

RESEALED ERYTHROCYTE DRUG DELIVERY: A REVIEW

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ABSTRACT

Among the various carriers used for targeting drugs to various body tissues, the cellular carriers meet several criteria desirable in clinical applications, among the most important being biocompatibility of carrier and its degradation products. Leucocytes, platelets, erythrocytes, nanoerythrocytes, hepatocytes, and fibroblasts etc. have been proposed as cellular carrier systems. Among these, the erythrocytes have been the most investigated and have found to possess greater potential in drug delivery. Biopharmaceuticals, therapeutically significant peptides and proteins, nucleic acid-based biological, antigens, anticancer drug and vaccines, are among the recently focused pharmaceuticals for being delivered using carrier erythrocytes. Erythrocytes, also known as red blood cells, and have been extensively studied for their potential carrier capabilities for the delivery of drugs. The biocompatibility, non-pathogenicity, non-immunogenicity and biodegradability make them unique and useful carriers. Carrier erythrocytes are prepared by collecting blood sample from the organism of interest and separating erythrocytes from plasma. By using various methods the cells are broken and the drug is entrapped into the erythrocytes, finally they are resealed and the resultant carriers are then called "resealed erythrocytes". So many drugs like aspirin, steroid, cancer drug which having many side effects are reduce by resealed erythrocyte. Current review highlights isolation, drug loading methods, Evaluation methods and applications of resealed erythrocytes for drug delivery.

Keywords: Resealed Erythrocytes, carrier, Isolation, Applications.

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.²

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.³

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.⁴

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 (O₂) 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.⁵

Anatomy, physiology and composition of RBCs

RBCs have shapes like biconcave discs with a diameter of 7.8 μm and thickness near 2.2 μm . Mature RBCs have a simple structure. It is also in elastic in nature. Their plasma membrane is both strong and flexible, which allows them to deform without rupturing as they squeeze through narrow capillaries. RBCs lack a nucleus and other organelles and can neither reproduce nor carry on extensive metabolic activities. RBCs are highly specialized for their oxygen transport function, because their mature RBCs have no nucleus, all their internal space is available for oxygen transport. Even the shape of RBC facilitates its function. A biconcave disc has a much greater surface area for the diffusion of gas molecules into and out of the RBC than would; say a sphere or a cube. The red blood cell membrane, a dynamic, semi permeable components of the cell, associated with energy metabolism in the maintenance of the permeability characteristic of the cell of various cations (Na^+ , K^{++}) and anions (Cl^- HCO_3^-). Each RBC contains about 280 million hemoglobin molecules. A hemoglobin molecule consists of a protein called globin, composed of four polypeptide chains; a ring like non-protein pigment called a heme, is bound to each of the four chains. At the center of the heme ring combine reversibly with one oxygen molecule, allowing each hemoglobin molecule to bind four oxygen molecules. RBCs include water (63%), lipids (0.5), glucose (0.8%), mineral (0.7%), non-hemoglobin protein (0.9%), meth hemoglobin (0.5%), and hemoglobin (33.67%).^{2, 6, 7}

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)⁹

ADVANTAGES

1. Biocompatible, particularly when autologous cells are used hence no possibility of triggered immune response.

2. Biodegradability with no generation of toxic products.
3. Considerable uniform size and shape of carrier.
4. Relatively inert intracellular environment can be encapsulated in a small volume of cells.
5. Isolation is easy and large amount of drug can be loaded.
6. Prevention of degradation of the loaded drug from inactivation by endogenous chemical.
7. Entrapment of wide variety of chemicals can be possible.
8. Entrapment of drug can be possible without chemical modification of the substance to be entrapped.
9. Possible to maintain steady-state plasma concentration, decrease fluctuation in concentration.
10. Protection of the organism against toxic effect of drug.
11. Targeting to the organ of the RES.
12. Ideal zero-order drug release kinetic.
13. Prolong the systemic activity of drug by residing for a longer time in the body.¹¹⁻²⁰

DISADVANTAGES

1. They have a limited potential as carrier to non-phagocyte target tissue.
2. Possibility of clumping of cells and dose dumping may be there.^{21, 22}

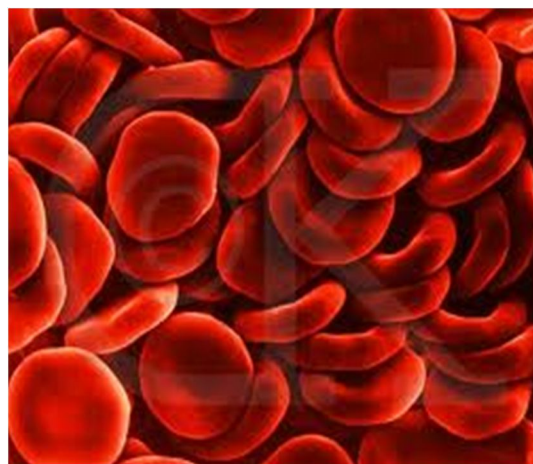


Fig. 1: Erythrocytes¹⁰

Erythrocytes can be used as carriers in two ways

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.²³

ISOLATION OF ERYTHROCYTES:

- Blood is collected into heparin zed tubes by venipuncture.
- 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 $4 \pm 1^{\circ}\text{C}$ 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 4°C 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.^{18, 24}

Various condition and centrifugal force used for isolation of erythrocytes²⁵

Sr.no	Species	Washing Buffer	Centrifugal force (g)
1.	Rabbit	10mmol $\text{KH}_2\text{PO}_4/\text{NaHPO}_4$	500-1000
2.	Dog	15mmol $\text{KH}_2\text{PO}_4/\text{NaHPO}_4$	500-1000
3.	Human	154mmol NaCl	<500
4.	Mouse	10mmol $\text{KH}_2\text{PO}_4/\text{NaHPO}_4$	100-500
5.	Cow	10-15mmol $\text{KH}_2\text{PO}_4/\text{NaHPO}_4$	1000
6.	Horse	2mmol MgCl_2 , 10mmol glucose	1000
7.	Sheep	10mmol $\text{KH}_2\text{PO}_4/\text{NaHPO}_4$	500-1000
8.	Pig	10mmol $\text{KH}_2\text{PO}_4/\text{NaHPO}_4$	500-1000

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.²⁶⁻³¹

METHODS OF DRUG LOADING IN ERYTHROCYTES

1) Hypo- osmosis lysis method

In this process, the intracellular and extracellular solute of erythrocytes is exchange by osmotic lysis and resealing. The drug present will be encapsulated within the RBCs by this process.³²

a) Hypotonic dilution

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.³³

b) Hypotonic Dialysis method

This method was first reported by Klibansky in 1959 and was used in 1977 by Deloach, Ihler and Dale for loading enzymes and lipids.

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.³⁴

c) Hypotonic Pre swelling method

This method was developed by Rechsteiner in 1975 and was modified by Jenner et al. for drug loading.

This method based on the principle of first swelling the erythrocytes without lysis by placing them in slightly hypotonic solution. The swollen cells are recovered by centrifugation at low speed. Then, relatively small volumes of aqueous drug solution are added to the point of lysis. The slow swelling of cells results in good retention of the cytoplasmic constituents and hence good survival *in vivo*. 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, methotrexate, insulin, metronidazole, levothyroxine, enalaprilat & isoniazid.³⁵⁻³⁷

d) Isotonic osmotic lysis method

This method was reported by Schrier et al in 1975. 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 sealed at 37°C.³⁸⁻⁴²

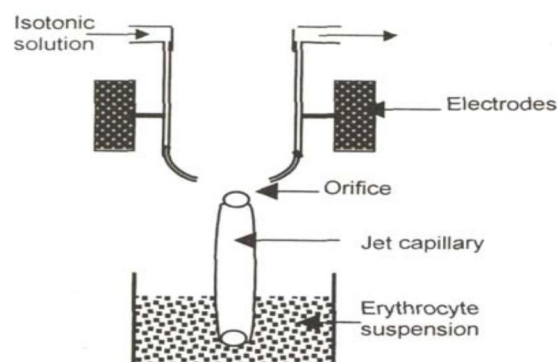
Comparison between percent drug loading, advantages as well as disadvantages of different osmosis based systems⁴³

Method	% Loading	Advantages	Disadvantages
Dilution	20-40	Fastest and simplest especially for low molecular weight Drugs	Entrapment efficiency is less.
Dialysis	30-45	Better <i>in vivo</i> survival of erythrocytes better structural integrity and membrane. Good retention of cytoplasm and good survival <i>in vivo</i> .	Time consuming, heterogeneous size distribution of resealed erythrocytes.
Presswell	30-90		-
Isotonic osmotic Lysis	-	Better <i>in vivo</i> survival.	Impermeable only large molecules, process is time Consuming.

2) Electro-insertion or Electro encapsulation method

In 1973, Zimmermann tried an electrical pulse method to encapsulate bioactive molecules. Also known as electroporation, the method is based on the observation that electrical shock brings about irreversible changes in an erythrocyte membrane.

This method is also called as electroporation. In this method erythrocyte membrane is open by a dielectric breakdown; subsequently the pore of erythrocyte can be resealed by incubation at 37°C in an isotonic medium. The various chemical encapsulated into the erythrocytes are primaquin and related 8-amino quinolone, vinblastin chlorpromazine and related phenothiazine, propranolol, tetracaine and vitamin A.⁴⁴⁻⁴⁶



Electroencapsulation⁴⁷

3) Entrapment by endocytosis

This method was reported by Schrier et al in 1975. This method involves the addition of one volume of washed packed erythrocytes to nine volume of buffer containing 2.5MM ATP,

2.5MM mgcl₂ and 1MM CaCl₂, followed by incubation for 2 minute at room temperature. The pores created by this method are resealed by using 154MM of NaCl and incubate at 37⁰C for 2 minute. Several chemicals are entrapped in erythrocytes by this method are primaquine and related 8- aminoquinoline, vinblastin, chlorpromazine, and related phenothiazines, hydrocortisone, tetracaine and vitamin A.⁴⁸⁻⁵⁰

4) 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 erythrocyte 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. However, these methods induce irreversible destructive changes in the cell membrane and hence are not very popular.⁵¹⁻⁵³

5) Lipid fusion method

The lipid vesicles containing a drug can be directly fuse to human erythrocytes, which lead to an exchange with a lipid 161 entrapped drug. The methods are useful for entrapping inositol monophosphate to improve the oxygen carrying capacity of cells and entrapment efficiency of this method is very low (~1%).⁴⁴

6) 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.⁵⁴

7) Use of red cell loader

Novel method was developed for entrapment of non diffusible 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 hem filter 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.⁵⁵

STORAGE

Store encapsulated preparation without loss of integrity when suspended in hank's balanced salt solution [HBSS] at 4⁰C 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 storage.⁵⁶

EVALUATION OF RESEALED ERYTHROCYTES

After loading of therapeutic agent on erythrocytes, the carrier cells are exposed to physical, cellular as well as biological evaluations.

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.⁵⁷

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.¹¹

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.⁵⁸

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.^{56, 59, 60}

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.²⁵

6. Determination of entrapped magnetite

Atomic absorption spectroscopic method is reported for determination of the concentration of particular metal in the 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.²⁵

7. In vitro stability

The stability of the loaded erythrocytes is assessed by means of the incubation of the cells in the autologous plasma or in an isoosmotic buffer, setting hematocrit between 0.5% and 5% at temperatures of 4°C and 37°C.⁶¹

8. Haemoglobin release

The content of haemoglobin of the erythrocytes may be diminished by the alterations in the permeability of the membrane of the red blood cells during the encapsulation procedure. Furthermore, the relationship between the rate of haemoglobin and rate of drug release of the substance encapsulated from the erythrocytes. The haemoglobin leakage is tested using a red cell suspension by recording absorbance of supernatant at 540nm on a spectrophotometer.⁶¹

9. In-vitro drug release and Hb content

The cell suspensions (5% hematocrit in PBS) are stored at 4°C in ambered colour glass container. Periodically clear supernatant are drawn using a hypodermic syringe equipped with 0.45 μ m filter, deproteinized using methanol and were estimated for drug content. The supernatant of each sample after centrifugation collected and assayed, %Hb release may be calculated using formula % Hb release = $\frac{A_{540} \text{ of sample} - A_{540} \text{ of background}}{A_{540} \text{ of } 100\% \text{ Hb}}$.²⁵

10. Osmotic shock

For osmotic shock study, erythrocytes suspension (1 ml 10% hct) was diluted with distilled water (5 ml) and centrifuge at 300 rpm for 15 minutes. The supernatant was estimated for % haemoglobin release analytically.⁶²

11. Miscellaneous

Resealed erythrocyte can also be characterized by cell sizes, mean cell volume, energy metabolism, lipid composition, membrane fluidity, rheological properties, and density gradient separation.²⁵

APPLICATIONS OF RESEALED ERYTHROCYTES

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.¹³

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.⁶³

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.⁶⁴

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 sulphhydryl.
- Surface chemical crosslinking.
- Surface modification with carbohydrates such as sialic acid.⁶⁵

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 P-glucosidase, P-glucuronidase, and P-galactosidase. The disease caused by an accumulation of glucocerebrosidase in the liver and spleen can be treated by glucocerebrosidase-loaded erythrocytes.³³

5) Treatment of parasitic disease

The ability of resealed erythrocytes to selectively accumulate within RES organs make them useful tool during the delivery of anti parasitic 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 anti malarial, anti leishmanial and anti amoebic drugs.^{11, 18, 56}

6) Removal toxic agents

Cannon *et al.* reported inhibition of cyanide intoxication with murine carrier erythrocyte containing bovine rhodanase and sodium thiosulphate. Antagonization of organophosphorus intoxication by resealed erythrocyte containing a recombinant phosphodiesterase also has been reported.⁶⁶

7) Treatment of hepatic tumors

Antineoplastic drugs such as metotrexate (MTX), bleomycin, asparaginase and adiramycin have been successfully delivered by erythrocytes. E.g. in a study, the MTX showed a preferential drug targeting to liver followed by lungs, kidney and spleen.⁶⁷

8) Delivery of antiviral agents

Several reports have been cited in the literature about antiviral agents entrapped in

resealed erythrocytes for effective delivery and targeting. Because most antiviral drugs are nucleotides or nucleoside analogs, their entrapment and exit through the membrane needs careful consideration.⁶⁸

9) Enzyme therapy

Many metabolic disorders related to deficient or missing enzymes can be treated by administering these enzymes as resealed erythrocytes. E.g. β -Glucosidase, β -glucouronidase, β -galactosidase.^{14, 31, 69}

10) Removal of RES iron overloads

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.⁶³

11) Targeting Non RES

Erythrocytes loaded with drugs have also been used to target organs outside the RES. The various approaches for targeting non-RES organs include:

- Entrapment of paramagnetic particles along with the drug.
- Entrapment of photosensitive material.
- Use of ultrasound waves.
- Antibody attachment to erythrocytes membrane to get specificity of action.
- Other approaches include fusion with liposome, lectin pre-treatment of resealed cells etc.^{63, 70}

ROUTE OF ADMINISTRATION

Intra peritoneal injection reported that survival of cells in circulation was equivalent to the cells administered by i.v. injection. They reported that 25% of resealed cell remained in circulation for 14 days they also proposed this method of injection as a method for extra vascular targeting of RBCs to peritoneal macrophages. Subcutaneous route for slow release of entrapped agents. They reported that the loaded cell released encapsulated molecules at the injection site.⁷¹⁻⁷³

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.⁷⁴⁻⁷⁵

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.⁷⁶⁻⁷⁷

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