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Review Article

Drug Targeting By Erythrocytes: A Carrier System

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Abstract: Carrier erythrocytes is resealed erythrocytes loaded by a drug or by other therapeutic Agents, have been exploited extensively in recent years for both temporally and spatially controlled delivery of a wide variety of drugs and other bioactive agent sowing to their remarkable degree of biocompatible, biodegradability and a series of potential advantages. Biopharmaceuticals, therapeutically significant peptides and proteins, nucleic acid-based biological, antigens and vaccines, are among the recently focused pharmaceuticals for being delivered using carrier erythrocytes. In this articles the potential application of erythrocytes in drug deliver drug deliver have been reviewed with a particular stress on the studies and laboratory experiences on successful erythrocyte loading and characterization of different classes of drugs.
Keywords: Resealed Erythrocytes, Cellular Carriers, Carrier Erythrocytes, Carrier RBCs.

INTRODUCTION

Drug delivery is now entering quite an exciting and challenging era. Significantly high cost involves in the development of new drug molecules has compelled scientists all over the world to search for alternative ways of administering, the existing drug molecules with enhanced effectiveness. Improper drug administration in-side the biological system not only causes the distress to the other body tissues but also demands more therapeutic molecules to elicit the appropriate response. Among the various carriers used for targeting drug 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 fibroblast etc. have been proposed as cellular carrier systems [1,2]. 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 to cure of many chronic diseases, including cancer, diabetes mellitus, rheumatoid arthritis, HIV infection, and drug addiction.

ERYTHROCYTES

The mature human erythrocytes is a circular, biconcave, non-nucleated disc. The edges are rounded and thicker than the centre. Hence, the central portion appears to have a lighter shade. When view from side it look like a dum-bell. The mature red cells is soft and flexible and can readily squeeze through narrow capillaries. Inside the corpuscles there is a framework, chiefly composed of protein and lipids. The meshes of this framework remain filled up with haemoglobin. Under the microscope a single red cell seems to have a light brown or yellowish colour. But when seen in bulk, the red cells appear to be red [3].

Composition of Erythrocytes

Each cell is composed of a colourless envelope enclosing semiliquid material, 65% water and 35% solids of which 33% is haemoglobin bound to 2% stromal messwork of protein, phospholipid, cholesterol, cholesterol esters and neutral fat. Other organic substances, such as urea, amino acids, creatinine, adenyl pyrophosphates, diphosphoglycerates, etc., are also present, but in very small amounts. Of total lipids 60% is phospholipid, 30% free cholesterol and 10% fats and cholesterol esters. Of the salts in the corpuscles, potassium phosphate is the chief [3].

Resealed Erythrocyte

Erythrocytes, the most abundant cells in the human body, have potential carrier capabilities for the delivery of drugs. Erythrocytes are biocompatible, biodegradable, possess long circulation half lives, and can be loaded with a variety of biologically active compounds using various chemical and physical methods.

Erythrocytes have been extensively studied for their potential carrier capabilities for the delivery of drugs and drug-loaded microspheres. Such drug-loaded carrier erythrocytes are simply prepared by collecting blood samples from the interested organism, separating erythrocytes from plasma, entrapping drug in erythrocytes, and resealing the resultant cellular carriers. Hence, these carriers are called resealed erythrocytes. The overall process is based on the response of these cells under osmotic conditions. Upon

reinjection, the drug-loaded erythrocytes serve as slow circulating depots and target the drugs to a reticuloendothelial system [RES].

Sources 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 [4].

Isolation of Erythrocytes

To isolate erythrocytes, blood is collected in heparinized tubes by venipuncture. Fresh whole blood is typically used for loading purposes because the encapsulation efficiency of the erythrocytes isolated from fresh blood is higher than that of the aged blood. Fresh whole blood is the blood that is collected and immediately chilled to 4 _C and stored for less than two days. The erythrocytes are then harvested and washed by centrifugation. The washed cells are suspended in buffer solutions at various hematocrit values as desired and are often stored in acid–citrate–dextrose buffer at 4ºC for as long as 48 h before use[4, 5].

Advantages of Erythrocytes As Drug Carriers

1. Their biocompatibility, particularly when autologous cells are used, hence no possibility of triggered immune response [4, 6, 8, 14,19]
2. Their biodegradability with no generation of toxic products [4, 6, 11, 14, 19, 20]
3. The considerably uniform size and shape of the carrier [9, 10, 21]
4. Relatively inert intracellular environment [22]
5. Prevention of degradation of the loaded drug from inactivation by endogenous chemicals [14, 18, 20, 23, 24]
6. The wide variety of chemicals that can be entrapped [14, 23, 25–27]
7. The modification of pharmacokinetic and pharmacodynamic parameters of drug [8, 17, 20, 23]
8. Attainment of steady-state plasma concentration decreases fluctuations in concentration [6, 8, 16, 31, 32]
9. Protection of the organism against toxic effects of drugs [e.g. antineoplastics, 23].
10. Their ability to circulate throughout the body [4]
11. The availability of the techniques and facilities for separation, handling, transfusion, and working with erythrocytes [6, 14]
12. The prevention of any undesired immune response against the loaded drug [22]
13. Their ability to target the organs of the RES [4, 6, 14, 30]
14. The possibility of ideal zero-order drug-release kinetics [24]
15. The lack of occurrence of undesired immune response against encapsulated drug [4]
16. The large quantity of drug that can be encapsulated within a small volume of cells ensures dose sufficiency [4, 14, 19]
17. A longer life span in circulation as compared with other synthetic carriers [7, 18, 28], and optimum conditions may result in the life span comparable to that of normal erythrocytes [15, 29, 30]
18. Easy control during life span ranging from minutes to months [18]
19. A decrease in side effects of drugs [6, 17, 32]
20. A considerable increase in drug dosing interval with drug residing in therapeutic window region for longer time periods [6, 17, 30, 31].

Disadvantages

1. Co-introduction of the erythrocyte membranes, viral envelopes, viral RNA and residual haemoglobin may have unpredicted effects on the cells.
2. A comparatively larger amount of test material is desired than that for the microcapillary method
3. Direct injection into the cell nucleus is not feasible.
4. They have a limited potential as carrier to non phagocyte target tissue.
5. Possibility of clumping of cells and dose dumping nay be there.[99,100]

Erythrocytes Can Be Used As Carriers In Two Ways

1. Targeting potential tissue/organ
2. For continuous or prolonged release of drugs.

For targeting, only the erythrocyte membrane is used. This is obtained by splitting the cell in hypotonic solution and after introducing the drug into the cells, allowing them to reseal into sphere.

Methods Of Preparation Of Resealed Erythrocytes

1.Hypotonic hemolysis
2.Hypotonic dilusion
3. Hypotonic preswelling
4. Hypotonic dialysis
5. Isotonic osmotic lysis
6. Chemical perturbation of the membrane
7. Electro insertion
8. Entrapment by endocytosis
9. Loading by electric cell fusion
10. Loading by lipid fusion.

1. 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 mosm/kg, above which the membrane ruptures, releasing the cellular contents. At this point [just before cell lysis], some transient pores of 200–500 Å are generated on the membrane. After cell lysis, cellular contents are depleted. The remnant is called an *erythrocyte ghost* [8, 26, 34, 35].

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 [26, 34, 35].

Use of Red Cell Loader

Magnani et al. developed a novel method for entrapment of nondiffusible drugs into erythrocytes. They developed a piece of equipment called a "red cell loader" [**36**].With as little as 50 mL of a blood sample, different biologically active compounds were entrapped into erythrocytes within a period of 2 h at room temperature under blood banking conditions. The process is based on two sequential hypotonic dilutions of washed erythrocytes followed by concentration with a hemofilter and an isotonic resealing of 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.

2. Hypotonic dilution

Hypotonic dilution was the first method investigated for the encapsulation of chemicals into erythrocytes [12] and is the simplest and fastest [8]. 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 [12, 26]. The major drawbacks of this method include a low entrapment efficiency [12, 14, 37–39] and a considerable loss of hemoglobin and other cell components [7, 20, 25]. 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 [12, 26]. Hypotonic dilution is used for loading enzymes such as -galactosidase and -glucosidase [12], asparginase [15, 40], and arginase [22], as well as bronchodilators such as salbutamol [41].

3. Hypotonic preswelling

This method was developed by Rechsteiner [42] in 1975 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 100–120 _L 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 37ºC to reanneal the resealed erythrocytes [26, 37]. Such cells have a circulation half life comparable to that of normal cells [8, 26, 37, 43]. This method is simpler and faster than other methods, causing minimum damage to cells. Drugs encapsulated in erythrocytes using this method include propranolol [16], asparginase [21], cyclopohphamide, cortisol-21-phosphate [32, 37], antitrypsin [37], methotrexate, insulin [37, 45], metronidazole [39], levothyroxine [43], enalaprilat [44], and isoniazid [46].

4. Hypotonic dialysis

This method was first reported by Klibansky [47] in 1959 and was used in 1977 by Deloach and Ihler [38], and Dale [48] for loading enzymes and lipids. 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 [26, 38]. 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 [24, 26, 49, 40] or by adding the drug to a dialysis bag after the stirring is complete [34, 38, 51-54]. The use of standard hemodialysis equipment for loading a drug in erythrocytes was reported by Roper et al. [55]. In this method, the erythrocyte suspension and the drug to be loaded was 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 [34, 50, 55–60]. The loaded cells exhibit the same circulation half life as that of normal cells [7, 8]. Also, this method has high entrapment efficiency on the order of 30–50% [7, 14, 34, 38], cell recovery of 70–80%, high-loading capacity [51, 55], and is amenable to automation with control of process variables [34, 61]. The drawbacks include a

long processing time [7, 14, 38] and the need for special equipment [27]. This method has been used for loading enzymes such as - galactosidase, glucoserebrosidase [37], asparginase [50], inositol hexaphosphatase [57, 59, 60], as well as drugs such as gentamicin [24], adriamycin [49], pentamidine and furamycin [52], interlukin-2 [53], desferroxamine [51, 55, 56, 58], and human recombinant erythropoietin [62].

5.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 [63], polyethylene glycol [64], 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] [65]. The suspension was diluted with an isotonic-buffered drug solution. After the cells were separated, they were resealed at 37ºC.

6.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 [66]. In 1980, this method was used successfully by Kitao and Hattori to entrap the antineoplastic drug daunomycin in human and mouse erythrocytes [67]. Lin et al. [68] used halothane for the same purpose.However, these methods induce irreversible destructive changes in the cell membrane and hence are not very popular.

7. Electro-insertion or electroencapsulation

In 1973, Zimmermann tried an electrical pulse method to encapsulate bioactive molecules [13]. 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 [69]. The erythrocyte membrane is opened by a dielectric breakdown. Subsequently, the pores can be resealed by incubation at 37 _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 [7, 30]. The optimum intensity of an electric field is between 1–10 kW/cm and optimal discharge time is between 20–160 _s [6, 7, 71–73]. An inverse relationship exists between the electric-field intensity and the discharge time [5, 30]. 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 [30, 71, 104]. 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., tetrasaccharidestachyose and bovine serum albumin] and ribonucleose. One advantage of this method is a more uniform distribution of loaded cells in comparison with osmotic methods [7].

The main drawbacks are the need for special instrumentation and the sophistication of the process [6, 14, 19, 34]. Entrapment efficiency of this method is _35% [31], and the life span of the resealed cells in circulation is comparable with that of normal cells [7, 30]. Various compounds such as sucrose [30, 70], urease [70], methotrexate [73], isoniazid [74], human glycophorin [75], DNA fragments, and latex particles of diameter 0.2 _m [7] 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 [76].

8. Entrapment by endocytosis.

This method was reported by Schrier et al. in 1975 [77]. Endocytosis involves the addition of one volume of washed packed erythrocytes to nine volumes of buffer containing 2.5 mM ATP, 2.5 mM MgCl2, and 1mM CaCl2, followed by incubation for 2 min at room temperature. The pores created by this method are resealed by using 154 mM of NaCl and incubation at 37 _C for 2 min. The entrapment of material occurs by endocytosis. The vesicle membrane separates endocytosed material from cytoplasm thus protecting it from the erythrocytes and vice-versa. 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 [69, 78, 79].

9. 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 [73, 80]. 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.

10. Loading by lipid fusion

Lipid vesicles containing a drug can be directly fused to human erythrocytes, which leads to an exchange with a lipid-entrapped drug. This technique was used for entrapping inositol monophosphate to improve the oxygen carrying capacity of cells [81]. However, the entrapment efficiency of this method is very low [-1%].

***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.

Parameter Method/instrument used

I. Physical characterization

Shape and surface morphology Transmission electron microscopy, scanning electron microscopy, phase contrast microscopy, optical microscopy. Vesicle size and size distribution Transmission electron microscopy, optical microscopy. Drug release Diffusion cell, dialysis Drug content Deproteinization of cell membrane followed by assay of resealed drug, radiolabelling Surface electrical potential Zeta potential measurement Surface pH pH-sensitive probes Deformability Capillary method

II. Cellular characterization

% Hb content Deproteinization of cell membrane followed by hemoglobin assay Cell volume Laser light scattering % Cell recovery Neubaur's chamber, hematological analyzer Osmotic fragility Stepwise incubation with isotonic to hypotonic saline solutions and determination of drug and hemoglobin assay Osmotic shock Dilution with distilled water and estimation of drug and hemoglobin Turbulent shock 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 ESR methods

III. Biological characterization

Sterility test Pyrogenicity Rabbit method, LAL test Animal toxicity Toxicity tests rameters and the techniques applied for their determination. The morphology of erythrocytes decides their life span after administration. Light microscopy reveals no observable change in resealed cells [19, 38] but in few cases spherical erythrocytes [spherocytes] are detected [31, 46]. Scanning electron microscopic studies have shown that a majority of the cells maintain their biconcave discoid shapes after the loading procedure [54], and few stomatocytes—a form of spherocytes with an invagination in one point—are formed [62]. In some cases, cells of smaller size [microcyte] are also observed [60]. 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 [51]. The deformability is measured by passage time of definite volume of cells through capillary of 4 _m diameter or polycarbonate filter with average pore size of 45 _m [51, 55]. Another indirect approach is to evaluate chlorpromazine induced shape changes turbidimetrically [82]. 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 [19, 82]. In most cases, osmotic fragility of resealed cells is higher than that of the normal cells [19, 31, 39, 46, 62, 82] 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 gauge] [19, 31, 39, 46] or vigorously shaking the cell suspension [82]. In both cases, hemoglobin and drug released after the procedure are determined. The turbulent fragility of resealed cells is found to be higher [19, 31, 39, 46, 82]. 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 [12, 15, 50, 54, 55, 61, 62, 82]. Drug content of the cells determines the entrapment efficiency of the method used. The process involves deproteinization of packed, loaded cells [0.5 mL] with 2.0 mL acetonitrile and centrifugation at 2500 rpm for 10 min. The clear supernatant is analyzed for the drug content [83]. The most important parameters for evaluation of resealed erythrocytes is 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 [6], phenytoin, dexamethasone [17], primpquin [19], and vitamin B12 [84]. 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 can not be released by mere diffusion. Polar drugs such as gentamicin [17, 24], heparin [17], and enalaprilat [35], and enzymes such as

asparginase [14, 21, 40, 50], peptides, including urogasterone and l-lysine-l-phenylalanine [85] follow such pattern.

- The rate of drug release lies between the above mentioned two extremes; for example, propranolol [17], isoniazid [31, 46], metronidazole [39], and recombinant human erythropoietin [62].

The two factors that determine the drug release pattern are size and polarity of the drug molecule [17]. The release rate can be modified by cross-linking cell membrane with gluteraldehyde [29, 39], which results in a slower drug release. This can also be achieved by entrapping biodegradable prodrug such as o-acetyl propranolol, o-pivaloyl propranolol [16], cortisol-21-phosphate [32, 37], prednisolone-21-sodium succinate [32], and cytosine arabinoside monophosphate [86]. The complexation of a drug with macromolecules such as dextran and albumin also retard the release rate [35].

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 methotrixate, phenytoin, dexamethasone, primpquin, and vitamin B12. Cell lysis is not essential for the release of such drugs.

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***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 [16]. The most common storage media include Hank's balanced salt solution [6, 14, 20, 82, 85] and acid–citrate–dextrose [7] at 4 _C. Cells remain viable in terms of their physiologic and carrier characteristics for at least 2 weeks at this temperature [85].

The addition of calcium-chelating agents [16] or the purine nucleosides [16, 85] 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 [8, 19, 39, 41]. 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 [19], high glycerol freezing technique [55, 56], and reversible immobilization in alginate or gelatin gels [6, 7, 20].

***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 [7]. The various methods used to determine in vivo survival time include labeling of cells by 51Cr or fluorescent markers such as fluorescin isothiocyanate or entrapment of 14C sucrose or gentamicin [24, 37, 43].

The circulation survival kinetics of resealed erythrocytes show typical bimodal behavior with a rapid loss of cells during the first24 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 [7, 8, 14, 40, 54, 55, 60, 88]. 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 [4].

Mechanism of drug release of released erythrocytes [118]

1. Phagocytosis
2. Diffusion through the cell membrane
3. Using a specific transport system the rate of diffusion depends upon the rate at which a particular molecule penetrates through a lipid bilayer. Many substances enter cells by a specific 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 intracellular drug concentration [first order kinetics]. By incorporating polymer into erythrocytes, the release pattern may be modified. The drug however, could be released from macrophages after phagocytosis if the linkage is susceptible to lysosomal enzymes.

Application 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 [6, 8, 56, 89–91].

1. Slow drug release

Erythrocytes have been used as circulating depots for the sustained delivery of antineoplastics [15, 21, 40,49, 50, 92–95], antiparasitics [18, 38, 52], veterinary antiamoebics[14], vitamins [84], steroids [29, 32, 36], antibiotics [31,46, 66], and cardiovascular drugs [44, 82, 96].

The various mechanisms proposed for drug release include

- Passive diffusion
- Specialized membrane associated carrier transport
- Phagocytosis of resealed cells by macrophages of RES, subsequent accumulation of drug into the macrophage interior, followed by slow release [8, 19].
- Accumulation of erythrocytes in lymph nodes upon subcutaneous administration followed by hemolysis to release the drug [53].

2. Drug targeting

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

3. Targeting RES organs

The various approachesto modify the surface characteristics of erythrocytes include

surface modification with antibodies
surface modification with gluteraldehyde
surface modification with carbohydrates such as sialic acid [98]
surface modification with sulphydryl
surface chemical cross-linking

4. Targeting the liver- enzymes deficient replacement therapy

The enzymes used include -glucosidase, _-glucoronidase, _-galactosidase [14, 24, 38]. The disease caused by an accumulation of glucocerebrosides in the liver and spleen can be treated by glucocerebrosidase-loaded erythrocytes [101].

5. Treatment of hepatic tumors

Antineoplastic drugs such as methotrexate [7, 14], bleomycin [13], asparginase [14, 15], and adriamycin [14, 49, 102, 103] have been successfully delivered by erythrocytes.

6. Treatment of parasitic disease

For erythrocytes loaded with antimalarial [18], antileishmanial [18, 24, 52], and antiamoebic drugs [14, 39].

7. Removal of RES iron overload

Desferrioxamine-loaded erythrocyteshave been used to treat excess iron accumulated because of multiple transfusions to thalassemic patients [14, 52].

8. Removal of toxic agents

Cannon et al. reported inhibition of cyanide intoxication with murine carrier erythrocytes containing bovine rhodanase and sodium thiosulfate [105]. Antagonization of organophosphorus intoxication by resealed erythrocytes containing a recombinant phosphodiestrase also has been reported.

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 along with the drug
- entrapment of photosensitive material
- the use of ultrasound waves
- antibody attachment to erythrocyte membrane to get specificity of action

10. Delivery of antiviral agents

Resealed erythrocytes have been used to deliver deoxycytidine derivatives [35], recombinant herpes simplex virus type 1 [HSV-1] glycoprotein B [106], azidothymidine derivatives [107], azathioprene, acyclovir [108], and fludarabine phosphate [109].

11. Enzyme therapy

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 [4, 11]. An injection of resealed erythrocytes loaded with aminolevulinate dehydrogenase [ALA–D] to lead intoxicated animal significantly reduces toxic manifestations [112].

Other enzymes used for loading resealed erythrocytes include urease [70], galactose-1-phosphate uridyl transferase [90], uricase [110], and acetaldehyde dehydrogenase [111].

12. Improvement in oxygen delivery to tissue

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.

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 hemoglobin
- increased radiosensitivity of radiation-sensitive tumors
- restoration of oxygen-delivery capacity of stored blood
- ischemia of myocardium, brain, or other tissues [7, 59, 60, 113].

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] [3, 114–116]. 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 [117]. In microinjection, erythrocytes are used as microsyringes for injection to the host cells [7].

Novel approaches

Novel system includes

1. Nanoerythrosomes
2. An erythrocyte based new drug carrier, named nanoerythrosome has been developed which is prepared by extrusion of erythrocyte ghosts to produce small vesicles having an average diameter of 100 nm.
3. Erythrosomes
4. Erythrosomes are specifically engineered vesicular systems in which chemically cross-linked human erythrocyte cytoskeletons are used as a support upon a lipid bilayer is coated.

Future perspectives

1. A large amount of valuable work is needed so as to utilize the potentials of erythrocytes in passive as well as active targeting of drugs.
2. Diseases like cancer could surely find its cure.
3. Genetic engineering aspects can be coupled to give a newer dimension to the existing cellular drug carrier concept.

Present investigation reports

The present investigation reports the formulation of resealed erythrocytes [RBCs] as carriers for the anticancer agent, 5-fluorouracil [5-FU]. The preswell dilution technique was adopted for loading and various formulation conditions like preswelling point, drug concentration, time of contact, point of lysis, resealing point, incubation period and cross linking conditions were optimized.

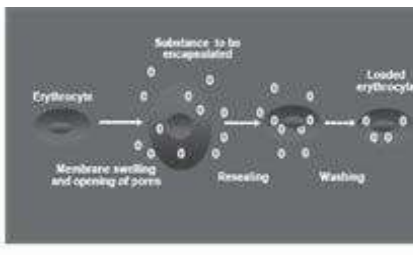

Figure 1

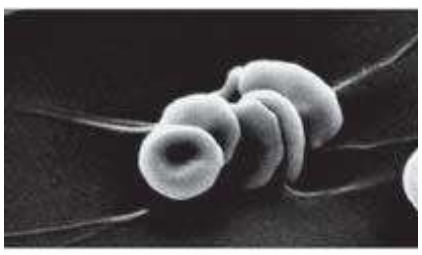

Figure 2

Figure 3

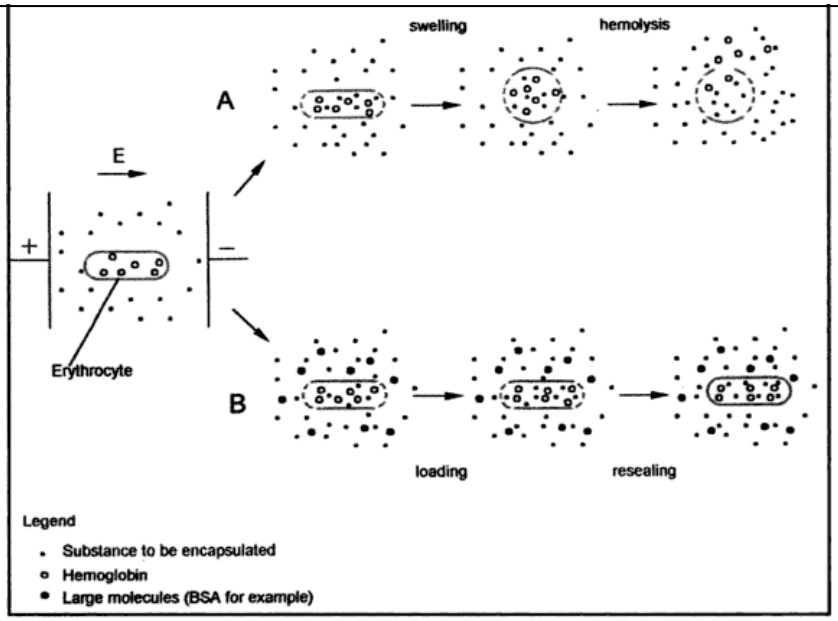

Figure 1

Table 1.Comparison of various hypo-osmotic lysis methods

Method	%Loading	Advantages	Disadvantages
Dilution method	20-40%	Fastest and simplest especially For low molecular weight drug	Entrapment efficiency is low
Dialysis	30-40%	Better in vivo survival of erythrocytes better structural Integrity membrane	Time consuming Heterogeneous size distribution of released erythrocytes
Preswell dilution	30-90%	Good retention of cytoplasm Constituent and good survival in vivo.	–
Isotonic osmotic lysis	–	Better in vivo surveillance	Impermeable only to large molecule, process is time consuming

CONCLUSION

During the past decade numerous application have been proposed for the use of resealed erythrocytes as carrier for drugs, enzyme replacement therapy etc. until other carrier system come of age released erythrocytes technology will remain an active area for further research. The commercial medical applications of carrier erythrocytes are currently being tested in Europe by a recently formed company that is developing products for human use. The coming years represent a critical time in this field as commercial application are explored in near future. Erythrocytes based delivery system with their ability to provide controlled and sit specific drug delivery will revolutionize disease management. The international society for the use of resealed erythrocytes [ISURE] through its biannual meetings provides an excellent forum for exchange of information to the scientist in the existing and rewarding field of research. For the present, it is concluded that erythrocyte carriers are "Golden Eggs In Novel Drug Delivery Systems" considering their tremendous potential.

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