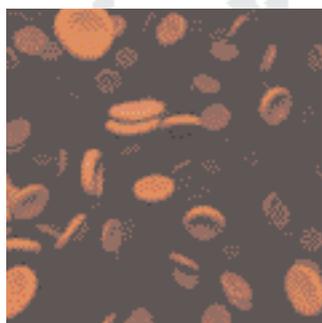


Resealed Erythrocytes: A Review

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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.

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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 (1–3). 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 (1). 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) (2–5).

Morphology and physiology of erythrocytes

Erythrocytes are the most abundant cells in the human body (~5.4 million cells/mm³ blood in a healthy male and ~4.8 million cells/mm³ blood in a healthy female). These cells were described in human blood samples by Dutch Scientist Lee Van Hock in 1674. In the 19th century, Hope Seyler identified hemoglobin and its crucial role in oxygen delivery to various parts of the body (6). Erythrocytes are biconcave discs with an average diameter of 7.8 μm, a thickness of 2.5 μm in periphery, 1 μm in the center, and a volume of 85–91 μm³ (7). The flexible, biconcave shape enables erythrocytes to squeeze through narrow capillaries, which may be only 3 μm wide. Mature erythrocytes are quite simple in structure. They lack a nucleus and other organelles. Their plasma membrane encloses hemoglobin, a heme-containing protein that is responsible for O₂-CO₂ binding inside the erythrocytes. The main role of erythrocytes is the transport of O₂ from the lungs to tissues and the CO₂ produced in tissues back to lungs. Thus, erythrocytes are a highly specialized O₂ carrier system in the body. Because a nucleus is absent, all the intracellular space is available for O₂ transport. Also, because mitochondria are absent and because energy is generated anaerobically in erythrocytes, these cells do not consume any of the oxygen they are carrying.

Erythrocytes live only about 120 days because of wear and tear on their plasma membranes as they squeeze through the narrow blood capillaries. Worn-out erythrocytes are removed from circulation and destroyed in the spleen and liver (RES), and the breakdown products are recycled. The process of erythrocyte forma-

tion within the body is known as *erythropoiesis*. In a mature human being, erythrocytes are produced in red bone marrow under the regulation of a hemopoietic hormone called *erythropoietin* (8).

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. 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 4 °C for as long as 48 h before use. Jain and Vyas have described a well-established protocol for the isolation of erythrocytes (9).

In 1953, Gardos tried to load erythrocyte ghost using adenosine triphosphate (ATP) (10). In 1959, Marsden and Ostting reported the entrapment of dextran (molecular weight 10–250 kDa). In 1973, the loading of drugs in erythrocytes was reported separately by Ihler et al. (11) and Zimmermann (12). In 1979, the term *carrier erythrocytes* was coined to describe drug-loaded erythrocytes (5).

Advantages of erythrocytes as drug carriers

Advantages include:

- their biocompatibility, particularly when autologous cells are used, hence no possibility of triggered immune response (3, 5, 9, 13–18)
- their biodegradability with no generation of toxic products (3, 8, 9, 13, 18, 19)
- the considerably uniform size and shape of the carrier (6, 7, 20)
- relatively inert intracellular environment (21)
- prevention of degradation of the loaded drug from inactivation by endogenous chemicals (13, 17, 19, 22, 23)
- the wide variety of chemicals that can be entrapped (13, 22, 24–26)
- the modification of pharmacokinetic and pharmacodynamic parameters of drug (5, 16, 19, 22)
- attainment of steady-state plasma concentration decreases fluctuations in concentration (3, 5, 15, 30, 31)
- protection of the organism against toxic effects of drugs (e.g. antineoplastics, 22).

Other advantages are:

- their ability to circulate throughout the body (9)
- the availability of the techniques and facilities for separation, handling, transfusion, and working with erythrocytes (3, 13)
- the prevention of any undesired immune response against the loaded drug (21)
- their ability to target the organs of the RES (3, 9, 13, 29)
- the possibility of ideal zero-order drug-release kinetics (23)
- the lack of occurrence of undesired immune response against encapsulated drug (9)

- the large quantity of drug that can be encapsulated within a small volume of cells ensures dose sufficiency (9, 13, 18)
- a longer life span in circulation as compared with other synthetic carriers (4, 17, 27), and optimum conditions may result in the life span comparable to that of normal erythrocytes (14, 28, 29)
- easy control during life span ranging from minutes to months (17)
- a decrease in side effects of drugs (3, 16, 31)
- a considerable increase in drug dosing interval with drug residing in therapeutic window region for longer time periods (3, 16, 29, 30).

Methods of drug loading

Several methods can be used to load drugs or other bioactive compounds in erythrocytes, including physical (e.g., electrical-pulse method) osmosis-based systems, and chemical methods (e.g., chemical perturbation of the erythrocytes membrane). Irrespective of the method used, the optimal characteristics for the successful entrapment of the compound requires the drug to have a considerable degree of water solubility, resistance against degradation within erythrocytes, lack of physical or chemical interaction with erythrocyte membrane, and well-defined pharmacokinetic and pharmacodynamic properties (32).

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* (5, 25, 33, 34).

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 (25, 33, 34).

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” (35). 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.

Hypotonic dilution. Hypotonic dilution was the first method investigated for the encapsulation of chemicals into erythrocytes (11) and is the simplest and fastest (5). 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 (11, 25). The major drawbacks of this method include a low entrapment efficiency (11, 13, 36–38) and a considerable loss of hemoglobin and other cell components (4, 19, 24). 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 (11, 25). Hypotonic dilution is used for loading enzymes such as β -galactosidase and β -glucosidase (11), asparaginase (14, 39), and arginase (21), as well as bronchodilators such as salbutamol (40).

Hypotonic preswelling. This method was developed by Rechester (41) 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 μ 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 37 °C to reanneal the resealed erythrocytes (25, 36). Such cells have a circulation half life comparable to that of normal cells (5, 25, 36, 42). This method is simpler and faster than other methods, causing minimum damage to cells. Drugs encapsulated in erythrocytes using this method include propranolol (15), asparaginase (20), cyclophosphamide, cortisol-21-phosphate (31, 36), ω 1-antitrypsin (36), methotrexate, insulin (36, 44), metronidazole (38), levothyroxine (42), enalaprilat (43), and isoniazid (45).

Hypotonic dialysis. This method was first reported by Kliban-sky (46) in 1959 and was used in 1977 by DeLoach and Ihler (37), and Dale (47) 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 (25, 37). 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 (23, 25, 48, 49) or by adding the drug to a dialysis bag after the stirring is complete (33, 37, 50–53).

The use of standard hemodialysis equipment for loading a

drug in erythrocytes was reported by Roper et al. (54). 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 (33, 49, 54–59).

The loaded cells exhibit the same circulation half life as that of normal cells (4, 5). Also, this method has high entrapment efficiency on the order of 30–50% (4, 13, 33, 37), cell recovery of 70–80%, high-loading capacity (50, 54), and is amenable to automation with control of process variables (33, 60). The drawbacks include a long processing time (4, 13, 37) and the need for special equipment (25).

This method has been used for loading enzymes such as β -galactosidase, glucocerebrosidase (36), asparaginase (49), inositol hexaphosphatase (56, 58, 59), as well as drugs such as gentamicin (23), adriamycin (48), pentamidine and furamycin (51), interleukin-2 (52), desferroxamine (50, 54, 55, 57), and human recombinant erythropoietin (61).

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 (62), polyethylene glycol (63), 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) (64). The suspension was diluted with an isotonic-buffered drug solution. After the cells were separated, they were resealed at 37 °C.

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

Electro-insertion or electroencapsulation. In 1973, Zimmermann tried an electrical pulse method to encapsulate bioactive molecules (12). 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 Kinoshita suggested the use of transient electrolysis to generate desirable membrane permeability for drug loading (68). 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

Table I: Summary of characterization parameters and their determination for resealed erythrocytes

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	Sterility test
Pyrogenicity	Rabbit method, LAL test
Animal toxicity	Toxicity tests

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 (4, 29). The optimum intensity of an electric field is between 1–10 kW/cm and optimal discharge time is between 20–160 μ s (3, 4, 70–72). An inverse relationship exists between the electric-field intensity and the discharge time (4, 29). 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 (2, 29, 70).

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 (4). The main drawbacks are the need for special instrumentation

and the sophistication of the process (3, 13, 18, 33). Entrapment efficiency of this method is ~35% (30), and the life span of the resealed cells in circulation is comparable with that of normal cells (4, 29).

Various compounds such as sucrose (29, 69), urease (69), methotrexate (72), isoniazid (73), human glycoporphin (74), DNA fragments, and latex particles of diameter 0.2 μ m (4) 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 (75).

Entrapment by endocytosis. This method was reported by Schrier et al. in 1975 (76). Endocytosis involves the 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. 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 (68, 77, 78).

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 (72, 79). 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.

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 (80). 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. Table I summarizes the various evaluation pa-

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 (18, 37) but in few cases spherical erythrocytes (spherocytes) are detected (30, 45). Scanning electron microscopic studies have shown that a majority of the cells maintain their biconcave discoid shapes after the loading procedure (53), and few stomatocytes—a form of spherocytes with an invagination in one point—are formed (61). In some cases, cells of smaller size (microcyte) are also observed (59).

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 (50). 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 (50, 54). Another indirect approach is to evaluate chlorpromazine induced shape changes turbidimetrically (81).

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 (18, 81). In most cases, osmotic fragility of resealed cells is higher than that of the normal cells (18, 30, 38, 45, 61, 81) 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) (18, 30, 38, 45) or vigorously shaking the cell suspension (81). In both cases, hemoglobin and drug released after the procedure are determined. The turbulent fragility of resealed cells is found to be higher (18, 30, 38, 45, 81).

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 (11, 14, 49, 53, 54, 59, 61, 81).

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 (82).

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 (3), phenytoin, dexamethasone (16), primiquin (18), and vitamin B₁₂ (83). 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 (16, 23), heparin (16), and enalaprilat (34), and enzymes such as asparaginase (13, 20, 39, 49), peptides, including urogasterone and l-lysine-l-phenylalanine (84) follow such pattern.
- The rate of drug release lies between the above mentioned two extremes; for example, propranolol (16), isoniazid (30, 45), metronidazole (38), and recombinant human erythropoietin (61).

The two factors that determine the drug release pattern are size and polarity of the drug molecule (16). The release rate can be modified by cross-linking cell membrane with glutaraldehyde (28, 38), which results in a slower drug release. This can also be achieved by entrapping biodegradable prodrug such as o-acetyl propranolol, o-pivaloyl propranolol (15), cortisol-21-phosphate (31, 36), prednisolone-21-sodium succinate (31), and cytosine arabinoside monophosphate (85). The complexation of a drug with macromolecules such as dextran and albumin also retard the release rate (34).

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 (15).

The most common storage media include Hank's balanced salt solution (3, 13, 19, 81, 84) and acid-citrate-dextrose (4) at 4 °C. Cells remain viable in terms of their physiologic and carrier characteristics for at least 2 weeks at this temperature (84). The addition of calcium-chelating agents (15) or the purine nucleosides (15, 84) 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-bis-propionamide, glutaraldehyde, toluene-2-4-diisocyanate followed by lyophilization or sintered glass filtration has been reported to enhance their stability upon storage (5, 18, 38, 40). 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 (18), high glycerol freezing technique (54, 55), and reversible immobilization in alginate or gelatin gels (3, 4, 19).

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 (4).

The various methods used to determine in vivo survival time include labeling of cells by ^{51}Cr or fluorescent markers such as fluorescein isothiocyanate or entrapment of ^{14}C sucrose or gentamicin (23, 36, 42).

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 (4, 5, 13, 39, 53, 54, 59, 87).

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 (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 (3, 5, 55, 88–90).

Slow drug release. Erythrocytes have been used as circulating depots for the sustained delivery of antineoplastics (14, 20, 39, 48, 49, 91–94), antiparasitics (17, 37, 51), veterinary antiamoebics (13), vitamins (83), steroids (28, 31, 35), antibiotics (30, 45, 65), and cardiovascular drugs (43, 81, 95).

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 (5, 18).
- accumulation of erythrocytes in lymph nodes upon subcutaneous administration followed by hemolysis to release the drug (52).

Routes of administration include intravenous, which is the most common, followed by subcutaneous, intraperitoneal, intranasal, and oral (5). Studies regarding the improved efficacy of various drugs given in this form in animal models have been published. Examples include an enhancement in anti-inflammatory effect of corticosteroids in experimentally inflamed rats (28, 31), increase in half life of isoniazid (30, 45), levothyroxine (42), cytosine arabinoside (92), and interleukin-2 (52); prolongation of plasma half life of erythro-

poietin from 30 min to 35 h in mice (61), and can increase in mean survival time of mice with experimental hepatoma after injecting methotrexate loaded erythrocytes (91).

Thalasemic patients, because of multiple blood transfusions, are prone to hemosydrosis, a disease state associated with an excess storage of iron (96). This state is treated using SC or IV injections of iron-chelating compound desferrioxamine, which causes severe adverse effects in case of multiple injections (55). This agent was loaded on to erythrocytes and the performance of these cells upon reinjection was observed and found to be promising (54, 55). This therapeutic method is approved in the United States as regular management tool of hemosydrosis since 1984 (19).

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 (97). However, resealed erythrocytes also can be used to target organs other than those of RES.

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 glutaraldehyde
- surface modification with carbohydrates such as sialic acid (97)
- surface modification with sulphhydryl
- surface chemical cross-linking e.g. delivery of ^{125}I -labeled carbonic anhydrase loaded in erythrocytes cross-linked with *bis* (sulfosuccinimidyl) suberate and 3,3' dithio (sulfosuccinimidyl propionate) (98).

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 β -glucosidase, β -glucuronidase, β -galactosidase (13, 23, 37). The disease caused by an accumulation of glucocerebrosides in the liver and spleen can be treated by glucocerebrosidase-loaded erythrocytes (100).

Treatment of hepatic tumors. Hepatic tumors are one of the most prevalent types of cancer. Antineoplastic drugs such as methotrexate (4, 13), bleomycin (12), asparaginase (13, 14), and adriamycin (13, 48, 101, 102) 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 (103) as a spacer. The resealed erythrocytes loaded with carboplatin show localization in liver (104).

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 (17), antileishmanial (17, 23, 51), and antiamebic drugs (13, 38).

Removal of RES iron overload. Desferrioxamine-loaded erythrocytes have been used to treat excess iron accumulated because of multiple transfusions to thalassemic patients (13, 51). 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.

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 phosphodiesterase also has been reported (106).

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

Zimmermann (71) 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. The loading of ferrofluids (colloidal suspension of magnetite) has been reported by Sprandel et al. (107). Jain and Vyas (108) reported entrapment of the antiinflammatory 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 (109). Chiarantini et al. have reported in vitro targeting of erythrocytes to cytotoxic T-cells by coupling of Thy-1.2 monoclonal antibody (110).

Price et al. reported delivery of colloidal particles and erythrocytes to tissue through microvessel ruptures created by targeted microbubble destruction with ultrasound (111). 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 (4), intraperitoneal injection of resealed erythrocytes for drug targeting to peritoneal macrophages (112), and lectin pretreatment of resealed cells loaded with antineoplastic drugs to improve targeting tumor cells (113).

Delivery of antiviral agents. Several reports have been cited in the literature about antiviral agents entrapped in resealed erythrocytes for effective delivery and targeting (34). Because most antiviral drugs are either 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 (34), recombinant herpes simplex virus type 1 (HSV-1) glycoprotein B (110), azidothymidine derivatives (114), azathioprene, acyclovir (115), and fludarabine phosphate (116).

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 (44). 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). These cells then release enzymes into circulation upon hemolysis (4, 14); act as a "circulating bioreactors" in which substrates enter into the cell, interact with enzymes, and generate products (4, 13, 20, 21, 55, 69, 117, 118); or accumulate enzymes in RES upon hemolysis (3, 11, 37) for future catalysis.

The first report of successful clinical trials of the resealed erythrocytes loaded with enzymes for replacement therapy is that of β -glucoserebrosidase for the treatment of Gaucher's disease (90). The disease is characterized by inborn deficiency of lysosomal β -glucoserebrosidase in cells of RES thereby leading to accumulation of β -glucoserebrosides in macrophages of the RES.

The most important application of resealed erythrocytes in enzyme therapy is that of asparaginase loading for the treatment of pediatric neoplasms. This enzyme degrades asparagine, an amino acid vital for cells. This treatment prevents remission of pediatric acute lymphocytic leukemia (119). There are reports of improved intensity and duration of action in animal models (14, 20, 39, 49) as well as humans (5).

To treat lead poisoning, the concentration of β -aminolevulinic dehydrogenase (ALA-D) in erythrocytes decreases. This leads to an accumulation of β -aminolevulinic acid in tissues, blood, and urine. This state leads to acute porphyria and CNS-related problems (120). An injection of resealed erythrocytes loaded with ALA-D to lead intoxicated animal significantly reduces toxic manifestations (121).

Other enzymes used for loading resealed erythrocytes include urease (69), galactose-1-phosphate uridyl transferase (89), uricase (118), and acetaldehyde dehydrogenase (119).

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 (122).

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 (56). Inositol hexophosphate (IHP) is one of the strongest effectors of this type (123). However, because of its ionization at physiologic pH, it cannot enter erythrocytes (59, 123). 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 (4). As a result, the oxygen pressure corresponding to 50% of the total binding capacity of hemoglobin to oxygen (P_{50} value) increases from 26–27 mm Hg to >50 mm Hg (59).

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 β chain of hemoglobin 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 (59, 124). 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 (58). An isolated perfused-heart model showed reduction in coronary blood flow with increased oxygen consumption by myocardium upon administration of IHP-loaded erythrocytes (125–127). The same results are reported when intact animal models were used (128).

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 (4, 58, 59, 125).

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, 129–131). 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 (132). In microinjection, erythrocytes are used as microsyringes for injection

to the host cells (4).

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 (4, 132, 133). 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 (3), arginase (134, 135), proteins, nucleic acids, ferritin, latex particles, bovine and human serum albumin, and enzyme thymidine kinase (131) 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, unpredictable effects on cell resulting from the cointroduction of various components. Hence, this method is limited to mainly cell biological applications rather than drug delivery (132).

Novel approaches

Erythroosomes. 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 (136–138).

Nanoerythroosomes. These are prepared by extrusion of erythrocyte ghosts to produce small vesicles with an average diameter of 100 nm. Daunorubicin was covalently conjugated to nanoerythroosomes using gluteraldehyde spacer. This complex was more active than free daunorubicin alone, both in vitro and in vivo (139, 140).

Conclusion

The use of resealed erythrocytes looks promising for a safe and sure delivery of various drugs for passive and active targeting. However, the concept needs further optimization to become a routine drug delivery system. The same concept also can be extended to the delivery of biopharmaceuticals and much remains to be explored regarding the potential of resealed erythrocytes.

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