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Review Article

Resealed Erythrocytes: Potential Carrier for Delivery of Drugs

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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. Such drug-loaded 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. Therefore, these carriers are called resealed erythrocytes. The entire process is based on the response of these cells under osmotic conditions. Upon re-injection, the drug-loaded erythrocytes serve as slow circulating depots and target the drugs to reticulo-endothelial system. Several methods can be used to load drugs or other bioactive compounds in erythrocytes, including physical osmosis-based systems, and chemical methods. 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. Slow drug release, drug targeting, targeting RES organ, targeting the lever, enzyme therapy, targeting organs other than those of RES, improvement in oxygen delivery in tissues, delivery of antiviral agents are some of the applications. Erythrosomes and nanoerythrosomes are the recent novel approaches for drug delivery. In near future, erythrocytes based delivery system with their ability to provide controlled and site specific drug delivery will revolutionize disease management. Considering their tremendous potential it is concluded that erythrocyte carriers are “golden cells in novel drug delivery systems” The use of resealed erythrocytes looks promising for a safe and sure delivery of various drugs for passive and active targeting. The same concept also can be extended to the delivery of biopharmaceuticals and much remains to be explored regarding the potential of resealed erythrocytes.

Key Words: nanoerythrosomes, golden cells, resealed erythrocytes, Drug Carriers.

INTRODUCTION

Blood contains different type of cells like erythrocytes (RBC), leucocytes (WBC) and platelets, among them erythrocytes are the most interesting carrier and posses great potential in drug delivery due to their ability to circulate throughout the body, zero order kinetics, reproducibility and ease of preparation1 primary aim for the development of this drug delivery system is to maximize therapeutic performance, reducing undesirable side effects of drug as well as increase patient compliance. The overall process is based on the response of these cells under osmotic conditions. Upon reinjection, the drug-loaded erythrocytes

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serve as slow circulating depots and target the drugs to disease tissue or organ. Present pharmaceutical scenario is aimed at development of drug delivery systems which maximize the drug targeting along with high therapeutic benefits for safe and effective management of diseases. Targeting of an active bio molecule from effective drug delivery where pharmacological agent directed specifically to its target site. Drug targeting can be approaches by either chemical modification or by appropriate carrier.

Erythrocytes

Red blood cells (also referred to as erythrocytes) are the most common type of blood cells and the vertebrate organism's principal means of delivering oxygen (O_2) to the body tissues via the blood flow through the circulatory system. The cells develop in the bone marrow and circulate for about 100–120 days in the body before their components are recycled by macrophages. Each circulation takes about 20 seconds. Approximately a quarter of the cells in the human body are red blood cells.

1.2 Resealed Erythrocytes 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 carriers8. 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).

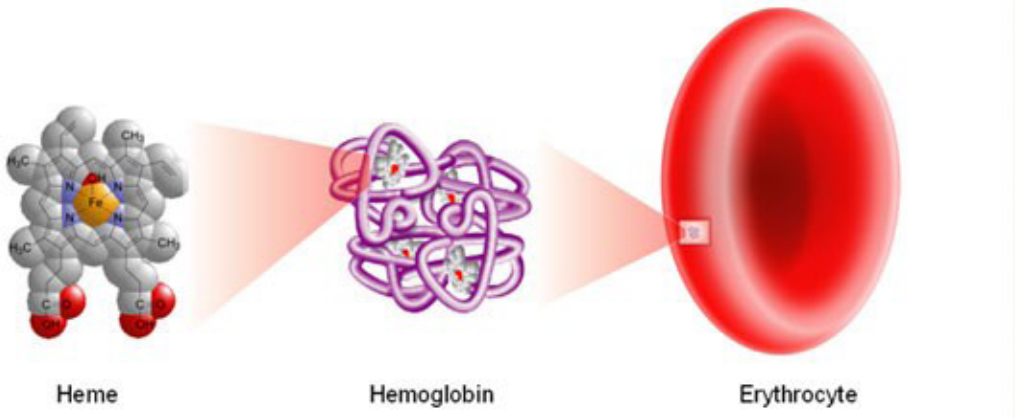

Fig: 1 Introduction of erythrocytes.

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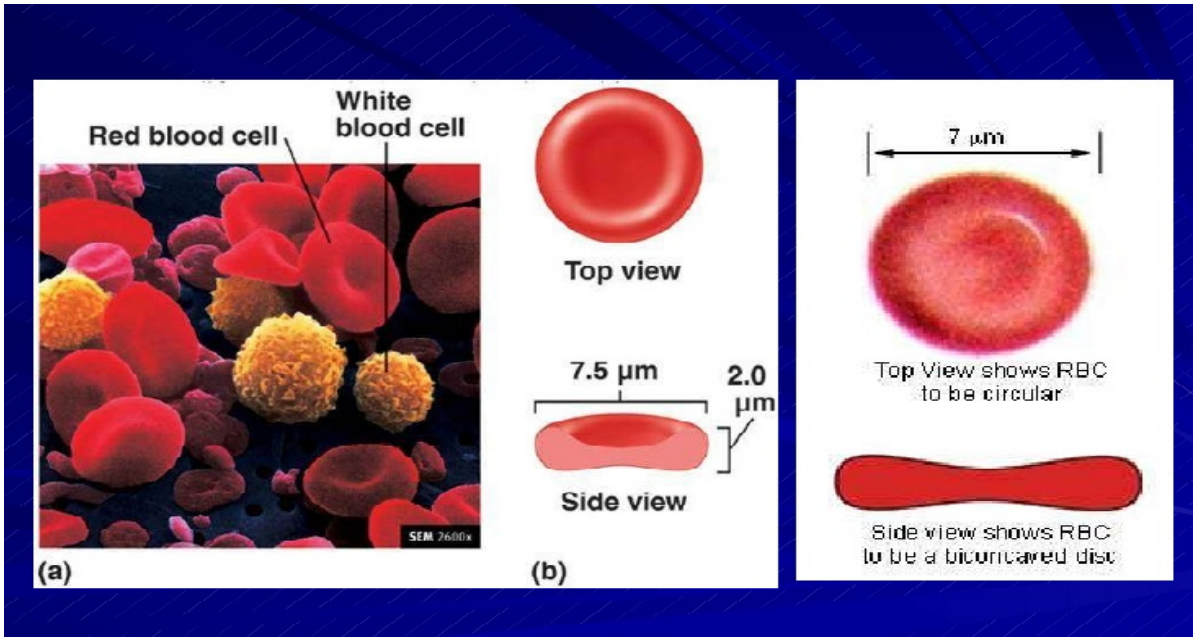

Fig.:2 Anatomy of erythrocytes

A. Composition of Erythrocytes [2]

- Blood contains about 55% of fluid portion (plasma) 45% of corpuscles or formed elements.
- Normal blood cells have extensile, elastic, biconcave and non nucleated configuration with a diameter ranging from 6-9 µ and the thickness is nearly 1-2 µ.
- Erythrocytes have a solid content of about 35% most of which is Hb and rest 65% being water.
- Lipid content of erythrocytes includes cholestrol, lecithin and cephaelins.

B.Electrolyte composition of erythrocytes: Although qualitatively similar to that of plasma however, quantitatively it differs from that of plasma.

- The concentration of K^+ is more in erythrocytes and Na+ in plasma.
- The osmotic pressure of the interior of the erythrocytes is equal to that of the plasma and termed as isotonic (0.9% NaCl or normal physiological saline.)
- Changes in the osmotic pressure of the medium surrounding the red blood cells changes the morphology of the cells.

C. Haematocrit value [3]

- If blood is placed into a tube and centrifuged, the cells and the plasma will separate.
- The erythrocytes, which are heavy, will settle down to the bottom of the tube, while

the plasma rises up to the top and the leukocytes and platelets will form a thin layer (buffy coat) between the erythrocytes and the plasma.

- The haematocrit is defined as the percentage of whole blood made up of erythrocytes.

Males..... 40-50%

Females..... 38-45%

D. Source, Fractionation and Isolation of Erythrocytes

- Different mammalian erythrocytes have been used for drug loading, resealing and subsequent use in drug and enzyme delivery. e.g. mice, cattle, pigs, dogs, sheep, goats, monkeys, chicken, rats, and rabbits etc.
- EDTA or heparin can be used as anticoagulants agents.

Advantages of Resealed Erythrocytes as Drug Carriers[4]

The resealed erythrocytes should have the following advantages:

- Their biodegradability with no generation of toxic products.
- The considerably uniform size and shape of the carrier.
- Relatively inert intracellular environment.
- Prevention of degradation of the loaded drug from inactivation by endogenous chemicals.
- The wide variety of chemicals that can be entrapped.
- The modification of pharmacokinetic and pharmacodynamic parameters of drug.
- Attainment of steady-state plasma concentration decreases fluctuations in concentration. Protection of the organism against toxic effects of drugs (e.g. antineoplastics).
- They are ability to circulate throughout the body and facilities for separation, handling, transfusion, and working with erythrocytes the availability of the techniques.

Disadvantages[5]

1. 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.

2. The rapid leakage of certain encapsulated substances from the loaded erythrocytes.

3. Several molecules may alter the physiology of the erythrocyte.

4. 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.

5. 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.

6. 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.

Erythrocytes as a Carrier[6]

1. Targeting particular tissue/organ

For targeting, only the erythrocyte membrane is used. This is obtained by splitting the cell in hypotonic solution and after introducing the drug into the cells, allowing them to reseal into spheres. Such erythrocytes are called Red cell ghosts.

2. 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.

Isolation of Erythrocytes: [7]

Blood is collected into heparin zed tubes by venipunture. Blood is withdrawn from cardiac/splenic puncture (in small animal) and through veins (in large animals) in a syringe containing a drop of anti-coagulant.

The whole blood is centrifuged at 2500 rpm for 5 min. at 4 ±10C in a refrigerated centrifuge. The serum and Buffy coats are carefully removed and packed cells washed three times with phosphate buffer saline (pH=7.4). The washed erythrocytes are diluted with PBS and stored at 40C for as long as 48 h before use. 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.

Various condition and centrifugal force used for isolation of erythrocytes.[8]

Requirement for encapsulation

Variety of biologically active substance (5000-60,000dalton) can be entrapped in erythrocytes. Non-polar molecule may be

entrapped in erythrocytes in salts. Example: tetracycline HCl salt can be appreciably entrapped in bovine RBC. Generally, molecule should be Polar & Non polar molecule also been entrapped. Hydrophobic molecules can be entrapped in erythrocyte by absorbing over other molecules.

Once encapsulated charged molecule are retained longer than uncharged molecule. The size of molecule entrapped is a significant factor when the molecule is smaller than sucrose and larger than B galactosidase.

Factors which considering resealed erythrocytes as carrier [9]

Its shape and size to permit the passage through the capillaries. Its specific physico-chemical properties by which a prerequisite site can be recognized.

- Its biocompatible and minimum toxicity character.
- Its degradation product, after release of the drug at the target site, should be biocompatible.
- Low leaching/leakage of drug should take place before target site is reached.
- Its drug released pattern in a controlled manner.
- High drug loading efficiency for broad spectrum of drugs with different properties
- Physico-chemical compatibility with the drug.
- The carrier system should have an appreciable stability during

Method of Drug Loading and Storage[10]

In general, the potential use of erythrocytes depends on their ability to encapsulate exogenous enzymes or other substances into erythrocytes. 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 erythrocytes membrane, and well defined pharmacokinetic and pharmacodynamic properties. The following methods are used for entrapment of therapeutic agent into erythrocytes:

The following are the types of drug loading:-

1- Hypo- osmosis lysis method

a- Hypotonic Dilution method

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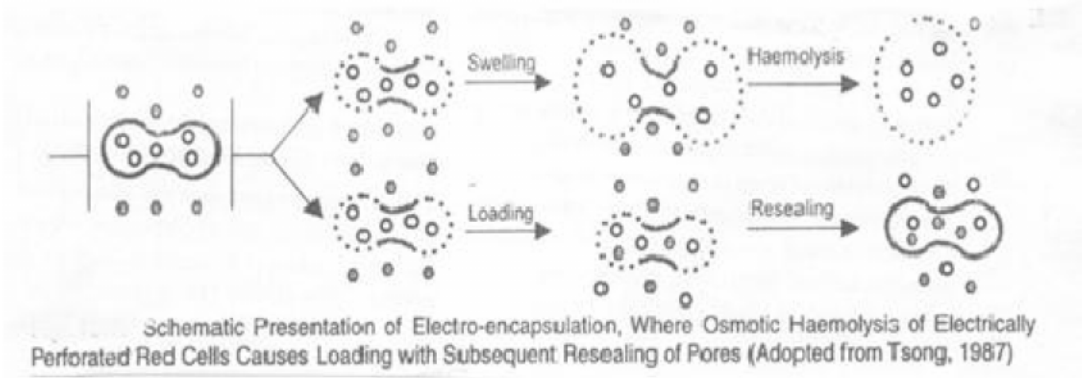

Fig: 8 Isotonic osmotic lysis.

b- Hypotonic Dialysis method
c- Hypotonic Pre swelling method
d- Isotonic osmotic lysis method.

2- Electro-insertion or Electro encapsulation Method.

3- Entrapment by endocytes.

4- Chemical perturbation of the membrane.

5- Lipid fusion method.

1. Hypotonic hemolytic

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.[11]

2. Use of red cell loader

Novel method for entrapment of non-diffusible drugs into erythrocytes Piece of equipment called a “red cell loader”. With as little as 50 mL of a blood sample, different biologically active compounds were entrapped into erythrocytes within a period of 2h at Room temperature The process is based on two sequential hypotonic dilutions of washed erythrocytes followed by concentration with a hemo filter and an

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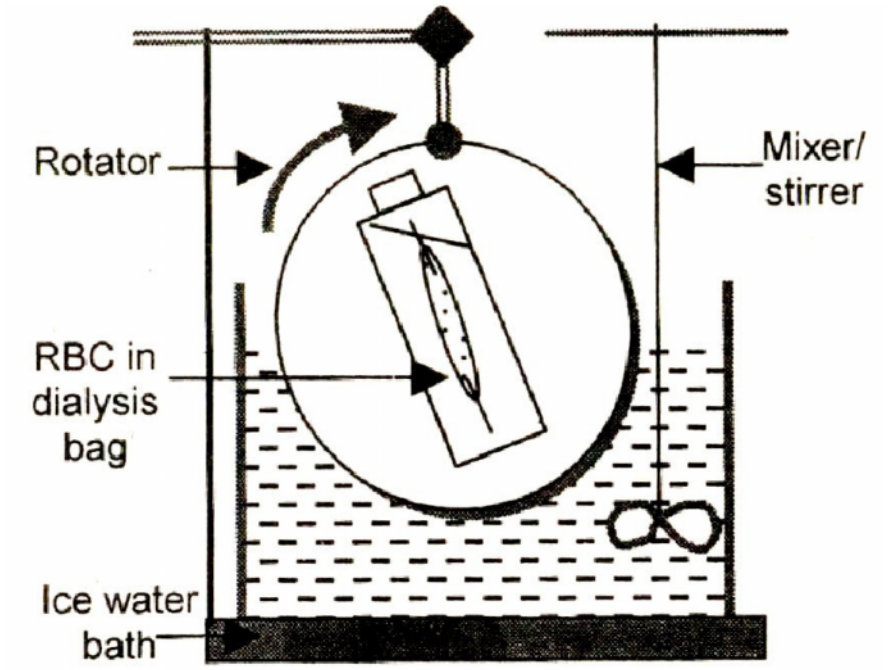

Fig: 9 Hypotonic dialysis

isotonic resealing of the cells. There was ~30% drug loading with 35–50% cell recovery.

3. 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.[12]

4. Hypotonic preswelling

This method was developed 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 values. The supernatant is discarded and the cell fraction is brought to the lysis point by L portions of an aqueous solution of the drug to be encapsulated. Adding 100–120. 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.

5. Isotonic osmotic lysis

This method, also known as the osmotic pulse method, involves isotonic hemolysis that is achieved by physical or chemical means. The isotonic solutions may or may not be 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.

6. Hypotonic dialysis

Several methods are based on the principle that semipermeable dialysis membrane maximizes the intracellular: extracellular volume ratio for macromolecules during lysis and resealing. In the process, an isotonic, buffered suspension of erythrocytes with a hematocrit value of 70–80 is prepared and placed in a conventional dialysis tube immersed in 10–20 volumes of a hypotonic buffer. The medium is agitated slowly for 2 h. The tonicity of the dialysis tube is restored by directly adding a calculated amount of a hypertonic buffer to the surrounding medium or by replacing the surrounding medium by isotonic buffer[13].

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.

7. Chemical perturbation of the membrane

This method is based on the increase in membrane permeability of erythrocytes when the cells are exposed to certain chemicals that the permeability of erythrocytic membrane increases upon exposure to polyene antibiotic such as amphotericin B. In1980, this method was used successfully by Kitao and Hattori to entrap the antineoplastic drug daunomycinin human and mouse erythrocytes, in *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.

Storage

Store encapsulated preparation without loss of integrity when suspended in Hank's

balanced salt solution [HBSS] at 4°C for two weeks. Use of group 'O' [universal donor] cells and by using the preswell or dialysis technique, batches of blood for transfusion. Standard blood bag may be used for both encapsulation and after loading of therapeutic agent on erythrocytes, the carrier cells are exposed to physical, cellular as well as biological evaluations[14]

Evaluation of Resealed Erythrocytes[15]

1. Shape and Surface Morphology

The morphology of erythrocytes decides their life span after administration. The morphological characterization of erythrocytes is undertaken by comparison with untreated erythrocytes using either transmission (TEM) or Scanning electron microscopy (SEM). Other methods like phase contrast microscopy can also be used.

2. Drug Content

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 by spectrophotometrically.

3. Cell Counting and Cell Recovery

This involves counting the number of red blood cells per unit volume of whole blood, usually by using automated machine it is determined by counting the no. of intact cells per cubic mm of packed erythrocytes before and after loading the drug.

4. Turbulence Fragility

It is determined by the passage of cell suspension through needles with smaller internal diameter (e.g., 30 gauges) or vigorously shaking the cell suspension. In both cases, haemoglobin and drug released after the procedure are determined. The turbulent fragility of resealed cells is found to be higher.

5. 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 α, β 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.

Applications of Resealed Erythrocytes [16,17]

In Vitro Applications

Carrier RBCs have proved to be useful for a variety of in vitro tests. For in vitro phagocytosis cells have been used to

facilitate the uptake of enzymes by phagolysosomes. An inside to this study showed that enzymes Content within carrier RBC could be visualized with the help of cytochemical technique. The most frequent in vitro application of RBC mediated microinjection. A protein or nucleic acid to be injected into eukaryotic cells by fusion process similarly, when antibody molecules are introduced using erythrocytic carrier system, they immediately diffuse throughout the cytoplasm. Antibody RBC auto Injected into living cells have been used to confirm the site of action of fragment of diphtheria toxin.

***In Vivo* Applications**

This includes the following

1) Slow drug release

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

2) Drug targeting

Ideally, drug delivery should be site specific and target oriented to exhibit maximal therapeutic index with minimum adverse effects. Resealed erythrocytes can act as drug carriers and targeting tools as well. Surface modified erythrocytes are used to target organs of mononuclear phagocytic system/ RES because the change in the membrane is recognized by macrophages.

3) Targeting reticuloendothelial system (RES) organs

Surface modified erythrocytes are used to target organs of mononuclear phagocytic systems/ reticuloendothelial system because the changes in membrane are recognized by macrophages. The various approaches used include

- Surface modification with antibodies (coating of loaded erythrocytes by anti-Rh or other types of antibodies)
- Surface modification with glutaraldehyde.
- Surface modification with sulphydryl.
- Surface chemical crosslinking.
- Surface modification with carbohydrates such as sialic acid.

4) Targeting the liver-deficiency/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 Pglucosidase, P-

glucoronidase, and Pgalactosidase. The disease caused by an accumulation of glucocerebrosidaes in the liver and spleen can be treated by glucocerebrosidase-loaded erythrocytes.

Novel Approaches[18, 19,20]

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

Nanoerythrosomes: These are prepared by extrusion of erythrocyte ghosts to produce small vesicles with an average diameter of 100 nm. Daunorubicin was covalently conjugated to nanoerythrosomes using gluteraldehyde spacer. This complex was more active than free daunorubicin alone.

Other

Significant advances have been made with the use of erythrocyte for specific targeting to cells of the immune system.antiviral drugs can be pretreated to deliver drug directly to macrophages. Several laboratory techniques have developed for the encapsulation of allosteric effector of hemoglobin, inisitol hexaphosphate, which are effective at oxygen delivery, much more effective than normal erythrocytes.[21, 22, 23]

Future Perspective [24, 25]

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

CONCLUSION AND SUMMARY [26, 27, 28]

During the past decade, numerous applications have been proposed for the use of resealed erythrocytes as carrier for drugs, enzyme replacement therapy etc.

Until other carrier systems come of age, resealed erythrocytes technology will remain an active arena for the further research. The commercial medical applications of carrier erythrocytes are currently being tested in Europe by a recently formed company that is developing products for human use. The coming years represent a critical time in this field as commercial applications are explored. In near future, erythrocytes based delivery system with their ability to provide controlled and site specific drug delivery

will revolutionize disease management.

The International Society for the use of Resealed Erythrocytes (ISURE) through its biannual meetings provides an excellent forum for exchange of information to the scientist in this exiting and rewarding field of research. For the present, it is concluded that erythrocyte carriers are “golden eggs in novel drug delivery systems” considering their tremendous potential.

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