MECHANISM OF DRUG LOADING, EVALUATION AND APPLICATIONS OF ERYTHROCYTES AS CARRIERS FOR DRUG TARGETTING

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ABSTRACT

Resealed Erythrocytes are of appropriate size and shape to carry drugs. They are biocompatible and they have minimum toxic side effects. Minimum leakage can be observed before they reach the target site and they are able to carry broad spectrum of drugs. Carrier erythrocytes and resealed erythrocytes are being used for continuous implementation of safe and effective delivery of various drugs for passive and active targeting. Further optimization is required to become a routine drug delivery system which has been extended to the delivery of biopharmaceuticals and to be explored continuously regarding the potential of encapsulated erythrocytes. Resealed erythrocytes and loaded erythrocytes allow controlled drug release and increased specificity of delivery to the targeted organ. This review focus on various methods of encapsulated erythrocytes preparation, techniques of drug loading such as electro-encapsulation, chemical perturbation, entrapment by endocytosis etc, mechanism of resealed erythrocyte release and evaluation resealed erythrocytes.

Keywords: Resealed erythrocytes, controlled drug release, electro-encapsulation, chemical perturbation.

1. INTRODUCTION

Drug delivery is now entering quite an exciting and challenging era. Significant high costs involved in the development of new drug molecule has compelled scientists all over the world to search for alternative ways of administering the existing drug molecules with enhanced effectiveness. Improper drug administration inside the biological system not only causes distress to other body tissues but also demands more therapeutic molecules to elicit the appropriate response. 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 (Gothoskar AV, 2004) (Rossi L, 1996). Among these, the erythrocytes have been the most investigated and have found to possess greater potential in drug delivery. Therapeutic uses of a variety of drug carrier systems have significant impact on the treatment and potential cure of many chronic diseases, including cancer, diabetes mellitus, rheumatoid arthritis, HIV infection, and drug addiction. Erythrocytes are natural products of the body, biodegradable in nature, isolation of these is easy and large amount of drug can be loaded in small volume of cells, non immunogenic in action and can be targeted to disease tissue or organ, prolong the systemic activity of the drug while residing for a longer time in the body (Lejeune A, 1997), protest the premature degradation, inactivation and excretion of proteins and enzymes, act as a carrier for number of drugs, target the drugs within the reticuloendothelial system (RES) as well non RES organs/sites. Moreover, the possibility of targeting carrier erythrocytes to non-RES organs has been exploited in recent years, e.g., using homing devices such as IgG or IgM. Also these cells are non-immunogenic and biodegradable; they freely circulate throughout the body and offer ease of preparation; they have the capacity to carry large amounts of drug; and can behave as a slow-release long-acting system (Al-Achi A, 1990). Also, lungs to tissues and the CO_2 produced in tissues back to lungs. Thus, erythrocytes are a highly specialized O_2 carrier system in the body. Because as aging erythrocytes are normally phagocytized by cells of the reticuloendothelial system, thus, these cells could serve as a natural target for delivery of their pay load to these organs. Potential clinical indications for "RES targeting" include iron over-storage diseases, parasitic diseases, hepatic tumors (Jain S, 1997) (Jain S, 1995) (Guyton CA, 1996) and lysosomal storage diseases.

Erythrocytes, also known as red blood cells, have been extensively studied for their potential carrier capabilities for the delivery of drugs and drug-loaded microspheres. Such drug-loaded carrier erythrocytes are prepared simply by collecting blood samples from the organism of interest, separating erythrocytes from plasma, entrapping drug in the erythrocytes, and resealing the resultant cellular carriers. Hence, these carriers are called resealed erythrocytes. Upon re injection, the drug-loaded erythrocytes serve as slow circulating depots and target the drugs to a reticuloendothelial system (RES). Disadvantages include the possibility of leakage of drug from the cells & dose dumping.

2. SOURCE AND ISOLATION OF ERYTHROCYTES

Various types of mammalian erythrocytes have been used for drug delivery, including erythrocytes of mice, cattle, pigs, dogs, sheep, goats, monkeys, chicken, rats, and rabbits. To isolate erythrocytes, blood is collected in heparinized tubes by venipuncture (Gaudreault B, 1989). Fresh 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. Freshly collected blood is immediately freezed to 4^0C and stored. It can be used for a period of two days. The erythrocytes are then harvested and washed by centrifugation. The washed cells are suspended in buffer

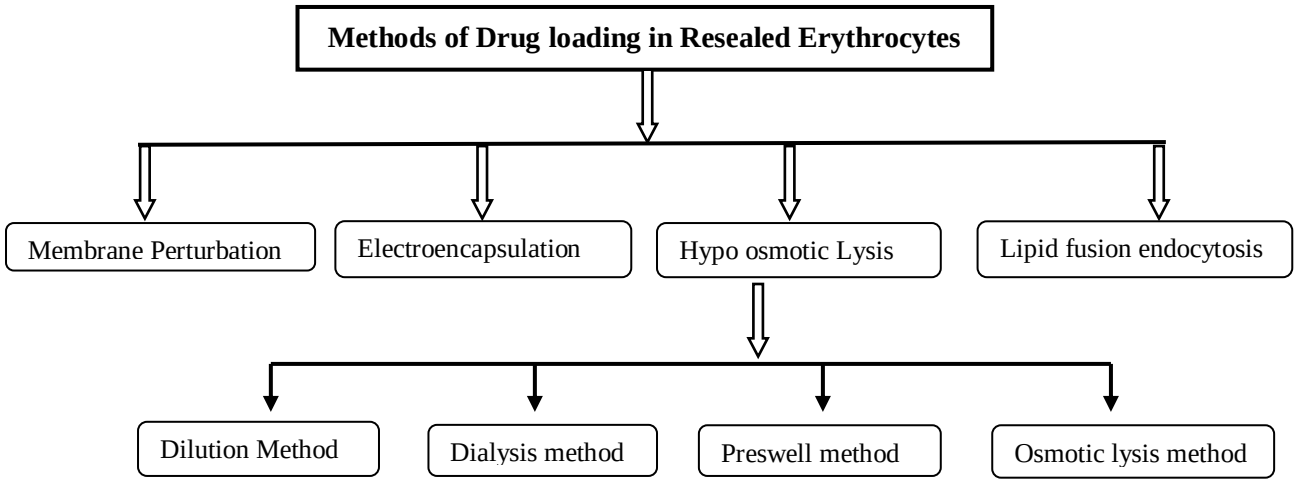
solutions at various hematocrit values as desired and are often stored in acid–citrate–dextrose buffer at 4^0C for a period of 48 hr before use (Banker GS, Rhodes, 2002).

3. ERYTHROCYTES CAN BE USED AS CARRIERS IN TWO WAYS

3.1. 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 intospheres. Such erythrocytes are called Red cell ghosts. Ghosts do not remain in the circulation for a long time as they are quickly sequestered and rapidly phagocytosed by the reticuloendothelial cells in the liver and spleen. The disadvantage of using ghosts is that they can be targeted only to those tissues which contain phagocytic cells (liver and spleen) and not to all other tissues in the body.

3.2. Continuous or prolonged release of drugs: Alternatively, erythrocytes can be used as a continuous or prolonged release system, which provides 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 (Mitchell DH, 1990).

4. VARIOUS METHODS OF PREPARATION OF RESEALED ERYTHROCYTES



When erythrocytes are osmotically lysed and then resealed, there is an exchange of intracellular and extracellular solutes. Therefore, a drug added during the lysis procedures will be encapsulated within the membrane envelope of erythrocytes.

4.1. Methods of drug loading: Several methods can be used to load drugs or other bioactive compounds in erythrocytes, including physical (electrical pulse method) osmosis-based systems, and chemical methods (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 (Pitt E, 1983).

Table 1: Comparison of various hypo-osmotic lysis methods

Method	Percent loading	Advantages	Disadvantages
Dilution method	30-40%	Fastest and simplest especially for low molecular weight drug	Entrapment efficiency is less
Dialysis	30-45%	Better *in vivo* survival of erythrocytes better structural integrity of membrane	Time consuming hetero- genous size distribution of resealed erythrocytes
Preswell dilution	30-90%	Good retention of cytoplasm constituents and good survival in vivo	-
Isotonic Osmotic lysis	-	Better in vivo surveillance	Impemeable only to large molecules, process is time consuming

4.2. Hypotonic hemolysis: This method is based on the ability of erythrocytes to undergo reversible swelling in a hypotonic solution. Erythrocytes have an exceptional capability for reversible 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 milli osmoles /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 (Alvarez, F.J., 1998).

4.3. Use of red cell loader: Novel methods of entrapment of non-diffusible drugs are used to load in to 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 hemofilter and an isotonic resealing of the cells. There was ~30% drug loading with 35–50% cell recovery. The processed erythrocytes had normal survival in vivo. The same cells could be used for targeting by improving their recognition by tissue macrophages (Gutierrez Millan, C, 2004).

4.4. Hypotonic dilution: Hypotonic dilution was the first method investigated for the encapsulation of chemicals into erythrocytes and is the simplest and fastest. In this method, a volume of packed erythrocytes is diluted with 2–20 volumes of aqueous solution of a drug. The solution tonicity is then restored by adding a hypertonic buffer. The resultant mixture is then centrifuged, the supernatant is discarded, and the pellet is washed with isotonic buffer solution. The major drawbacks of this method include low entrapment efficiency and a considerable loss of hemoglobin and other cell 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 ß- galactosidase and ß- glycosidase, asparginase,and arginase, as well as bronchodilators such as salbutamol (Tajerzadeh, H., 2000).

4.4. Hypotonic dialysis: Several methods are based on the principle that semipermeable 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 hematocrit value of 70–80 is prepared and placed in a conventional dialysis tube immersed in 10–20 volumes of a hypotonic buffer. The medium is agitated slowly for 2 h. The tonicity of the dialysis tube is restored by directly adding a calculated amount of a hypertonic buffer to the surrounding medium or by replacing the surrounding medium by isotonic buffer. The drug to be loaded can be added by either dissolving the drug in isotonic cell suspending buffer inside a dialysis bag at the beginning of the experiment or by adding the drug to a dialysis bag after the stirring is complete. 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. This method has been used for loading enzymes such as ß-galactosidase, glucoserebrosidase,asparginase, inositol hexaphosphatase, as well as drugs such as Gentamicin, Adriamycin, Pentamidine and Furamycin, Interlukin-2, Desferroxamine, and human recombinant erythropoietin (Hamidi M, 2003).

4.5. Hypotonic preswelling: This method is simpler and faster than other methods causing minimum damage to cells, drug in capsulated in erythrocytes. This method was developed by Rechsteiner in1975 and was modified by Jenner et al. for drug loading. The technique is based upon initial controlled swelling in a hypotonic buffered solution. This mixture is centrifuged at low g values. The supernatant is discarded and the cell fraction is brought to the lysis point by adding 100–120µL portions of an aqueous solution of the drug to be encapsulated. The mixture is centrifuged between the drug-addition steps. The lysis point is detected by the disappearance of a distinct boundary between the cell fraction and the supernatant upon centrifugation. The tonicity of a cell mixture is restored at the lysis point by adding a calculated amount of hypertonic buffer. Then, the cell suspension is incubated at 37 ºC to re anneal the resealed erythrocytes. Such cells have a circulation half life comparable to that of normal cells. Drugs encapsulated in erythrocytes using this method include Propranolol, Methotrexate, Insulin, Metronidazole, Levothyroxine, Elaprnailat, and Isoniazid (Jaitely V, 1996).

4.6. Isotonic osmotic lysis: This method, also known as the osmotic pulse method, involves isotonic hemolysis that is achieved by physical or chemical means. The isotonic solutions may or may not be isoionic. If erythrocytes are incubated in solutions of a substance with high membrane permeability, the solute will diffuse into the cells because of the concentration gradient. This process is followed by an influx of water to maintain osmotic equilibrium. Chemicals such as urea solution, polyethylene glycol, and ammonium chloride have been used for isotonic hemolysis. However, this method also is not immune to changes in membrane structure composition. In 1987, Franco et al. developed a method that involved suspending erythrocytes in an isotonic solution of dimethyl sulfoxide (DMSO). The suspension was diluted with an isotonic-buffered drug solution. After the cells were separated, they were resealed at 37^{o}C (Pei L, 1994).

4.7. Chemical perturbation of the membrane: This method is based on the increase in membrane permeability of erythrocytes when the cells are exposed to certain chemicals. In 1973, Deuticke *et al* showed that the permeability of erythrocytic membrane increases upon exposure to polyene antibiotic such as amphotericin B. In 1980, this method was used successfully by Kitao and Hattori to entrap the antineoplastic drug Daunomycin in human and

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 (Li, L.H., 1996).

4.8. Electro-insertion or electro encapsulation: 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 20-160µs.An inverse relationship exists between the electric-field intensity and the discharge time. The compound to be entrapped is added to the medium in which the cells are suspended from the commencement of the experiment. The characteristic pore diameter created in the membrane depends upon the intensity of electric field, the discharge time, and the ionic strength of suspending medium. The colloidal macromolecules contents of the cell may lead to cell lysis because of the increase in osmotic pressure. This process can be prevented by adding large molecules (e.g., tetrasaccharide stachyose and bovine serum albumin) and ribonucleose.One advantage of this method is a more uniform distribution of loaded cells in comparison with osmotic methods. The main drawbacks are the need for special instrumentation and the sophistication of the process. Entrapment efficiency of this method is ›35%, and the life span of the resealed cells in circulation is comparable with that of normal cells. Various compounds such as sucrose, urease, Methotrexate, Isoniazid, DNA fragments, and latex particles of diameter 0.2µm can be entrapped within erythrocytes by this method. Mangal and Kaur achieved sustained release of a drug entrapped in erythrocytes with the use of electroporation.

4.9. Entrapment by endocytosis: This method was reported by Schrier *et al*. in1975. Endocytosis involves the addition of one volume of washed 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. The pores created by this method are resealed by using 154 mM of NaCl and incubation at 37^{o}C for 2 min. The entrapment of material occurs by endocytosis. The vesicle membrane separates endocytosed material from cytoplasm thus protecting it from the erythrocytes and vice-versa. 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 (Alvarez-Guerra M, 1998).

4.10. 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 (Hamidi M, 2003).

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

5. EVALUATION

5.1. *In-vitro* properties of loaded erythrocytes

5.1.1. Cell counting and cell recovery: This involves counting the number of red blood cells per unit volume of whole blood, usually by automated counting. Red cell recovery may be calculated on the basis of the differences in the hematocrit and the volume of the suspension of erythrocytes before and after loading. The goal is to minimize the loss during the encapsulation procedure to maximize cell recovery (Hamidi M, 2001).

5.1.2. Morphological aspect: The morphological examination of these ghost erythrocytes is undertaken by comparison with untreated erythrocytes using either transmission (TEM) or scanning (SEM) electron microscopy. By means of electron microscopy observation may be made of the morphological changes in the erythrocytes induced by osmosis-based encapsulation methods, when they are subjected to solutions of different osmolality. Thus, when rat erythrocytes are subjected to isotonic solutions (300 milli osmoles / kg) they reveal the typical morphology of discocyte (biconcave). This evolves to a morphology of stomatocyte (uniconcave) when they are subjected to solutions of 200 milli osmoles/kg, attaining the spherocytic shape (the most fragile of the three) when the solution is of 150 mosM/kg (Gupta A, 2010).

5.1.3. Osmotic fragility: Osmotic fragility is a test to detect abnormal fragility of red blood cells. Untreated or loaded erythrocytes are tested by exposure to hypotonic solutions, making them swell, in order to determine the relative fragility of the red cells (Gupta A, 2010).

5.1.4. Turbulence shock: Turbulence shock enables an evaluation to be made of the stability of the loaded erythrocytes against the turbulence stress exerted by the cells against *in-vivo* circulation turbulence (De Loach R, 1983) (Gupta A, 2010). Where by the suspension of cells are passed several times through a 22-gauge needle.

5.1.5. *In-vitro* drug or peptide release: The encapsulation of many drugs in erythrocytes can give rise to a sustained release of the drug that influences the pharmacokinetic behaviour in vivo of the loaded erythrocytes. In-vitro leakage of the drug from loaded erythrocytes is tested using autologous plasma or an iso-osmotic buffer at 37°C with a hematocrit adjusted between 0.5 % and 50 %. The supernatant is removed at the time intervals previously programmed and replaced by an equal volume of autologous plasma or buffer. Certain authors recommend performing in vitro the release studies from loaded erythrocytes using a dialysis bag. The molecular weight and liposolubility of the substance constitute two factors that have a decisive bearing on the release profile of the active principle from the loaded erythrocytes. Liposoluble drugs may be released from the red cells by a mechanism of passive diffusion. Other drugs may become attached to cell structures and are not released by the diffusion mechanism, requiring the lysis of the cell. Band 3 and glycophorin A are proteins present in high density on the extra-cellular surface of erythrocytes and which may act as potential targets for anchoring via covalent bond formation with different substances. Band 3 plays an important role as a carrier protein for anions (Jaitely V, 1996).

5.1.6. Haemoglobin release: The content of hemoglobin of the erythrocytes may be diminished by the alterations in the permeability of the membrane of the red cells during the encapsulation procedure. Furthermore, the relationship between the rate of hemoglobin and the rate of drug release contributes to interpreting the mechanisms involved in the release of the substance encapsulated from the erythrocytes. The hemoglobin leakage is tested using a red cell suspension by recording the absorbance of supernatant at 540 nm on a spectrophotometer (Vyas SP, 1999).

5.1.7. *In vitro* stability: The stability of the loaded erythrocytes is assessed by means of the incubation of the cells in autologous plasma or in an iso-osmotic buffer, setting the hematocrit between 0.5% and 5% at temperatures of 4^0 and 37^0C. The mechanism of resealed erythrocytes shows potential for a safer and sure delivery of various drugs for active and passive targeting. The authors have reviewed the explanations of the different method of drug loading and their characterization parameters for resealed erythrocytes. However more research and inputs are required for the cell based drug delivery systems to open a new perspective to the possibility of using cells for therapeutic purposes. The authors are confident that erythrocytes have great potentialities in the field of drug delivery, since the key to success of much therapeutics greatly depends on the development of novel technologies to improve and control the delivery of drugs (Kravtzoff R, 1990).

5.1.8. *In vitro* characterization: The *in vivo* performance of resealed erythrocytes is affected to a great extent by their biological properties. Hence, in vitro characterization forms an important part of studies involving such cellular carriers.

5.1.8.1. Physical characterization: Shape, surface morphology, vesicle size and size distribution of drug loaded erythrocytes can be studied by using transmission electron microscopy, scanning electron microscopy, phase contrast microscopy, optical microscopy (DeLoach, 1983). Drug release from the erythrocytes can be carried using diffusion cell method. Surface charge of the erythrocytes can be measured by zeta potential measurement (Vyas SP and Khar RK, 2002).

5.1.8.2. Cellular characterization: Percentage Hb content is determined by deproteinization of cell membrane followed by hemoglobin assay. Percentage cell recovery is carried out by Neubaur's chamber and hematological analyzer. Osmotic fragility test involves stepwise incubation with isotonic to hypotonic saline solutions and determination of drug and hemoglobin assay. Osmotic shock is determined by dilution with distilled water and estimation of drug and hemoglobin. Turbulent shock is determined by Passage of cell suspension through 30-gauge hypodermic needle at 10 mL/min flow rate and estimation of residual drug and hemoglobin, vigorous shaking followed by hemoglobin estimation Erythrocyte sedimentation rate by ESR methods (Schrier SL, 1975).

5.2. Biological characterization: Biological characterization of the developed erythrocytes includes sterility test, pyrogenicity test, LAL test and toxicity tests. The morphology of erythrocytes decides their life span after administration. Light microscopy reveals no observable change in resealed cells but in few cases spherical erythrocytes (spherocytes) are detected. Scanning electron microscopic studies will show that a majority of the cells maintain their biconcave discoid shapes after the loading procedure, and few stomatocytes, a form of spherocytes. In some cases, cells of smaller size (microcyte) are also observed. Shape change (deformability) is another factor that affects the life span of the cells.

This parameter evaluates the ease of passage of erythrocytes through narrow capillaries and the RES. It determines the rheological behavior of the cells and depends on the 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. The osmotic fragility of resealed erythrocytes is an indicator of the possible changes in cell membrane integrity and the

resistance of these cells to osmotic pressure of the suspension medium. The test is carried out by suspending cells in media of varying sodium chloride concentration and determining the hemoglobin released. In most cases, osmotic fragility of resealed cells is higher than that of the normal cells because of increased intracellular osmotic pressure. The turbulence fragility is yet another characteristic that depends upon changes in the integrity of cellular membrane and reflects resistance of loaded cells against hemolysis resulting from turbulent flow within circulation. It is determined by the passage of cell suspension through needles with smaller internal diameter(e.g., 30 gauges) or vigorously shaking the cell suspension. In both cases, hemoglobin and drug released after the procedure are determined. The turbulent fragility of resealed cells is found to be higher. Routine clinical hematological tests also can be carried out for drug-loaded cells, including mean corpuscular volume, mean corpuscular hemoglobin, mean corpuscular hemoglobin content. Studies have shown that the average size and hemoglobin content of resealed cells is lower than that of normal cells (Bhaskaran, S., 1995).

5.3. Drug release kinetics: 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:

- The rate of drug release is considerably higher than that of hemoglobin. In other words, drug diffuses readily. Such a pattern is shown by lipophilic drugs, including Methotrexate, Phenytoin, Dexamethasone, Primpquin, and Vitamin B12. Cell lysis is not essential for the release of such drugs.
- The rate of drug release is comparable to that of hemoglobin. This indicates that cell lysis is essential for drug release and drug cannot be released by mere diffusion. Polar drugs such as Gentamicin, Heparin, and Enalaprilat, and enzymes such as Asparginase, Peptides, including Urogasterone and l-lysine-l-phenylalanine follow such pattern.
- The rate of drug release lies between the above mentioned two extremes; for example, propranolol, isoniazid, metronidazole, and recombinant human erythropoietin.

The two factors that determine the drug release pattern are size and polarity of the drug molecule. The release rate can be modified by cross-linking cell membrane with gluteraldehyde, which results in a slower drug release. This can also be achieved by entrapping biodegradable prodrug such as o-acetyl propranolol, o-pivaloyl propranolol, cortisol-21- phosphate, prednisolone-21-sodium succinate, and cytosine arabinoside monophosphate. The complexation of a drug with macromolecules such as dextran and albumin also retard the release rate.

5.3.1. Mechanism of release of resealed erythrocytes: The rate of diffusion depends upon the rate at which a particular molecule penetrates through a lipid bilayer. Many substances enter cells by as pecific membrane protein system because the carriers are proteins with many properties analogous to that of enzymes, including specificity e.g. nucleotides and nucleosides. Release of drugs from erythrocytes is rapid followed by sustained release profile and rate of exit is proportional to the instantaneous intra cellular drug concentration (first order kinetics). By incorporating polymer into erythrocytes, the release pattern may be modified. The drug, however, could be resealed from macrophages after phagocytosis if the linkage is susceptible to lysosomal enzymes (Rossi, L., 2005).

- Phagocytosis
- Diffusion through the membrane of cell
- By using a specific transport system.

6. IN VITRO STORAGE

The success of resealed erythrocytes as a drug delivery system depends to a greater extent on their in vitro storage. Preparing drug-loaded erythrocytes on a large scale and maintaining their survival and drug content can be achieved by using suitable storage methods. However, the lack of reliable and practical storage methods has been a limiting factor for the wide-spread clinical use of the carrier erythrocytes. The most common storage media include Hank's balanced salt solution and acid–citrate–dextrose at 4 ^{o}C. Cells remain viable in terms of their physiologic and carrier characteristics for at least 2 weeks at this temperature. The addition of calcium-chelating agents or the purine nucleosides improve circulation survival time of cells upon reinjection. Exposure of resealed erythrocytes to membrane stabilizing agents such as dimethyl sulfoxide, dimethyl, 3,3-di-thio-bispropionamide, gluteraldehyde, toluene-2-4-diisocyanate followed by lyophilization or sintered glass filtration has been reported to enhance their stability upon storage. The resultant powder was stable for at least one month without any detectable changes. But the major disadvantage of this method is the presence of appreciable amount of membrane stabilizers in bound form that remarkably reduces circulation survival time. Other reported methods for improving storage stability include encapsulation of a prodrug that undergoes conversion to the parent drug only at body temperature, high glycerol freezing technique, and reversible immobilization in alginate or gelatin gels (Price RJ, 1998).

7. CROSSLINKING, STABILITY AND *IN-VIVO* SURVIVAL OF RESEALED ERYTHROCYTES

The cells treated with dimethyl sulphoxide (DMSO), toluene 2, 4-di-isocyanate (TD1) and gluteraldehyde are even resistant to sonication, freezing and thawing. Chemically cross linking of erythrocytes renders a yield of 55-97% of non-lysed cells. An attempt was made to get drug loaded cells in lyophilized form. The dried powder was filled in amber color glass vials and stored at 4ϒC for one month. Improvement in shelf-life of the carrier

erythrocytes was achieved by storing the cells in powder form ready for reconstitution at 4ϒC. This is important in the large scale manufacturing of drug loaded erythrocytes (Mangal PC, 1991).

8. *IN VIVO* LIFE SPAN

The efficacy of resealed erythrocytes is determined mainly by their survival time in circulation upon reinjection. For the purpose of sustained action, a longer life span is required, although for delivery to target-specific RES organs, rapid phagocytosis and hence a shorter life span is desirable. The life span of resealed erythrocytes depends upon its size, shape, and surface electrical charge as well as the extent of hemoglobin and other cell constituents lost during the loading process. The various methods used to determine in vivo survival time include labeling of cells by ^{51}Cr or fluorescent markers such as fluorescin isothiocyanate or entrapment of ^{14}C sucrose or gentamicin. The circulation survival kinetics of resealed erythrocytes show typical bimodal behavior with a rapid loss of cells during the first 24 h after injection, followed by a slow decline phase with a half life on the order of days or weeks. The early loss accounts for ~15–65% loss of total injected cells. The erythrocytic carriers constructed of red blood cells of mice, cattle, pigs, dogs, sheep, goats, and monkeys exhibit a comparable circulation profile with that of normal unloaded erythrocytes. On the other hand, resealed erythrocytes prepared from red blood cells of rabbits, chickens, and rats exhibit relatively poor circulation profile (Talwar N, 1992).

9. APPLICATIONS OF RESEALED ERYTHROCYTES

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 (Moorjani M, 1996).

9.1. 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 (Magnani M, 1998).

9.2. 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 (Hamidi M, 2007).

9.3. 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, Gluteraldehyde, Sialic acid, Sulphydryl and Surface chemical cross-linking e.g. delivery of 125 I-labeled carbonic -anhydrase loaded in erythrocytes cross-linked with sulfosuccinmidyl propionate (Hamidi M, 2001).

9.4. 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 ß-glucosidase, ß-glucoronidase, ß-galactosidase. The disease caused by an accumulation of glucocerebrosides in the liver and spleen can be treated by glucocerebrosidase- loaded erythrocytes (Hamidi M, 2001).

9.5. Treatment of hepatic tumors: Hepatic tumors are one of the most prevalent types of cancer. Antineoplastic drugs such as methotrexate, bleomycin, asparginase and adriamycin have been successfully delivered by erythrocytes. Agents such as daunorubicin diffuse rapidly from the cells upon loading and hence pose a problem. This problem can be overcome by covalently linking daunorubicin to the erythrocytic membrane using gluteraldehyde or cis-aconitic acid as a spacer. The resealed erythrocytes loaded with carboplatin show localization in liver (Hamidi M, 2001).

9.6. 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.

9.7. Removal of res iron overload: Desferrioxamine-loaded erythrocytes have been used to treat excess iron accumulated because of multiple transfusions to thalassemic patients. Targeting this drug to the RES is very beneficial because the aged erythrocytes are destroyed in RES organs, which results in an accumulation of iron in these organs.

9.8. 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.
9.9. 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, photosensitive material along with the drug and Antibody attachment to erythrocyte membrane to get specificity of action. Zimmermann proposed that the entrapment of small paramagnetic particles into erythrocytes might allow their localization to a particular location under the influence of an external magnetic field. Jain and Vyas reported entrapment of the anti-inflammatory drugs Diclofenac sodium and Ibuprofen in magnetoresponsive erythrocytes. Photosensitized erythrocytes have been studied as a phototriggered carrier and delivery system for Methotrexate in cancer treatment. Rossi L *et al* have reported *in-vitro* targeting of erythrocytes to cytotoxic T-cells by coupling of Thy-1.2 monoclonal antibody. Price *et al.* reported delivery of colloidal particles and erythrocytes to tissue through microvessel ruptures created by targeted microbubble destruction with ultrasound. IV fluorescent erythrocytes were delivered to the interstitium of rat skeletal muscle through microvessel ruptures by insonifying microbubbles in vivo. This technique provides a noninvasive means for delivering resealed erythrocytes across the endothelial carrier to the target tissue. Other approaches for targeting organs outside the RES include the preparation of carrier erythrocytes fused to thermoresponsive liposomes and their localization using an external thermal source, intraperitoneal injection of resealed erythrocytes for drug targeting to peritoneal macrophages, and lectin pretreatment of resealed cells loaded with antineoplastic drugs to improve targeting tumor cells.
9.10. 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 where as 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, Acyclovir, and Fludarabine phosphate.
9.11. 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 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 ß-glucoserebrosidase for the treatment of Gaucher's disease. The disease is characterized by inborn deficiency of lysosomal ß-glucoserebrosidase in cells of RES there by leading to accumulation of ß-glucoserebrosides in macrophages of the RES. The most important application of resealed erythrocytes in enzyme therapy is that of asparginase loading for the treatment of pediatric neoplasms. This enzyme degrades aspargine, an amino acid vital for cells. This treatment prevents remission of pediatric acute lymphocytic leukemia. There are reports of improved intensity and duration of action in animal models as well as humans. To treat lead poisoning, the concentration of ß-aminolevulinate dehydrogenase (ALA-D) in erythrocytes decreases. This leads to an accumulation of ß-aminolevulinic acid in tissues, blood, and urine. This state leads to acute porphyria and CNS related problems. An injection of resealed erythrocytes loaded with ALA–D to lead intoxicated animal significantly reduces toxic manifestations. Other enzymes used for loading resealed erythrocytes include urease, galactose-1-phosphate uridyl transferase, uricase and acetaldehyde dehydrogenase.
9.12. Improvement in oxygen delivery to tissues: Hemoglobin is the protein responsible for the oxygen-carrying capacity of erythrocytes. Under normal conditions, 95% of hemoglobin is saturated with oxygen in the lungs, whereas under physiologic conditions in peripheral blood stream only ~25% of oxygenated hemoglobin becomes deoxygenated. Thus, the major fraction of oxygen bound to hemoglobin is recirculated with venous blood to the lungs. The use of this bound fraction has been suggested for the treatment of oxygen deficiency. 2, 3-Diphosphoglycerate (2, 3-DPG) is a natural effector of hemoglobin. The binding affinity of hemoglobin for oxygen changes reversibly with changes in intracellular concentration of 2, 3-DPG. This compensates for changes in the oxygen pressure outside of the body, as the affinity of 2, 3-DPG to oxygen is much higher than that of hemoglobin.

Other organic polyphosphates can serve as allosteric effectors of hemoglobin with binding affinities higher than those of 2, 3-DPG and can compete with 2, 3-DPG for binding to hemoglobin. Inositol hexophosphate (IHP) is one of the strongest effectors of this type. However, because of its ionization at physiologic pH, it cannot enter erythrocytes. Hence, it is entrapped by the electroporation process. Upon encapsulation, IHP irreversibly binds to hemoglobin, thereby decreasing the oxygen affinity to hemoglobin 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 50mm Hg. In the presence of IHP encapsulated in erythrocytes, the difference between the oxygen bound fraction of hemoglobin in lungs and tissues increases, thereby increasing the oxygen concentration in tissues. Also, the extent of carbamate formed in the N-terminal amine group of ß-chain of hemoglobin decreases, which is compensated by an uptake of H^+ and CO_2 that leads to increased formation of bicarbonate ion. Intravenous (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 used.

An application of IHP-loaded erythrocytes for improved oxygen supply is beneficial under the following conditions, such as high altitude conditions where the partial pressure of oxygen is low, reduction in the number of alveoli, where exchange surface of the lungs is decreased, increased resistance to oxygen diffusion in the lungs, reduction in oxygen transport capacity, mutation or chemical modification, which involves a decrease in oxygen affinity for hemoglobin, increased radiosensitivity of radiation-sensitive tumors, restoration of oxygen-delivery capacity of stored blood and ischemia of myocardium, brain, or other tissues.

9.13. Microinjection of macromolecules: Biological functions of macromolecules such as DNA, RNA, and proteins are exploited for various cell biological applications. Hence, various methods are used to entrap these macromolecules into cultured cells (e.g., microinjection). A relatively simple structure and a lack of complex cellular components (e.g., nucleus) in erythrocytes make them good candidates for the entrapment of macromolecules.In microinjection, erythrocytes are used as microsyringes for injection to the host cells. 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 cosuspended 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 a 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 (Kravtzoff R, 1990).

10. NOVEL APPROACHES

10.1. Erythrosomes: These are specially engineered vesicular systems that are chemically cross-linked to human erythrocytes support upon which a lipid bilayer is coated (Vyas SP and Dixit VK, 1999). This process is achieved by modifying a reverse-phase evaporation technique. These vesicles have been proposed as useful encapsulation systems for macromolecular drugs.

10.2. Nanoerythrosomes: These are prepared by extrusion of erythrocyte ghosts to produce small vesicles with an average diameter of 100 nm (Jaitely V, 1996). Daunorubicin was covalently conjugated to nanoerythrosomes using gluteraldehyde spacer. This complex was more active than free Daunorubicin alone, both *in vitro* and *in vivo.*

11. 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 routine drug delivery system. The same concept also can be extended to the delivery of biopharmaceuticals and much remains to be explored regarding the potential of resealed erythrocytes.

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