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Review Article

RESEALED ERYTHROCYTES AS CARRIERS AND ITS APPLICATION IN THERAPY

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Abstract

The aim of the present review is to focus on the various features, drug loading technology and biomedical application of resealed erythrocytes. Resealed erythrocytes are gaining more popularity because of their ability to circulate throughout the body, biocompatibility, zero order release kinetics, reproducibility and ease of preparation. 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 (Hypotonic dilution, Hypotonic hemolysis, Electro-insertion, entrapment by endocytosis, Hypo-osmotic lysis). Most of the resealed erythrocytes used as drug carriers are rapidly taken up from blood by macrophages of reticuloendothelial system (RES), which is present in liver, lung, and spleen of the body. So many drugs like aspirin, steroid, cancer drug which having many side effects are reduce by resealed erythrocyte. The present article gives information about morphology, isolation, properties, and methods of drug loading, characterization, mechanism, and applications of resealed erythrocytes.

Keywords: Resealed Erythrocytes, Isolation, Methods of drug loading, characterization, Applications.

INTRODUCTION

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. (Lewis DA, Ilher 1989) Such drug loaded carrier erythrocyte are prepared simply by collecting blood samples from the organism of interest, separating erythrocyte from plasma, entrapping the drug in the erythrocyte and resealing the resultant cellular carriers. Hence these carriers are called resealed erythrocyte. 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 disease tissue or organ. (Rajendra 2011)

ERYTHROCYTES

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, protect 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. They have the capacity to carry large amounts of drug; and can behave as a slow-release long acting system. Potential clinical indications for "RES targeting" include iron over-storage diseases, parasitic diseases, hepatic tumors, cancer and lysosomal storage diseases carriers. (Rajendra 2011)

ERYTHROCYTES CAN BE USED AS CARRIERS IN TWO WAYS:

Targeting particular tissue/organ

For targeting, only the erythrocyte membrane is used. This is obtained by splitting the cell in hypotonic solution

and after introducing the drug into the cells, allowing them to reseal into spheres. Such erythrocytes are called Red cell ghosts.

For continuous or prolonged release of drugs

Alternatively, erythrocytes can be used as a continuous or prolonged release system, which provide prolonged drug action. There are different methods for encapsulation of drugs within erythrocytes. They remain in the circulation for prolonged periods of time (up to 120 days) and release the entrapped drug at a slow and steady rate. (Rajendra 2011)

MORPHOLOGY AND PHYSIOLOGY OF ERYTHROCYTES

Erythrocytes are the most abundant cells in the human body (~5.4 million cells/mm³ bloods in a healthy male and ~ 4.8 million cells/mm³ bloods 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. 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 85–90 μm³. 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 aerobically 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 formation within the body is known as erythropoiesis.

In a mature human being, erythrocytes are produced in red bone marrow under the regulation of a hemopoietic hormone called erythropoietin. (Gothoskar 2004).

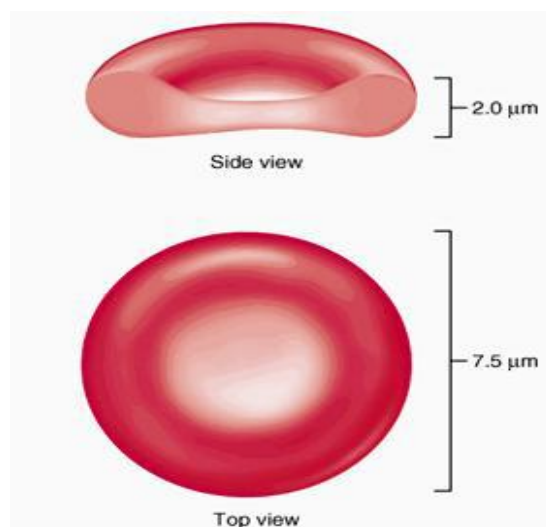


FIG 1: COMPOSITION OF ERYTHROCYTE

FACTORS CONSIDERING RESEALED ERYTHROCYTES AS CARRIER

1. Its shape and size to permit the passage through the capillaries.
2. Its specific physico-chemical properties by which a prerequisite site can be recognized.
3. Its biocompatible and minimum toxicity character.
4. Its degradation product, after release of the drug at the target site, should be biocompatible.
5. Low leaching/leakage of drug should take place before target site is reached.

6. Its drug released pattern in a controlled manner.
7. High drug loading efficiency for broad spectrum of drugs with different properties.
8. Physicochemical compatibility with the drug.
9. The carrier system should have an appreciable stability during storage. (Sha 2011)

ADVANTAGES OF ERYTHROCYTES AS DRUG CARRIERS:

Advantages include:

1. Their biocompatibility, particularly when autologous cells are used, hence no possibility of triggered immune response.
2. Their biodegradability with no generation of toxic products.
3. The considerably uniform size and shape of the carrier.
4. Relatively inert intracellular environment.
5. Prevention of degradation of the loaded drug from inactivation by endogenous chemicals.
6. The wide variety of chemicals that can be entrapped.
7. The modification of pharmacokinetic and pharmacodynamic parameters of drug.
8. Attainment of steady-state plasma concentration decreases fluctuations in concentration.
9. Protection of the organism against toxic effects of drugs (e.g. antineoplastics).

Other advantages are:

1. Their ability to circulate throughout the body.
2. The availability of the techniques and facilities for separation, handling, transfusion, and working with erythrocytes.
3. The prevention of any undesired immune response against the loaded drug.
4. Their ability to target the organs of the RES.
5. The possibility of ideal zero-order drug-release kinetics.
6. The lack of occurrence of undesired immune response against encapsulated drug.
7. The large quantity of drug that can be encapsulated within a small volume of cells ensures dose sufficiency.
8. A longer life span in circulation as compared with other synthetic carriers, and optimum conditions may result in the life span comparable to that of normal erythrocytes.
9. Easy control during life span ranging from minutes to months.
10. A decrease in side effects of drugs.
11. A considerable increase in drug dosing interval with drug residing in therapeutic

window region for longer time periods. (Gothoskar 2004)

DISADVANTAGES

1. Possibility of clumping of cells and dose dumping may be there.
2. They have a limited potential as carrier to non-phagocyte target tissue.
3. The major problem encountered in the use of biodegradable materials or natural cells as drug carriers is that they are removed in vivo by the RES as result of modification that occurred during loading procedure in cells. This, although expands the capability to drug targeting to RES, seriously limits their life-span as long-circulating drug carriers in circulation and, in some cases, may pose toxicological problems.
4. The rapid leakage of certain encapsulated substances from the loaded erythrocytes.
5. Several molecules may alter the physiology of the erythrocyte.
6. Given that they are carriers of biological origin, encapsulated erythrocytes may present some inherent variations in their loading and characteristics compared to other carrier systems.
7. The storage of the loaded erythrocytes is a further problem provided that there are viable cells and need to survive in circulation for a long time upon re-entry to the host body. Conditioning carrier cells in isotonic buffers containing all essential nutrients, as well as in low temperatures, the addition of nucleosides or chelators, lyophilization with glycerol or gel immobilization have all been exploited to overcome this problem.
8. Possible contamination due to the origin of the blood, the equipment used and the loading environment. Rigorous controls are required accordingly for the collection and handling of the erythrocytes. (Hamidi 2007)

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 vein puncture. (Chasis 1986) 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.

In 1953, Gardos tried to load erythrocyte ghost using adenosine triphosphate (ATP). 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. and Zimmermann. In 1979, the term carrier erythrocytes were coined to describe drug- loaded erythrocytes. (Gothoskar 2004)

PROPERTIES OF RESEALED ERYTHROCYTE OF NOVEL DRUG DELIVERY CARRIERS

1. The drug should be released at target site in a controlled manner.
2. It should be appropriate size, shape and should permit the passage through capillaries and minimum leakage of drug should take place.
3. It should be biocompatible and should have minimum toxic effect.
4. It should possess the ability to carry a broad spectrum of drug.
5. It should possess specific physicochemical properties by which desired target size could be recognized.
6. The degradation product of the carriers system, after release of the drug at the selected site should be biocompatible. It should be physiochemically compatible with drug.
7. The carrier system should have an appreciable stability during storage. (Shah 2011)

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).

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 m osm/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. (G.M. Ihler1983, Panchal R. 2006)

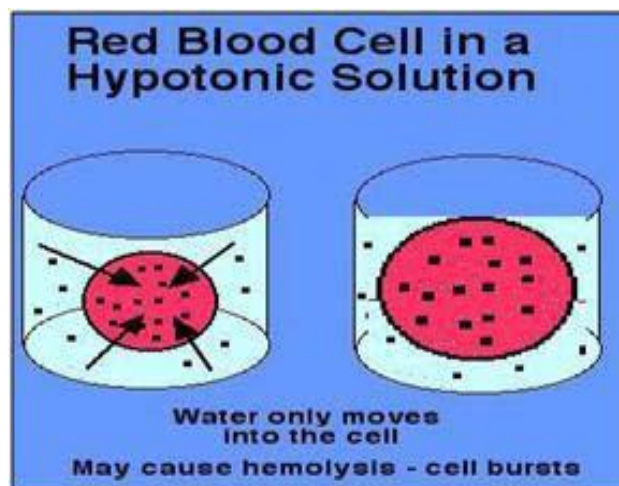


FIG.2; HYPNOTIC HEMOLYSIS

Use of red cell loader

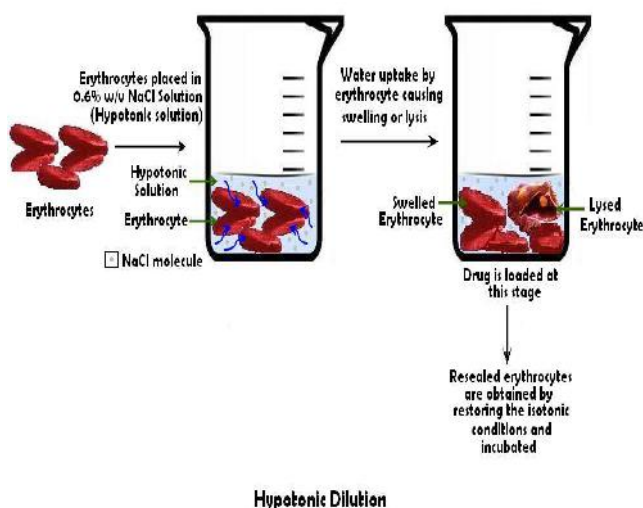
Magnani et al. developed a novel method for entrapment of nondiffusible drugs into erythrocytes. They developed a piece of equipment called a “red cell loader”. With as little as 50 mL of a blood sample; different biologically active compounds were entrapped into erythrocytes within a period of 2 h at 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. (E. Pitt1983).



FIG.2: RED CELL LOADE

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 -galactosidase and -glucosidase, asparaginase, and arginase as well as bronchodilators such as salbutamol. (Deloach JR 1977, Talwar N.1992)



Hypotonic Dilution

FIG.3: HYPOTONIC DILUTION METHOD

Hypotonic preswelling

This method was developed by Rechsteiner in 1975 and was modified by Jenner et al. for drug loading. The technique is based upon initial controlled swelling 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 μL 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. Such cells have a circulation half life comparable to that of normal cells. This method is simpler and faster than other methods, causing minimum damage to cells. Drugs encapsulated in erythrocytes using this method include propranolol, asparaginase, cyclophosphamide, cortisol-21-phosphate, 1-antitrypsin, methotrexate, insulin, metronidazole, levothyroxine, enalaprilat, and isoniazid. (Lewis DA 1984)

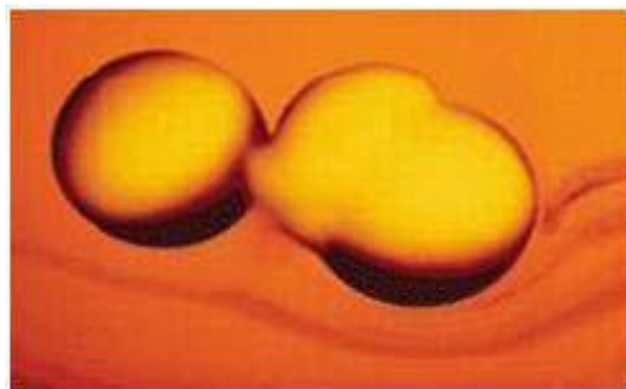


FIG.5; HYPOTONIC PRE-SWELLING

Hypotonic dialysis

This method was first reported by Klibansky in 1959 and was used in 1977 by Deloach and Ihler, and Dale 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. The drug to

be loaded can be added by either dissolving the drug in isotonic cell suspending buffer inside a dialysis bag at the beginning of the experiment or by adding the drug to a dialysis bag after the stirring is complete. The use of standard hemodialysis equipment for loading a drug in erythrocytes was reported by Roper et al. In this method, the erythrocyte suspension and the drug to be loaded were placed in the blood compartment and the hypotonic buffer was placed in a receptor compartment. This led to the concept of "continuous flow dialysis," which has been used by several others researchers. The loaded cells exhibit the same circulation half life as that of normal cells. Also, this method has high entrapment efficiency on the order of 30–50%, cell recovery of 70–80%, high-loading capacity, and is amenable to automation with control of process variables. The drawbacks include a long processing time and the need for special equipment. This method has been used for loading enzymes such as galactosidase, glucoserebrosidase, asparaginase, inositol hexaphosphatase as well as drugs such as gentamicin, Adriamycin, pentamidine and uramycin, interleukin-2, desferrioxamine, and human recombinant erythropoietin. (Gothoskar 2004)

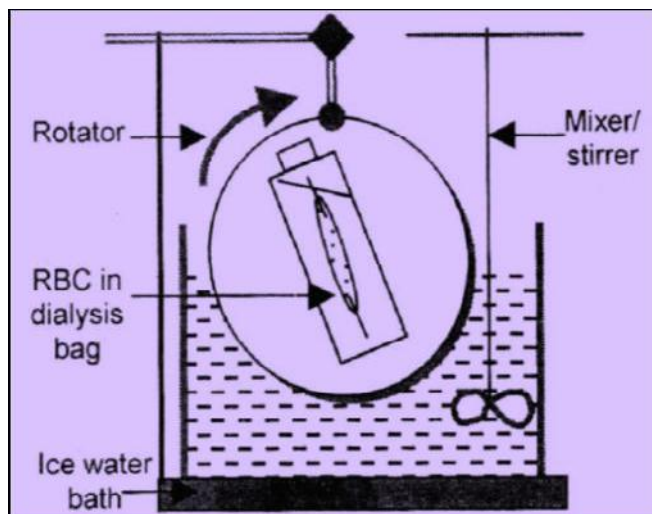


FIG 2: HYPOTONIC DIALYSIS METHOD

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 isotonic. 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°C. (Gothoskar 2004)

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 erythrocytes' 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. (Gothoskar 2004)

Electro-insertion or electro encapsulation

This method is also known as electroporation, the method consist of creating electrically induced permeability changes at high membrane potential differences. In 1977, Tsong and Kinoshita suggested the use of transient electrolysis to generate desirable membrane permeability for drug loading. Electrical breakdown is achieved by membrane polarization for microseconds using varied voltage of 2kV/cm is applied for 20μ sec. The potential difference across the membrane is built up either directly by inter and intracellular electrodes or indirectly by applying internal electric field to the cells. The extent of pore formation depends upon the electric field strength, pulse duration and ionic strength of suspending medium. Once membrane is perforated, regardless of the size of pores, ions rapidly distribute between the extra and intracellular space to attain Donnan equilibrium, however the membrane still remains impermeable to its cytoplasmic macromolecules. The colloidal osmotic pressure of its macromolecular contents. In the case of red blood cells, the colloidal osmotic pressure of hemoglobin is about 30 mOsm.

This pressure drives water and ion influx, as a result swelling of the cells occurs. The membrane is ruptured when the cell volume reaches 155% of its original volume. Thus, cell lysis is a secondary effect of electric modification of the membrane. Since the cell lysis is due to colloidal osmotic swelling, the rational to prevent lysis is to balance the colloidal osmotic pressure of cellular macromolecules. This can be affected by addition of large molecules (like tetrasaccharide stachyose or protein such as bovine

serum albumin) and ribonucleases. This helps to counteract the colloidal osmotic swelling of electrically perforated erythrocytes. Under this osmotically balanced condition pores stay open at 4°C for few days. If drug molecules are added at this point, they permeate into red blood cells. A suitable procedure could be subsequently used to reseal these pores. The various candidates entrapped by this method include primaquine and related 8- amino-quinolines, vinblastine, chlorpromazine and related phenothiazines, hydrocortisone, propranolol, tetracaine, and vitamin A. (Gothoskar 2004)

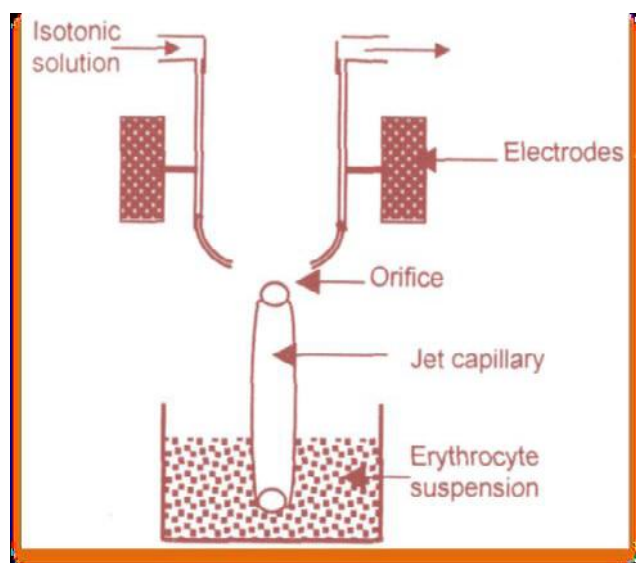


FIG 3: Electro-insertion OR Electro encapsulation

Entrapment by endocytosis

This method was reported by Schrier et al. in 1975 (Energized Endocytosis in Human Erythrocyte Ghosts 1975). 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. (Gothoskar 2004)

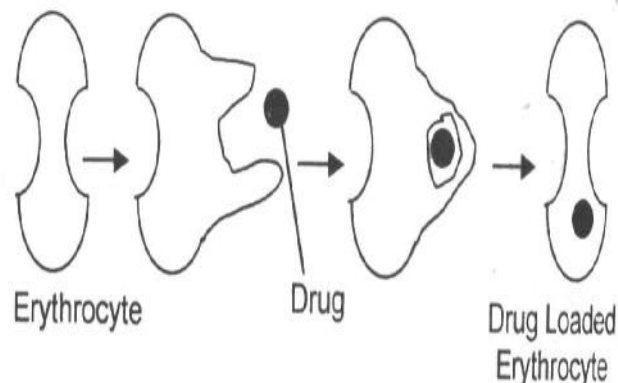


FIG 4: Entrapment by endocytosis

Loading by electric cell fusion

This method involves the initial loading of drug molecules into erythrocyte ghosts followed by adhesion of these cells to target cells. The fusion is accentuated by the application of an electric pulse, which causes the release of an entrapped molecule. An example of this method is loading a cell-specific monoclonal antibody into an erythrocyte ghost. An antibody against a specific surface protein of target cells can be chemically cross-linked to drug-loaded cells that would direct these cells to desired cells. (Gothoskar 2004)

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%). (Gothoskar 2004)

CHARACTERIZATION OF RESEALED ERYTHROCYTES

Table I summarizes the various evaluation parameters and the techniques applied for their determination. (Sha 2011)

Drug Content Quantification: To determine the drug content, packed loaded cells are deproteinized with acetonitrile after centrifugation at 3000 rpm for a fixed time interval. The clear supernatant liquid is analyzed spectrophotometrically. (Sha 2011)

In-vitro drug release and hemoglobin content study: In-vitro release of drug(s) and hemoglobin are monitored periodically from drug-loaded cells. The cell suspension (5% hematocrit in PBS) is stored at 4 °C in amber colored glass containers. Periodically the clear supernatant are withdrawn using a hypodermic

syringes equipped with 0.45 m filter, deproteinied using methanol and were estimated for drug content.(Sha 2011)

The supernatant of each sample after centrifugation is collected and assayed, % hemoglobin release may be calculated using the formula. (Li 1996)

$$\% \text{ hemoglobin release} = \frac{A_{540} \text{ of sample} - A_{540} \text{ of background}}{A_{540} \text{ of 100\% hemoglobin}}$$

Or

$$\text{Mean corpuscular hemoglobin (MCH (pg))} = \frac{\text{Hemoglobin (g/100ml)} \times 10}{\text{Erythrocyte count (millions/cu mm)}}$$

Where
An A540 refers to absorbance at 540nm.

Percent cell recovery and Morphological study:

Percent cell recovery may be determined by counting the no. of intact cells per cubic mm of packed erythrocytes before and after loading the drug. Phase contrast or electron microscope may be used for normal and drug loaded erythrocytes. (Sha 2011)

Osmotic fragility and Osmotic shock study

When red blood cells are exposed to solution of varying tonicities their shape change due to osmotic imbalance. To study the effect of different tonicities, drug loaded erythrocytes are incubated separately in normal saline solution at 37 ± 2 °C for 10 minutes, followed by centrifugation at 2000 rpm for 10 min. For osmotic shock study, dispersing the resealed erythrocyte suspension in distilled water and centrifuged at 300 rpm for 15 min. The supernatant was estimated for percent hemoglobin release spectrophotometrically. (Sha 2011).

Turbulence shock study

It is the measure of simulating destruction of loaded cells during injection. Normal and drug loaded cells are passed through a 23 gauge hypodermic needle at a flow rate of 10 ml/min which is comparable to the flow rate of blood. It is followed by collection of an aliquot and centrifugation at 2000 rpm for 10 minutes. The hemoglobin in withdrawn sample is estimated. Drug loaded erythrocytes appear to be less resistant to turbulence, probably indicating destruction of cells upon shaking. (Sha 2011)

Determination of entrapped magnetite

Atomic Absorption spectroscopic method is reported for determination of the concentration of a particular metal element in a sample. The HCl is added to a fixed amount of magnetite bearing erythrocytes and content are heated at 60°C for 2 hours. then 20%w/v

trichloro acetic acid is added and supernatant obtained after centrifugation is used to determine magnetite concentration using atomic absorption spectroscopy. (Shah 2011)

Erythrocyte sedimentation rate (ESR)

It is an estimate of the suspension stability of RBC in plasma and is related to the number and size of the red cells and to relative concentration of plasma protein, especially fibrinogen and globulins. This test is performed by determining the rate of sedimentation of blood cells in a standard tube. Normal blood ESR is 0 to 15 mm/hr. higher rate is indication of active but obscure disease processes. (Shah 2011)

Miscellaneous

Resealed erythrocyte can also be characterized by cell sizes, mean cell volume, energy metabolism, lipid composition, membrane fluidity, rheological properties, density gradient separation (Shah 2011)

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°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, glutaraldehyde, 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. (Gothoskar 2004)

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 ⁵¹Cr or fluorescent markers such as fluorescein isothiocyanate or entrapment of ¹⁴C 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 erythrocytes' 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. (Gothoskar 2004)

APPLICATIONS OF RESEALED ERYTHROCYTES:
(Gothoskar 2004)

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.

Slow drug release

Erythrocytes have been used as circulating depots for the sustained delivery of antineoplastics, antiparasitics, veterinary antiamebics, vitamins, steroids, antibiotics, and cardiovascular drugs. 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.

- Accumulation of erythrocytes in lymph nodes upon subcutaneous administration followed by hemolysis to release the drug.

Routes of administration include intravenous, which is the most common, followed by subcutaneous, intraperitoneal, intranasal, and oral. 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, increase in half life of isoniazid, levothyroxine, cytosine arabinoside, and interleukin-2; prolongation of plasma half life of erythropoietin from 30 min to 35 h in mice, and can increase in mean survival time of mice with experimental hepatoma after injecting methotrexate loaded erythrocytes. Thalassaemic patients, because of multiple blood transfusions, are prone to hemosiderosis, a disease state associated with an excess storage of iron. This state is treated using SC or IV injections of iron-chelating compound desferrioxamine, which causes severe adverse effects in case of multiple injections. This agent was loaded on to erythrocytes and the performance of these cells upon reinjection was observed and found to be promising. This therapeutic method is approved in the United States as regular management tool of hemosiderosis since 1984.

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.

Targeting RES Organ

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. (Franco 1998) 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
- Surface modification with sulphhydryl
- Surface chemical cross-linking e.g. delivery of ¹²⁵I-labeled carbonic anhydrase loaded in

erythrocytes cross-linked with bis (sulfosuccinimidyl) suberate and 3,3_ dithio (sulfosuccinimidyl propionate) . (Alvarez 1998)

Targeting the liver Enzyme

deficiency/replacement therapy

Many metabolic disorders related to deficient or missing enzymes can be treated by injecting these enzymes. (Jaitely 1996) However, the problems of exogenous enzyme therapy include a shorter circulation half life of enzymes, allergic reactions, and toxic manifestations. These problems can be successfully overcome by administering the enzymes as resealed erythrocytes. The enzymes used include -glucosidase, -glucuronidase, -galactosidase. The disease caused by an accumulation of glucocerebrosides in the liver and spleen can be treated by glucocerebrosidase- loaded erythrocytes.

Treatment of hepatic tumor

Hepatic tumors are one of the most prevalent types of cancer. Antineoplastic drugs such as methotrexate, bleomycin, asparaginase, 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 erythrocytes' membrane using gluteraldehyde or cisaconitic acid as a spacer. The resealed erythrocytes loaded with carboplatin show localization in liver.

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.

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.

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 phosphodiesterase also has been reported.

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 (U 1975) 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. Jain and Vyas (Jain and Vyas 1994) reported entrapment of the anti-inflammatory drugs diclofenac sodium and ibuprofen in magneto responsive erythrocytes. Photosensitized erythrocytes have been studied as a photo triggered carrier and delivery system for methotrexate in cancer treatment . Chiarantini 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 micro vessel ruptures created by targeted micro bubble destruction with ultrasound. IV fluorescent erythrocytes were delivered to the interstitium of rat skeletal muscle through micro vessel ruptures by insonifying micro bubbles 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 thermo responsive 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.

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 either nucleotides or nucleoside analogs, their entrapment and exit through the membrane need careful consideration. Nucleosides

are rapidly transported across the membrane whereas nucleotides are not, and thus exhibiting prolonged release profiles. The release of nucleotides requires conversion of these moieties to purine or pyrimidine bases. Resealed erythrocytes have been used to deliver deoxycytidine derivatives, recombinant herpes simplex virus type 1 (HSV-1) glycoprotein B, azidothymidine derivatives, azathioprene, acyclovir, and fludarabine phosphate.

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 α -glucoserebrosidase for the treatment of Gaucher's disease. The disease is characterized by inborn deficiency of lysosomal α -glucoserebrosidase in cells of RES thereby leading to accumulation of α -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. There are reports of improved intensity and duration of action in animal models as well as humans.

To treat lead poisoning, the concentration of α -aminolevulinic acid dehydrogenase (ALA-D) in erythrocytes decreases. This leads to an accumulation of α -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.

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 >50 mm 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 chain of hemoglobin decreases, which is compensated by an uptake of H⁺ and CO₂ that leads to increased formation of bicarbonate ion.

IV injection of IHP-loaded erythrocytes to piglets led to a decrease in cardiac output with a constant oxygen consumption by animals. This indicates that because of an increased extraction ratio of oxygen by tissues, a given amount of oxygen can be delivered in lower blood flow. In addition, these erythrocytes reduce ejection fraction, left ventricular diastolic volume, and heart rate. An isolated perfused-heart model showed reduction in coronary blood flow with increased oxygen consumption by myocardium upon administration of IHP-loaded erythrocytes. The same

results are reported when intact animal models were used. 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 radio sensitivity of radiation-sensitive tumors
- restoration of oxygen-delivery capacity of stored blood
- Ischemia of myocardium, brain, or other tissues

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 micro syringes 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 glycoprotein's 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 cointroduction of various components. Hence, this method is limited to mainly cell biological applications rather than drug delivery. (Gothoskar2004)

NOVEL APPROACHES:

These are specially engineered vesicular systems that are chemically cross-linked to human erythrocytes support upon which a lipid bilayer is coated. (Legha 1981) This process is achieved by modifying a reverse-phase evaporation technique. These vesicles have been proposed as useful encapsulation systems for macromolecular drugs. (rajendra 2011)

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. (Moorjani 1996) This complex was more active than free daunorubicin alone, both in vitro and in vivo. (rajendra 2011)

FUTURE PERSPECTIVES

The concept of employing erythrocytes as drug or bio active carrier still needs further optimization a large amount of valuable work is needed so us to utilize the potential of erythrocyte in passive and as well as active targeting of drugs. Disease like cancer would surely find it cure. Genetic engineering aspects can be coupled to give a newer dimension to the existing cellular drug concept. (Gothoskar 2004)

TABLE 1: VARIOUS CHARACTERIZATION PARAMETERS AND METHOD EMPLOYED FOR RESEALED ERYTHROCYTES (Sha 2011)

| PARAMETER | INSTRUMENT/METHOD USED |
|--|---|
| Physical parameter Shape, size, surface morphology | Transmission electron microscopy, scanning electron microscopy, optical microscopy, phase contrast 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 drug, radio labelling |
| Surface electrical potential spectroscopy | Zeta potential determination by photon correlation(PCS) |
| Surface pH | Ph sensitive probes |
| Deformity | Capillary method |
| II. Cellular parameter | |
| a. % Hb content | Deproteinization of cell membrane followed by haemoglobin assay of drug |
| b. Cell volume | Laser light scattering |
| c. % cell recovery | Neubaur's chamber, haematological analyser |
| d. Osmotic fragility | Stepwise incubation with isotonic to hypotonic saline solutions and determination of drug and haemoglobin assay |
| e. Osmotic shock | Dilution with distilled water and estimation of drug and haemoglobin |
| f. Turbulent shock | Passage of cell suspension to 30-gauge hypodermic needle at the rate of 10ml/min flow and estimation of residual drug and haemoglobin, vigorous shaking |
| g. Erythrocyte sedimentation rate | Determine by ESR technique |
| III. Biological parameter | |
| a) Pyrogenicity | LAL test, rabbit method |
| b) Sterility | Sterility testing method |
| c) Toxicity | Toxicity test method |

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