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A REVIEW ON RESEALED ERYTHROCYTES

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ABSTRACT

Carrier erythrocytes have been evaluated in thousands of drug administration in humans proving safety and efficacy of the treatments. Carrier erythrocytes, resealed erythrocytes loaded by a drug or other therapeutic agents, have been exploited extensively in recent years for both temporally and spatially controlled delivery of a wide variety of drugs and other bioactive agents owing to their remarkable degree of biocompatibility, biodegradability and a series of other potential advantages. Biopharmaceuticals, therapeutically significant peptides and proteins, nucleic acid-based biological, antigens and vaccines, are among the recently focused pharmaceuticals for being delivered using carrier erythrocytes. In this review article, the potential applications of erythrocytes in drug delivery have been reviewed with a particular stress on the studies and laboratory experiences on successful erythrocyte loading and characterization of the different classes of biopharmaceuticals.

KEYWORDS: Resealed erythrocytes, Drug targeting, characterization methods and Applications.

INTRODUCTION

At present, there are 30 main drug delivery products on the market. The total annual income for all of these is approximately US$33 billion with an annual growth of 15 % (based on global product revenue). The reasons for this increasing interest in drug delivery are due to the increasing need of safe drugs, capable of reaching the target and with minimal side effects. In fact the main problems associated with systemic drug administration are essentially related to the bio-distribution of pharmaceuticals throughout the body. This indiscriminate distribution means that, to achieve a required therapeutic concentration the

drug has to be administered in large quantities, the major part of which is just wasted in normal tissues. Ideally, a "perfect" drug should exert its pharmacological activity only at the target site, using the lowest concentration possible and without negative effects on non-target compartments.

The delivery systems currently available enlist carriers that are either simple, soluble macromolecules (such as monoclonal antibodies, soluble synthetic polymers, polysaccharides and particulate biodegradable polymers) or more complex multicomponent structures (microcapsules, Micro particles, cells, cell ghosts, lipoproteins, liposomes, erythrocytes (Jaitely V. *et al* 1996). Unfortunately, sometime the body recognizes the drug targeting system as non-self and unexpected toxicities could hamper the use of the same. This is the case of the first generation of monoclonal antibodies coupled to cytotoxic drugs or of other soluble carriers experimented at preclinical level. It has been envisaged that ideal drug delivery systems should be made of self-powered, computer-controlled medical nanorobot system, named pharmacyte, capable of precise transport, timing, and targeted delivery of pharmaceutical agents to specific target in the body. This ideal drug delivery system is not yet available but significant progress has been made in the last years over the traditional drug formulations and in is identified:

1. **Transduced cells**, capable of expressing pharmaceutically relevant agents.

2. **Cell carriers** which could be loaded with drugs or therapeutics. In this category the carrier cells could release he drug content in circulation or at selected sites or could target the drug to other relevant cells in the body.

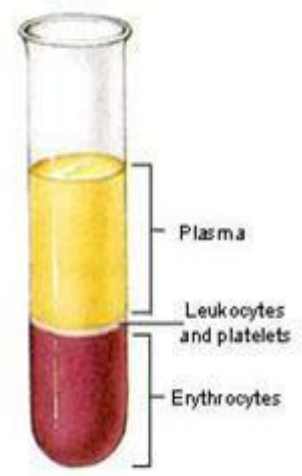
Erythrocytes also known as red blood cells have been extensively studied for their potential carrier capabilities for the delivery of drugs and drug-loaded microspheres. Such drug-loaded carrier erythrocytes are prepared simply by collecting blood samples from the organism of interest, separating erythrocytes from plasma, entrapping drug in the erythrocytes and resealing the resultant cellular carriers. Hence, these carriers are called resealed erythrocytes.

Source 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.

Isolation of Erythrocytes

Erythrocytes may be prepared as carriers from blood taken from human beings and from different animal species, such as rats , mice , rabbits , dogs , etc. Blood is taken from the human being, mouse, rat, or the animal species in question, using a suitable anti-coagulant. Application is normally made of EDTA, as it is the anticoagulant that best preserves the properties of blood cells. Freshly collected blood is centrifuged in a refrigerated centrifuge in order to separate packed erythrocytes. Several washes are subsequently performed. This is a process that normally involves repeated centrifugation with an sss solution to remove other blood components. The erythrocytes can be washed more efficiently by using a capillary hollow fibre plasma separator. The hematocrits employed may be variables ranging between 5 % and 95 % (Eichler HG. *et al* 1986). Although the most usual is to work with a haematocrit of 70 %. 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.

		
Separation process	**Isolated erythrocytes**	**Resealed erythrocytes**

Advantages & Disadvantages of Resealed Erythrocytes

ADVANTAGES	DISADVANTAGES
1. Biocompatibility; Biodegradability; inert. 2. No undesired immune responses against the entrapped drug. 3. Considerable protection of the organism against the toxic effects of the entrapped drug, e.g., antineoplasms. 4. Remarkably longer life-span. 5. Desirable size range and the considerably uniform size and shape. 6. Possibility of targeted drug delivery to the RES organs. 7. Possibility of ideal zero-order kinetics of drug release. 8. Wide variety of compounds with relatively high amount of drug and with the capability of being entrapped within the erythrocytes. 9. Possibility of using synthetic erythrocyte counterparts (artificial erythrocytes). 10. The entrapment of drug does not require the chemical modification of drugs.	1) 1) Being biodegradable, they are removed in vivo by the RES. This, although expands its capability to drug targeting , seriously limits their useful life as long circulating drug carriers and in some cases may pose toxicological problems. 2) Being from biological origin, entrapped erythrocytes may present variability and lesser standardization in their preparation, compared to other carrier systems. 3) Several molecules may alter the physiology of the erythrocyte. 4) Being from biological origin, entrapped erythrocytes may present variability and lesser standardization in their preparation, compared to other carrier systems. 5) Inaccessibility of many important therapeutic targets like solid tumors, extra vascular tissue components, and central nervous system. 6) Safety and technical concerns related to the storage of the loaded erythrocytes.

Erythrocyte Encapsulation Methods

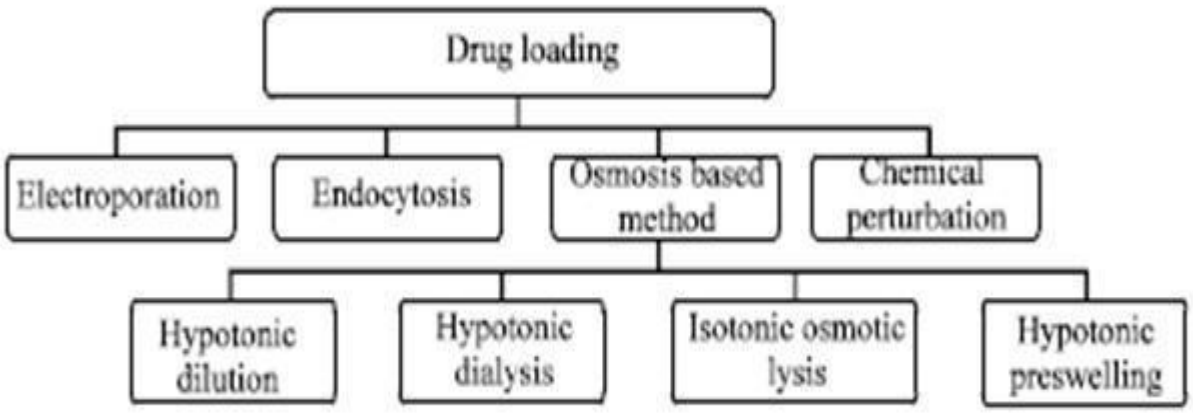


Fig .No.1 Schematic Illustration of Drug Loading in Erythrocytes.

1) Hypotonic Dialysis

This method was first reported by Klibansky (Zocchi E *et al* 1988). In 1959 and was used in 1977 by Deloach and Ihler *et al* (1977, 496, 136–145) (and Dale(Gaudreault RC, Bellemare B, Lacroix J *et al*, 1989, 9, 1201-1205) for loading enzymes and lipids. A desired Haematocrit is achieved by mixing washed erythrocyte suspension and phosphate buffer (pH 7.4) containing drug solution. This mixture is placed into dialysis bag and then both ends of

the bag are tied with thread. An air bubble of nearly 25 % of the internal volume is left in the tube. During dialysis bubble serves to blend the content. The tube is placed in a bottle containing 200 ml of lysis buffer solution and placed on a mechanical rotator at 4^{o} C for 2 hrs. The dialysis tube is then placed in 200 ml of resealing solution (isotonic PBS pH 7.4) at temperature 25 – 30^{o}C for resealing. The loaded erythrocytes thus obtained are then washed with cold PBC at 4^{o}C. The cells are finally resuspended in PBC.

Advantages

1. Good entrapment efficiency is obtained
2. The volume of extra cellular solution that equilibrates with the intracellular space of erythrocyte during lyses is considerably reduced.

Disadvantages

1. Time consuming method.
2. The size distribution of loaded ghosts is not found to be homogeneous as revealed by studies with hydro dynamically focusing particle analyzer.

Examples of encapsulated agents: gentamicin, adriamycine, erythropoietin, Pentamidine, furamycin A, interleukine-2, IgG

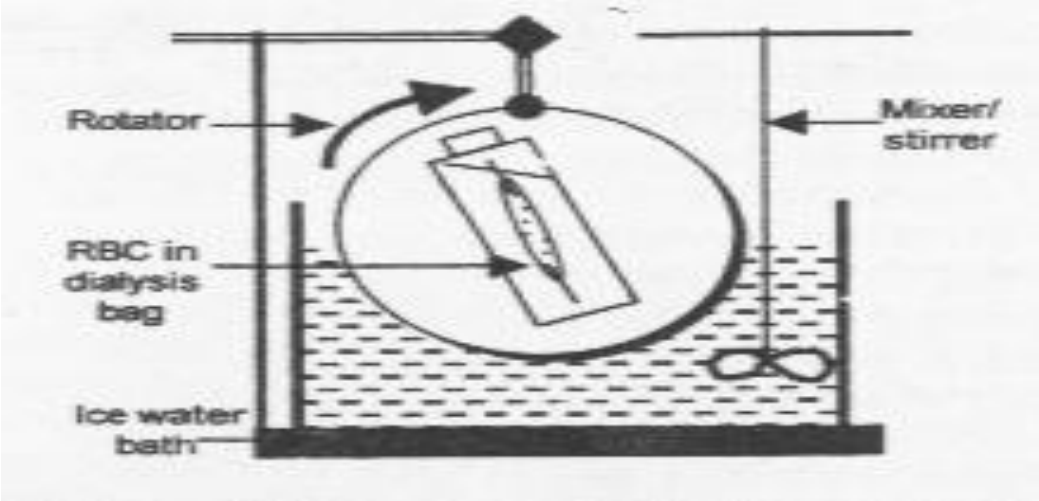


Fig No.2 Hypotonic Dialysis

2) Hypotonic hemolysis

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. An increase

in volume leads to an initial change in the shape from biconcave to spherical. This change is attributable to the absence of superfluous membrane; hence the surface area of the cell is fixed. The cells assume a spherical shape to accommodate additional volume while keeping the surface area constant. The volume gain is 25–50 %. The cells can maintain their integrity up to a tonicity of ~150 mosm/kg, above which the membrane ruptures, releasing the cellular contents. At this point (just before cell lysis), some transient pores of 200–500 Å are generated on the membrane. After cell lysis, cellular contents are depleted. The remnant is called an erythrocyte ghost.

Examples of encapsulated agents: Glucose oxidize (De Flora A, Guida L, Zocchi E, Tonetti M, Benatti U. *et al*, 1986, 35, 361- 367.) Glucose 6-phosphatase Gerli GC, Agostoni A, Fiorelli G *et al* , 1978, 34, 431- 432)anti-hexokinase IgG,(Magnani M, Rossi L, Bianchi M, Serafini G, Stocchi V *et al*, 1989, 82, 27-34) ATP(Gourley DHR *et al*).

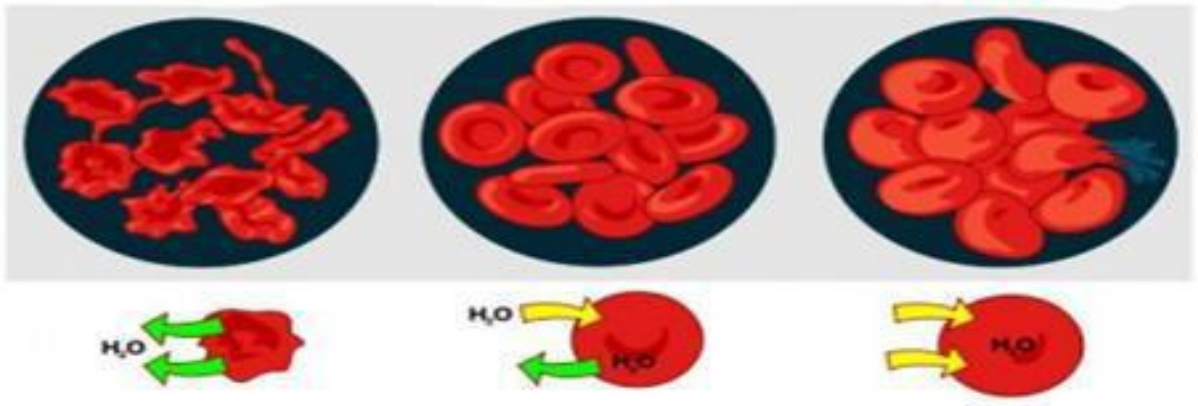


Fig. No. 3 Hypotonic hemolysis

3) Hypotonic Dilution

In this method, a volume of packed erythrocytes is diluted with 2–20 volumes of aqueous solution of a drug. The RBC'S are exposed to hypotonic solution (corresponding to 0.4 % Nacl), the erythrocytic membrane ruptures permitting escape of cellular contents and equilibrium is achieved with in one minute. The cells swell up to 1.6 times its original volume. The swelling results in the appearance of pores of 200 – 500 A^o in size. The length of time for which these pores remain open is not fixed. However at 0^oC the opening permits long enough to allow partial resealing of membrane. Increasing the ionic strength to isotonicity and incubating the cells at 37^o C causes the pores to close and restore osmotic properties of the RBC'S. This method was used to entrap b–giucosidase and b–galactosidase.

This method is simplest and fastest yet the capsulation efficacy is very low i. e. 1 – 8 %.Efficient for of low weight drugs.

Examples of encapsulate agents: β-glucosidase(Gopal VS, Kumar AR, Usha AN, Udupa AKN *et al* , 2007, 1, 18-3) asparaginase(Updike SJ, Wakarniya RT, Lightfoot EN *et al*, 1976, 193, 681-683 arginase (Adriaenssens K, Karcher D, Lowenthal A, Terheggen HG *et al* , 1976, 22, 323-326) salbutamol (Bhaskaran S, Dhir SS. *et al*).

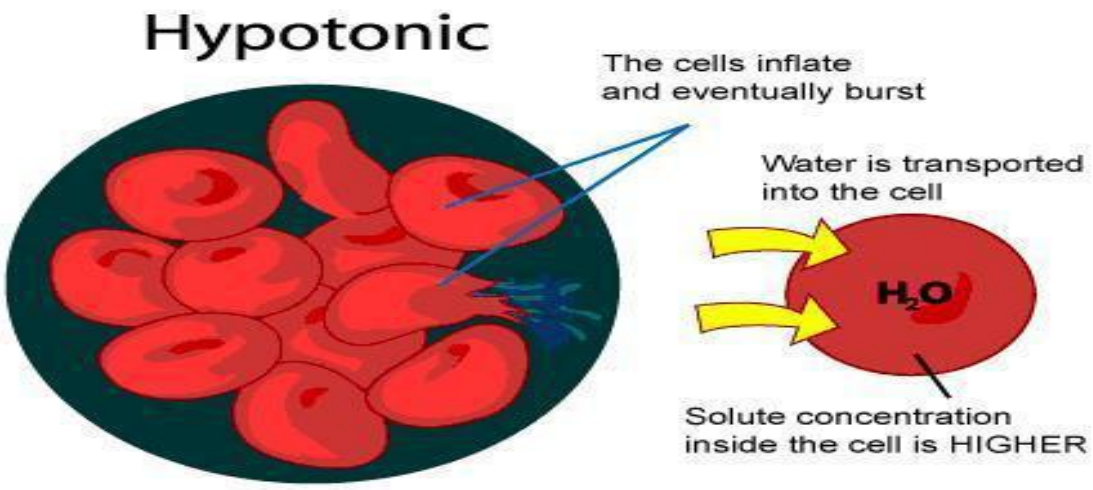


Fig. No.4 Hypotonic Dilution

4) Hypotonic preswell method

This method was investigated by Rechsteiner (Iher GM *et al*, 1973) in 1975 and was modified by Jenner et al for drug loading. This method was 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(Deuticke B *et al*, 1973, 345–359) asparginase(Kitao T. *et al*, 341, 1978, 94–95.) cyclopohphamide, cortisol-21-phosphate(Lin W *et al*, 369 (1), 1999, 78–88.) 1-antitrypsin (De Flora A, Guida L, Zocchi E, Tonetti M, Benatti U. *et al* , 1986, 35, 361- 367.) methotrexate, insulin(Gerli GC, Agostoni A, Fiorelli G. *et al* , 1978, 34, 431- 432.) metronidazole(Magnani M, Rossi L, Bianchi M, Serafini G, Stocchi V *et al* , 1989, 82, 27-34) levothyroxine(Gourley DHR.*et al* , 1957, 10)

enalaprilat(Gopal VS, Kumar AR, Usha AN, Udupa AKN *et al*, 2007, 1, 18-33.) and isoniazid(Updike SJ, Wakarniya RT, Lightfoot EN *et al* , 1976, 193, 681-6.

Examples of encapsulated agents: Enalaprilat (Tajerzadeh H, Hamidi M. *et al*, 2000, 26, 1247- 1257)(Hamidi M, Tajerzadeh H, Rouini MR, Dehpour AR, Ejtema ee-Mehr Sh *et al* , 2001, 8, 231- 237.)methotrexate(Pitt E, Johnson CM, Lewis DA, Jenner DA, Offord R *et al* , 1983, 22, 3359-3368.) (Bird J, Best R, Lewis DA *et al*, 1983, 35, 246-247) propranolol(Alpar HO, Irwin WJ. *et al*, 1987, 67, 1-9.) isoniazid insulin metronidazole (Talwar N, Jain NK. *et al*, 1992, 18, 1799-1812) asparaginase, primaquin(Talwar N, Jain NK *et al*, 1992, 20, 133-142.) levothyroxine (Field WN, Gamble MD, Lewis DA. *et al*, 1992, 20, 133-142.) cortisol- , 21-phsphate prednisolone-21-sodium , succinate, cyclophosphamide, α - 1, antitrypsin, interferon.

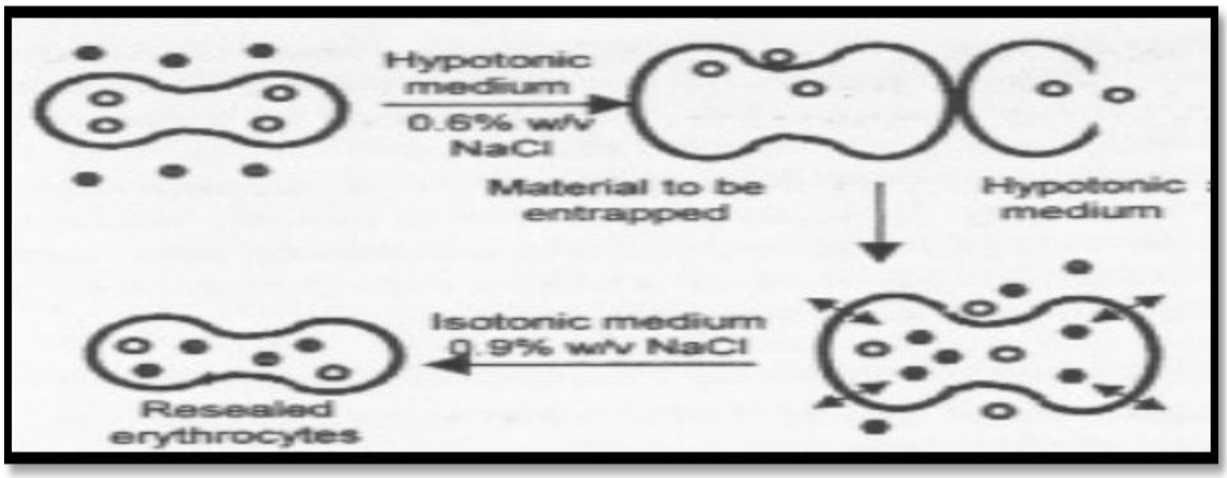


Fig No.5 Hypotonic Press well Method

5) Isotonic osmotic lysis

This method, also known as the osmotic pulse method, involves isotonic hemolysis. Erythrocytes are incubated in solutions of a substance with high membrane permeability; the solute will diffuse into the cells because of the concentration gradient. Chemicals such as urea solution(Adriaenssens K, Karcher D, Lowenthal A, Terheggen HG. *et al*, 1976) polyethylene glycol (Bhaskaran S, Dhir SS. *et al*) and ammonium chloride have been used for isotonic hemolysis. In 1987, Franco et al. developed a method that involved suspending erythrocytes in an isotonic solution of dim ethyl sulfoxide (DMSO).

*Examples of encapsulated agents: Inositol hexaphosphate (*Franco R, Barker R, Weiner M. *et al*, 1987).

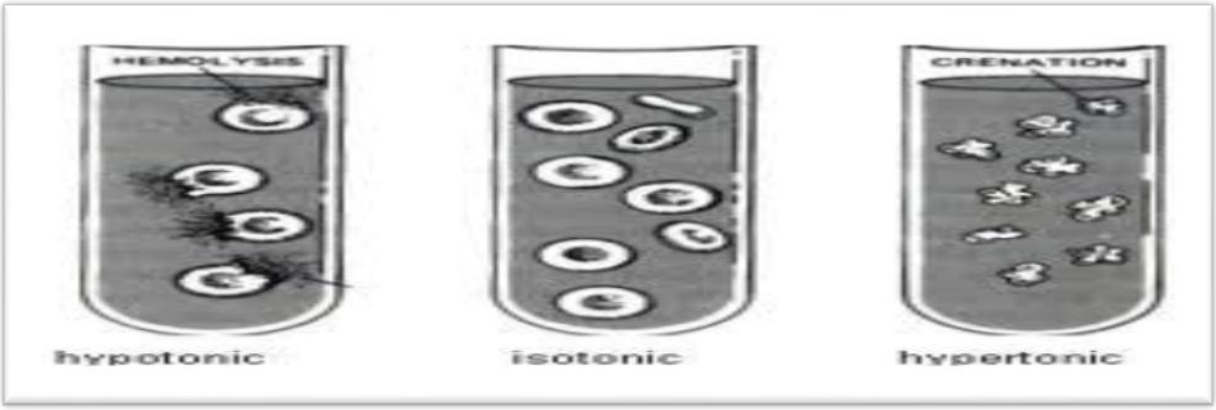


Fig.No.6 Isotonic Osmotic Lysis

6) Chemical Perturbation of the Membrane

This method is based on the increase in membrane permeability of erythrocytes when the cells are exposed to certain chemicals. In 1973, Deuticke showed that the permeability of erythrocytic 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.

Examples of encapsulated agents: Daunomycin (Deuticke B, Kim M, Zolinev C. *et al.*)

7) *Electrical breakdown method*

Electrical breakdown of a cell membrane is observed when the membrane is polarized very rapidly (in nano to micro seconds) using voltage of about 2kV/ cm for 20µ sec which leads to the formation of pores and entrapment of drugs. Electrical breakdown probably takes place in the lipoid regions or at the lipid protein junction in the membrane. Pores formed are stable and it is possible to control pore size. Subsequently the pores can be resealed by incubation at 37º C in Osmotic ally balanced medium. 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.

Examples of encapsulated agent: Sucrose (Kinosita K, Tsong TY. *et al*, 1978)

Urease (Zimmermann U, Riemann F, Pilwat G. et al , 1976, 436, 460–474.) methotrexate, interleukine (Iher GM *et el* , 1973).

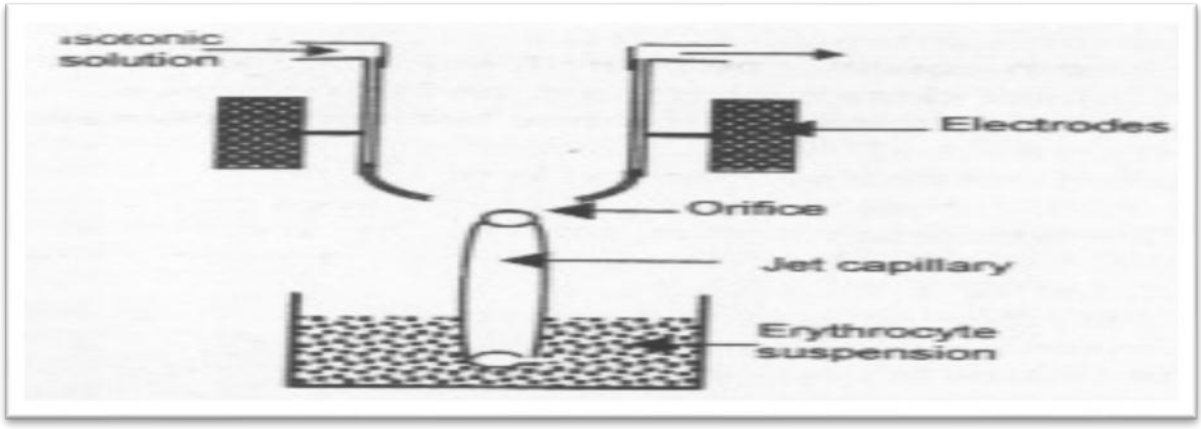


Fig.No.8 ***Electrical breakdown method***

8) ***Entrapment by Endocytosis***

This method was reported by Schrier et al. in 1975. Endocytosis involves the addition of one volume of washed packed erythrocytes to nine volumes of buffer containing2.5 mm ATP, 2.5 mm MgCl2, and1mM CaCl2, followed by incubation for2 min at room temperature. The pores created by this method are resealed by using 154 mm of Nacl and incubation at 370cfor 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. *Examples of encapsulated agents: Hydrocortisone, propranolol, vitamin A Primaquine (*Schrier Sl *et al,* 1987) *vinblastine, chlorpromazine (*Colin FC, Schrier SL *et al* , 1991).

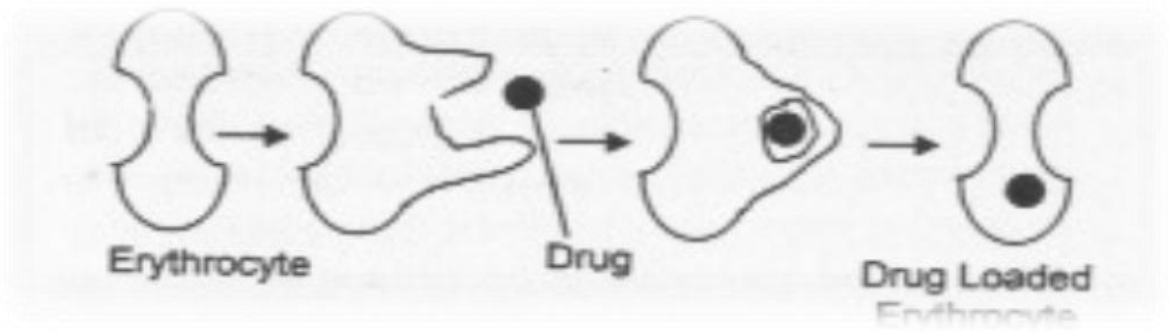


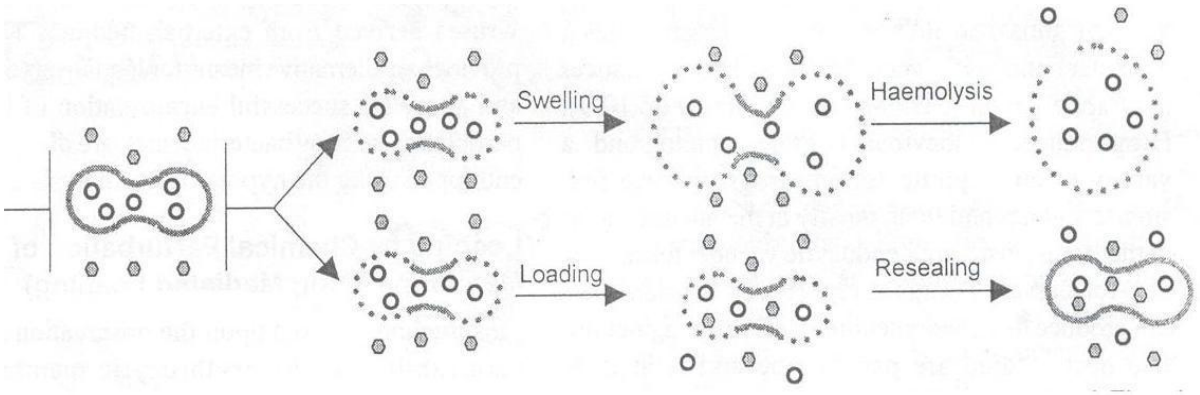
Fig.No.9Entrapment by endocytosis

9) ***Loading by electric cell fusion***

This method involves the initial loading of drug molecules into erythrocyte ghosts followed by adhesion of these cells to target cells. The fusion is accentuated by the application of an

electric pulse, which causes the release of an entrapped molecule. An example of this method is loading a cell-specific mono clonal 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.

Fig.No.10 ***loading by electric cell fusion***



10) ***Loading by lipid fusion***

In this method fused lipid vesicle containing bioactive molecule along with human erythrocytes leading to exchange of lipid entrapped drug molecule. This method provides very low encapsulation efficiency. Nicola and Gresonde fused lipid vesicle containing inositol hexaphosphate with human erythrocytes. The incorporated inositol hexaphosphate in erythrocytes provided a significant lowering of the oxygen affinity for hemoglobin in intact erythrocytes. Harrison et al reported resealing of tyrosine kinase into human erythrocytes by rapid freezing and thawing in liquid.

*Examples of encapsulated agents: Inositol monophosphate (*Nikolaou WB, Gersonde K *et al*, 1979)

Applications of Resealed Erythrocytes

(1) In-Vitro Application

For in vitro phagocytosis cells have been used to facilitate the uptake of enzymes. Enzymes within carrier RBC could be visualized with the help of cytochemical technique. The defects such as the glucose- 6- phosphate dehydrogenase (G6PD) deficiency can be useful tool for discerning the mechanism that eventually causes these effects. The most frequent in vitro application of RBC is that of micro- injection. A protein or nucleic acid was 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 has been used to confirm the site of action of fragment of toxin. Antibodies introduced using RBC mediated microinjection is recorded not to enter the nucleus, thus limiting the studies to the cytoplasmic level.

2) In-Vivo Application

A. Targeting of bioactive agents to RE System

Damaged erythrocytes are rapidly cleared from circulation by phagocyte Kuffer cells in liver and spleen. Resealed erythrocytes, by modifying their membranes, can therefore be used to target the liver and spleen. The various approaches to modify the surface characteristics of erythrocytes include surface modification with antibodies, gluteraldehyde, carbohydrates such as sialic acid and sulphydryl.

B. Targeting to sites as other than RES Organ

Resealed erythrocytes have the ability to deliver a drug or enzyme to the macrophage-rich. Organ targeting other than RES have been tried recently with resealed erythrocytes. Some of the representative approaches are discussed in brief.

C. *Targeting the liver*

Enzyme deficiency / replacement therapy. Many metabolic disorders related to deficient or missing enzymes can be treated by injecting these enzymes. However, the problems of exogenous enzyme therapy include a shorter circulation half life of enzymes, allergic reactions, and are toxic. These problems can be successfully overcome by administering the enzymes as resealed .The enzymes used include glycosidase, glucoronidase, and galactosidase4. The disease caused by an accumulation of glucocerebro-sides in the liver and spleen can be treated by glucocerebrosi-dase-loaded erythrocytes (Deloach J, Ihler et al, 1977).

D. *Treatment of hepatic tumors*

Hepatic tumors are one of the most prevalent types of cancer. Antineoplastic drugs such as Methotrexate, bleomycin, Asparginase and Adriamycin (Zocchi E. *et al*, 1988) have been successfully delivered by erythrocytes. Agents such as daunorubicin diffuse rapidly from the cells upon loading and hence pose a problem. This problem can be overcome by covalently linking daunorubicin to the erythrocytic membrane using gluteraldehyde or cisaconitic acid(

Gaudreault RC, Bellemare B, Lacroix J. *et al,* 1989) as a spacer .The resealed erythrocytes loaded with carboplatin show localization in liver(Tonetti M *et al,* 1990).

E. *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, anti leishmanial and antiamoebic drugs.

F. *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.

G. *Removal of toxic agents*

Cannon et al. reported inhibition of cyanide intoxication with murine carrier erythrocytes containing bovine rhodanase and sodium thiosulfate (Cannon EP. *et al,* 1994) .An-tagonization of organ phosphorus intoxication by resealed erythrocytes containing a recombinant phosphodiestrase also has been reported (Pei L. *et al,* 1994).

H. Erythrocytes as Circulating Bioreactors

Erythrocytes have been realized as carriers for enzymes to serve as circulating bioreactors. Sometimes it is desirable to decrease the level of circulating metabolites that can enter erythrocytes. Erythrocytes have also been used as circulating bioreactors for the controlled delivery of antiviral drugs.

I. *Erythrocytes as Carriers for Drugs*

Various bioactive agents encapsulated in erythrocytes are developed for the slow and sustained release in circulation to allow effective treatment of parasitic disease. Resealed erythrocytes serve as an ideal carrier for antineoplastic agents, antimicrobial drug and vitamins and steroids.

J. *Erythrocytes as Carriers for Enzymes*

Enzymes can be injected into the blood stream to replace a missing or deficient enzyme in metabolic disorders or to degrade toxic compounds accumulated in the blood due to a disease likewise, environmental, lysosomal storage disorders such as Gaucher's disease, hyperphenyl- alaninaemia and kidney failure are only few examples of metabolic disorders that can be treated by administration of enzymes.

Other applications of resealed erythrocytes include

1. Surface modification with antibodies
2. Surface modification with gluteraldehyde
3. Surface modification with carbohydrates such as salicylic acid
4. Entrapment of paramagnetic particles along with the drug
5. Entrapment of photosensitive material
6. Antibody attachment to erythrocyte membrane to get specificity of action of enzymes.
7. It is used for Delivery of antiviral agents such as azidothymidine derivatives, azathioprene, and acyclovir and fludarabine phosphate.
8. It is used for Improvement in oxygen delivery to tissues
9. It is used for Microinjection of macromolecules.

CONCLUSION

The use of resealed erythrocytes is having a very good result for a safe and sure delivery of various drugs for passive and active targeting. The concept needs further optimization to become a routine drug delivery system and we can also use the same concept for extended to the delivery of biopharmaceuticals and much remains to be explored regarding the potential of resealed erythrocytes. The preparation of resealed erythrocytes is very easy. There are several techniques identified now a day by which we can easily entrap the drug into erythrocytes. It is having both types of application *in-vitro* as well as *in-vivo.*

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