difference between the oxygen bound fraction of haemoglobin in lungs and tissues increases, thereby increasing the oxygen concentration in tissues. Also, the extent of carbamate formed in the N-terminal amine group chain of haemoglobin decreases, which is compensated by an uptake of H and CO_2 that leads to increased formation of bicarbonate ion. IV injection of IHP-loaded erythrocytes to piglets led to a decrease in cardiac output with a constant oxygen consumption by animals.

This indicates that because of an increased extraction ratio of oxygen by tissues, a given amount of oxygen can be delivered in lower blood flow. In addition, these erythrocytes reduce ejection fraction, left ventricular diastolic volume, and heart rate. An isolated perfused-heart model showed reduction in coronary blood flow with increased oxygen consumption by myocardium upon administration of IHP-loaded erythrocytes. The same results are reported when intact animal models were use. 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 haemoglobin
- Increased radiosensitivity of radiation-sensitive tumors
- Restoration of oxygen-delivery capacity of stored blood
- Ischemia of myocardium, brain, or other tissues.

B. ***In-vitro* Applications:** Most important in-vitro application is that of 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.

C. In microinjection, erythrocytes are used in micro-syringes for injection in the host cells. The microinjection process involves culturing host eukaryotic cells *in vitro*. The cells are coated with fusogenic agent and then suspended with erythrocytes loaded with the compound of interest in an isotonic medium. Sendai virus (hemagglutinating virus of Japan, HVJ) or its glycoproteins or polyethylene glycol have been used as fusogenic agents. The fusogen causes fusion of co-suspended erythrocytes and eukaryotic cells.

Thus, the contents of resealed erythrocytes and the compound of interest are transferred to host cell. This procedure has been used to microinject DNA fragments, arginase, proteins, nucleic acids, ferritin, latex particles, bovine and human serum

albumin,and enzyme thymidine kinase to various eukaryotic cells.

Advantages of this method:

- Include quantitative injection of materials into cells
- Simultaneous introduction of several materials into a large number of cells
- Minimal damage to the cell
- Avoidance of degradation effects of lysosomal enzymes
- And simplicity of the technique.

Disadvantages include:

- Need for a larger size of fused cells, thus making them amenable to RES clearance
- Adverse effects of fusogens
- And unpredictable effects on cell resulting from the co introduction of various components.

Hence, this method is limited to mainly cell biological applications rather than drug delivery.

Novel Approaches (Recent Developments) [20, 21]:

- **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. Moreover, the complex appears to be stable and maintain both cytotoxic and antineoplastic activity of daunorubicin against leukemia.

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