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RESEALED ERYTHROCYTE AS A SMARTER DRUG DELIVERY SYSTEM: A BRIEF OVERVIEW

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Abstract

Present pharmaceutical modernization innovation era aimed toward the development of such a drug delivery system which could maximizes the drug targeting alongside high therapeutic effect for safe and effective management of disease by reducing the adverse effect of the drug. The substances that have mechanisms to enhance the delivery and therefore not compromising with the effectiveness of medicine are termed as novel drug carriers. Drug carriers are utilized in assorted drug delivery systems such as: Controlled-release technology to increase *in-vivo* drug actions, diminish drug metabolism and reduce drug toxicity. These cellular carrier offer a greater prospective advantages related to their ability to circulate throughout the body, zero order kinetics, reproducibility and simple preparation. Primary aim for the event of this drug delivery system is to form best use of therapeutic performance, minimize the unwanted side effects of drug, eco-friendly, doesn't stimulate any immune reaction, co-exist with living tissues, alongside high drug loading efficiency. Encouraging the use of Resealed Erythrocytes in drug delivery include numerous advantages like as incredible degree of biocompatibility, thorough biodegradability, non-existence of toxic product, manageable life-span, diminishing drug side effects etc.

Keywords: Resealed Erythrocytes, Controlled-release technology, drug targeting, cellular carrier, Biodegradability.

1. Introduction

Drug delivery is that the method of formulation that aims transporting an active pharmaceutical ingredient to the target receptor at an organ within the body with no loss or compromise within the chemical integrity of the molecule and will be manipulated to effect the specified pharmacological action. Drug delivery technology plays around modification and improvement of varied pharmacokinetic parameters like drug release profile, absorption, distribution, and elimination, etc. The substances that are won't transport the drug to the target site were called as drug carriers and will aid the drug to prolong it's *in-vivo* actions, decrease metabolism, eliminates toxicity and with tailored pharmacokinetic parameters **[1, 2]**.

Drug carriers are substances that serve as transporters to deliver the drugs to target site and improve the effectiveness of drugs. New drug delivery systems have been developed to overcome the limitations associated with the conventional drug delivery systems in order to improve the patient compliance and safety.

They may be classified based on their size as:

(i) Microcarriers for example- Liposomes, Resealed Erythrocytes, Microspheres.

(ii) Nanocarriers for example- Niosomes, Pharmacosome, Aquasomes, Nanoparticles, Solid Lipid Nanoparticles (SLN), Miscelles.

(iii) Variable carriers for example- Nendrimers. **[1, 2]**

Resealed erythrocytes are prepared by the delivery of medicine and drug-loaded microspheres into the Erythrocytes which are extensively studied for one's potential carrier capabilities. Such drug-loaded carrier erythrocytes are prepared just by collecting blood samples from the organism of interest, separating erythrocytes from plasma, entrapping drug within the erythrocytes, and resealing the resultant cellular carriers. Hence, these carriers are called resealed erythrocytes **[1, 2]**.

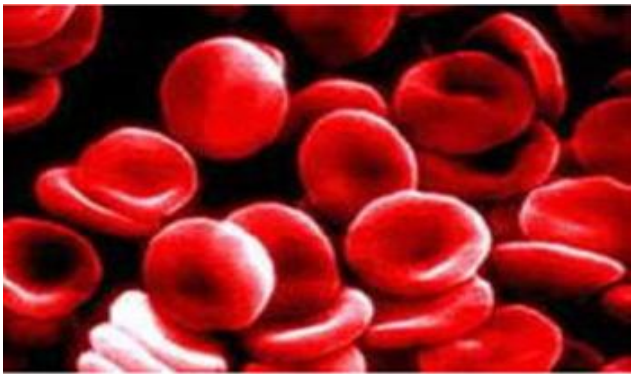

Fig. 1: Resealed erythrocytes [3]

1.1. Anatomy and Physiology of Resealed Erythrocytes:

RBCs are biconcave disc with a diameter of 7.8 µm and thickness near 2.2 µm. Mature RBCs have a simple structure and are elastic in nature their plasma membrane is strong and flexible so that they can squeeze through narrow capillaries without deformation. RBCs lack 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. The red blood cell membrane is associated with energy metabolism and maintains the permeability cell characteristics by various anions and cations. Each RBC contains about 280 million haemoglobin molecules which composed of protein called globin, containing four polypeptide chains, a ring like non-protein pigment called a heme, bound to each of the four chains. The heme ring combines reversibly with oxygen molecule **[4]**.

1.2. Composition of RBCs [5]:

RBCs include water (63%), lipids (0.5%), glucose (0.8%), mineral (0.7%), non-haemoglobin protein (0.9%), meth haemoglobin (0.5%), and haemoglobin (33.67%).

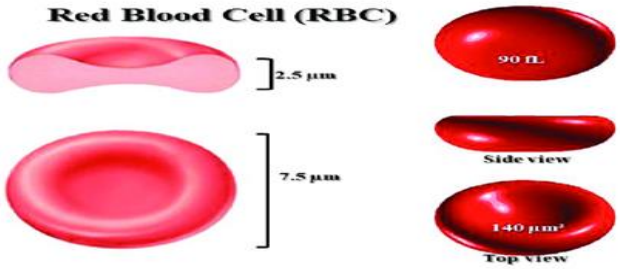

Fig. 2: Anatomy of RBC [6]

1.3. Source and isolation of Erythrocytes [7, 8, 9]:

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. In the process of isolating erythrocytes involves:-

1. Blood is collected into heparin zed tubes by venepuncture.

2. 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.

3. The whole blood is centrifuged at 2500 rpm for 5 min. at 4 ± 10^0C.

4.The serum and buffy coats are carefully removed and packed cells washed three times with phosphate buffer saline (pH=7.4) 4 ± 10^0C in a refrigerated centrifuge.

5. The washed erythrocytes are diluted with PBS and stored at 4°C until used.

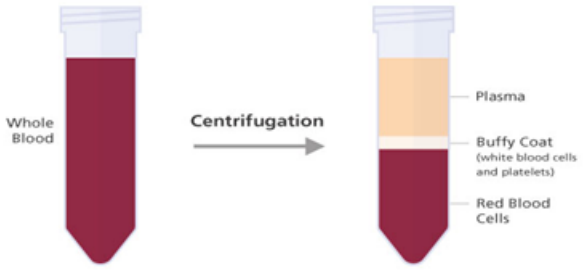

Fig. 3: Isolation of Erythrocytes [10]

2. Objective / Purpose of Resealing Erythrocytes [11]:

I. To improve existing dosage forms and development of more sophisticated dosage forms.

II. For Avoiding immune response or reaction on intravenous administration.

III. For Achieving maximum therapeutic benefit for safe and effective management of diseases.

IV. To have accurate dose on targeting sites and hence reducing the side effects.

V. To improve Patient compliance.

3. Desirable Properties Essential for Suitability of RBC as Drug Carrier [12, 13]:

I. Biodegradability : Resealed erythrocytes release the drug loaded in it on biodegradation , therefore the biodegraded product is of utter importance (90% RBC's are degraded by macrophages present in Reticulo-endothelial system (RES) and remaining 10% by haemolysis in circulation)

II. Circulate throughout the circulatory system (On addition of calcium chelating agents or purine nucleosides, the circulation is further enhanced)

III. Large quantity of materials or bioactive agents can be encapsulated within small volumes of cells.

IV. Can be utilized for organ targeting within RES.

V. Erythrocytes are biocompatible provided that compatible cells are used in patients.

VI. There should be no possibility of triggered immunological reaction.

VII. The flexibility of red blood cells to retain its shape and morphology when placed in isotonic saline also make them suitable carriers for drugs and enzymes.

VIII. It should have an appreciable stability during storage.

IX. It should have specific physicochemical properties by which a desired target site could be recognized.

X. Minimum leakage of drug should take place before target site is reached.

4.1. Advantages of Erythrocytes in Drug Loading [4, 6]:

I. A notable degree of biocompatibility, predominantly when the autologous cells are used for drug loading.

II. Wholly biodegradability and the lack of toxic product(s) resulting from the carrier biodegradation.

III. Significant protection of the organism against the toxic effects of the encapsulated drug, e.g. anti-neoplastic agents.

IV. An easily controllable life-span within a wide range from minutes to months.

V. Required size range and the considerably uniform size and shape.

VI. Possibility of targeted drug delivery to the RES organs.

VII. Possibility of ideal zero-order kinetics of drug release.

VIII. Wide variety of compounds with the capability of being entrapped within the erythrocytes.

IX. Remarkable reduction in concentration fluctuations in steady state in comparison to the conventional methods of drug administration, which is one of the common advantages for most of the novel drug delivery systems.

X. Considerable escalation in drug dosing intervals with drug concentration in the safe and effective level for a comparatively long time.

4.2. Disadvantages [4, 6]:

I. Natural cells as drug carriers are removed *in-vivo* by the RES as result of alteration that occurs during loading procedure in cells. This, although grows the capability to drug targeting to RES, seriously restricts their life-span as long-circulating drug carriers in circulation and, may create toxicological problems.

II. The rapid leakage of certain encapsulated drugs from the loaded erythrocytes.

III. Several molecules may modify the physiology of the erythrocyte.

IV. Encapsulated erythrocytes may show some inherent variations in their loading and characteristics compared to other carrier systems.

V. Potential contamination due to the derivation of the blood, the equipment used and the loading environment. Strict controls are required accordingly for the collection and handling of the erythrocytes.

5. METHODS OF ENCAPSULATION OF ERYTHROCYTES [14-20]

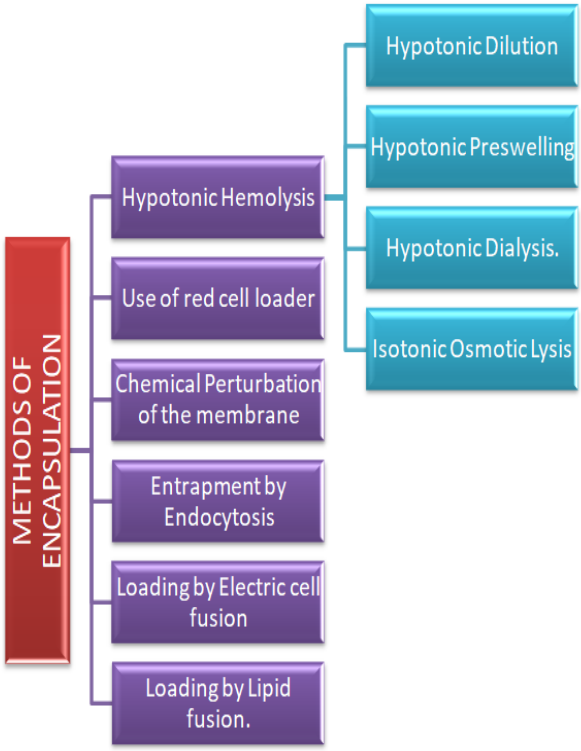

Chart 1: Methods of Encapsulation of Erythrocytes

I. Hypotonic haemolysis:

This method is based on the capability of erythrocytes to experience reversible swelling in a hypotonic solution. An exceptional capability of erythrocytes for reversible shape changes with or without accompanying volume change and for reversible deformation under stress. An initial change in the shape from biconcave to spherical is seen due an increase in the volume of the erythrocytes, resulting in fixed surface area. The volume gain is about 25-50%. The cells can maintain their integrity up to a tonicity of about 150 milli moles/kg, above which the membrane would rupture, there by releasing the cellular contents. Some intramembranous pores of 200-500 Å are generated just before cell lyses, the cellular contents are depleted after the cell lyses. The residue is known as an erythrocyte ghost. The principle of using these ruptured erythrocytes as drug carriers is based upon the fact that the ruptured membranes can be resealed by restoring isotonic conditions. After which the cells are incubated to let them resume their original biconcave shape and recover original impermeability. For example Glucose oxidase, glucose 6-phosphatase, anti-hexokinase IgG, ATP has been entrapped successfully by this method.

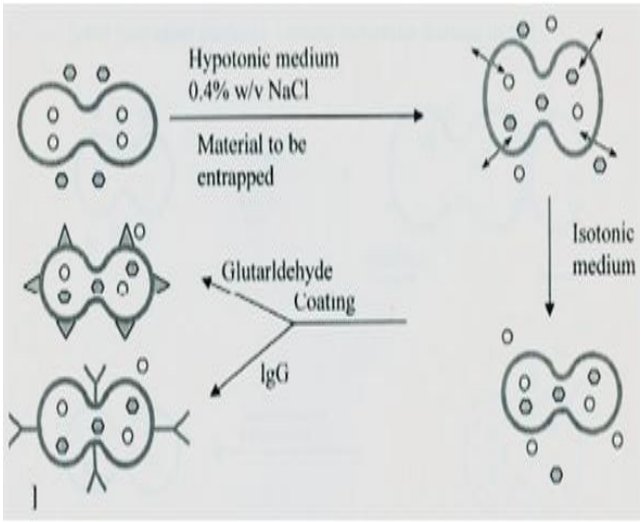

Fig. 4: Schematic representation of Hypotonic haemolysis [21]

I. (a) Hypotonic dilution:

Hypotonic dilution was the simplest and fastest method for the encapsulation of chemicals into erythrocytes. In this method, a 2-20 volume of packed erythrocytes is diluted with aqueous solution of a drug. A hypertonic buffer was added to restore solution tonicity. The consequential mixture is then centrifuged, the pellet is washed with isotonic buffer solution and the supernatant is discarded, which causes reduction of circulation half-life of the loaded cells. The major drawback of this method includes low entrapment efficiency and a significant trouncing of hemoglobin and other cell components. By RES macrophages, these cells are readily used for

targeting RES organ and phagocytosed. This method is used for loading of enzymes such as ß-galactosidase and ß-glycosidase, asparagines and arginase, as well as bronchodilators such as salbutamol.

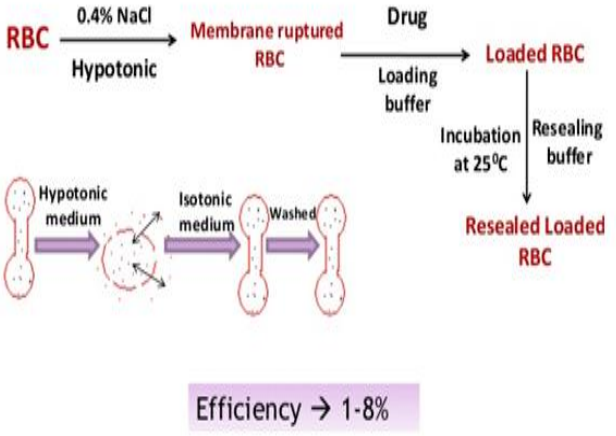

Fig. 5: Schematic representation of Hypotonic dilution [22]

I. (b) Hypotonic Preswell method:

This drug loading procedure was developed by Rechsteiner in1975. It causes minimal damage to cells, and it is very simple and faster than other methods, to encapsulate a drug in erythrocytes. The technique is based initially controlling swelling in a hypotonic buffered solution. At low gravitational force, 100-120 µl portions of an aqueous solution of the drug to be encapsulated at the lysis point and the supernatant were discarded. The mixture is centrifuged between the drug-addition steps. The detection of lysis point is observed by the disappearance of a distinct layer between the cell fraction and the supernatant upon centrifugation. By adding a calculated amount of hypertonic buffer, the tonicity of a cell mixture is restored at the lysis point. To re-anneal the resealed erythrocytes, the cell suspension is incubated at 37°C .Such cells have a similar circulation half-life comparable to that of normal cells. Drugs encapsulated in erythrocytes using this method include Propranolol, levothyroxine, Metronidazole, Levothyroxine, Enalaprilat, and Isoniazid cortisol-21-phsphate, prednisolone-21-sodium, cyclophosphamide, interferon alpha-2, insulin.

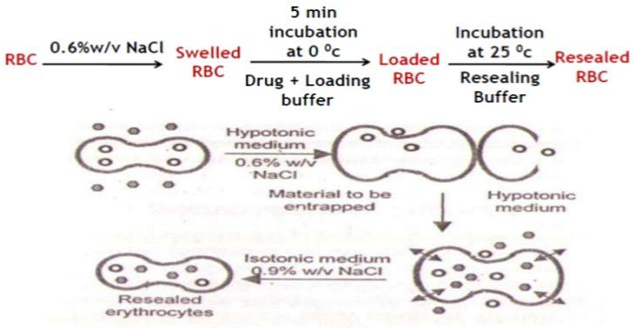

Fig. 6: Schematic representation of Hypotonic Preswell method [22]

I. (c) Hypotonic dialysis:

Principle of this method is based on semipermeable dialysis membrane maximises the intracellular to extracellular volume ration for macromolecules during lysis and resealing. In this method, 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 and the medium is agitated slowly for 2 hours. By adding a calculated amount of a hypertonic buffer to the surrounding medium, the tonicity of dialysis tube is restored. The drug to be loaded can be added by either dissolving the drug in isotonic cell suspending buffer within a dialysis bag at the initiation of the experiment or else at the completion of the stirring the drug is added to the dialysis bag. Under this method, the erythrocyte suspension and the drug to be loaded were placed in the blood compartment and in the presence of hypotonic buffer in a receptor compartment.

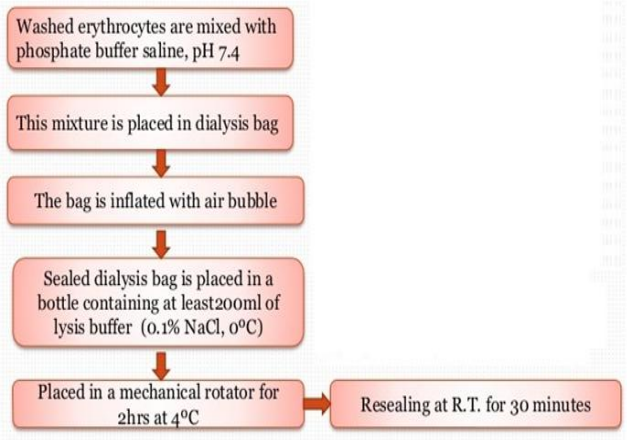

Fig. 7 [22]: Schematic representation of Hypotonic dialysis

Several researchers have used this method which leads to the concept of "Continuous flow dialysis". For example loading drugs such as pentamidine furamycin, interlukin-2, desferroxamine, gentamicin, adriamycin and human recombinant erythropoietin.

I. (d) Isotonic osmotic lysis:

This method is achieved by physical or chemical means and it is also known as the osmotic pulse method, because of the concentration gradient, if erythrocytes are incubated in solutions of a substance with high membrane permeability the solute will diffuse into the cells. To maintain osmotic equilibrium, this process follows an influx of water. For isotonic hemolysis chemicals such as urea solution, PEG, and ammonium chloride have been used. However, to make-out changes in membrane structure composition, this method is not immune. Later another method was developed which involved suspending erythrocytes in an isotonic solution of dimethyl sulfoxide. The suspension was diluted with an isotonically adjusted buffered drug solution. After cells separation took place, they were resealed at 37°C.

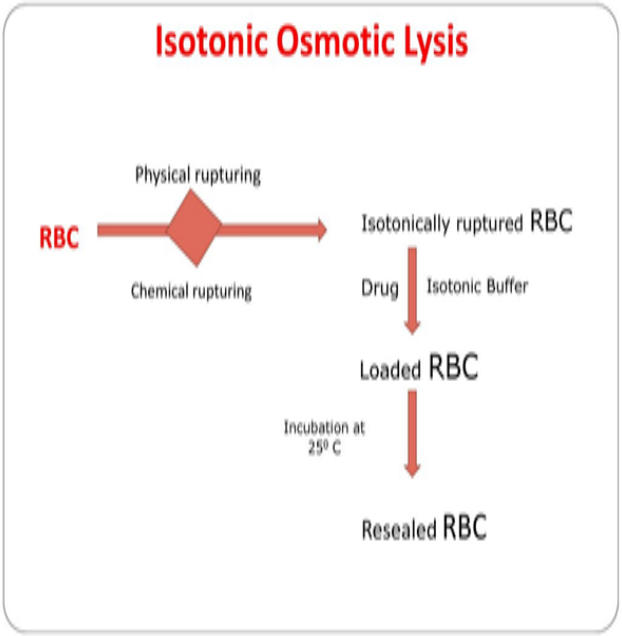

Fig. 7: Schematic representation of Isotonic osmotic lysis [23]

Table 1: Comparison Of Various Hypo-osmotic Lysis Methods [6]

Methods	%Loading	Advantages	Disadvantages
Dilution Method	1-8 %	For low molecular weight drugs it is the fastest and simplest method.	• Low entrapment efficiency • (1-8%). • Reduction in circulation half life of loaded cells is due loss of haemoglobin and other cell components.
Dialysis Method	30-45%	• Loaded cells have same circulation half-life as of normal cells. • Structural integrity to membrane imparts better *in vivo* survival of erythrocytes.	• Long processing time an requirement of special equipment, leading to increase costs. • Heterogeneous size distribution of loaded ghosts causes variable drug concentration.
Preswell Dilution	20-70%	• Good retention of Cytoplasmic constituents. • Decent in vivo survival.	-Nil-
Isotonic Osmotic lysis	--	Better in vivo surveillance of erythrocytes.	• Time consuming. • Presence of membrane lytic substances causes eliminates no changes in membrane structure and composition. • Obtained ghosts cells seem to have limited applications as carrier.

II. Use of Red Cell Loader:

A novel method developed for entrapment of non-diffusible drugs into erythrocytes. In this method a part of the equipment known as the "Red Cell Loader" was developed with which as small as 50ml of blood sample and different biologically active compounds were entrapped into erythrocytes within a period of 2 hours maintained at room temperature under blood banking conditions. This process is based on 2 sequential hypotonic dilutions of washed erythrocytes followed by concentrating it with a hemofilter and an isotonic resealing of cells.

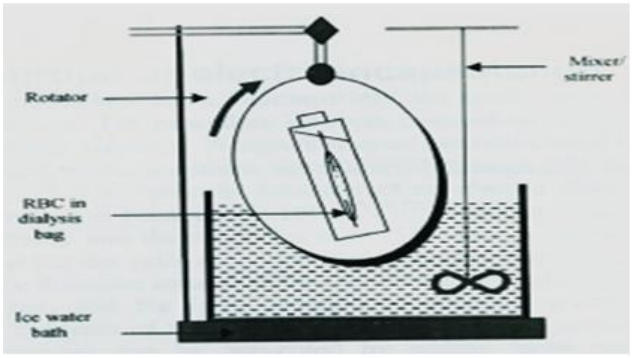

Fig. 8: Diagram of Red Cell Loader [21]

III. Chemical perturbation of the membrane:

This method is developed to reinforce the permeability of cells on exposure to varied chemicals. The permeability of erythrocyte membrane increases upon exposure to polyene antibiotic like amphotericin B. Danorubicin an antineoplastic agent are often delivered by this method. However, these methods induce irreversible destructive changes within the cell wall and hence not used widely.

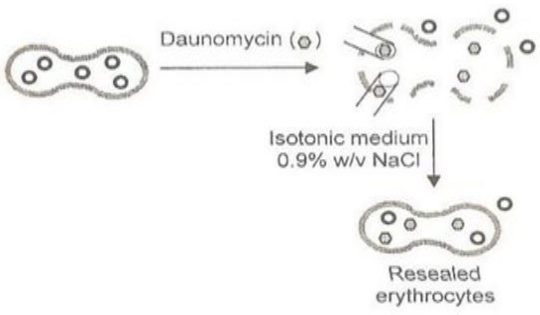

Fig. 9: Schematic representation of Chemical perturbation of the membrane [22]

IV. Entrapment by endocytosis:

This method involves the addition of 1 volume of washed erythrocytes was added to 9 volumes of buffer containing 2.5 mM ATP, 2.5 mM $MgCl_2$, and 1 mM $CaCl_2$, followed by incubation for 2 minutes at room temperature which causes pores in its membrane. Resealing of pores by using 154 mM of NaCl and incubation at 37°C for 2 minutes. By endocytosis the entrapment of material occurs. The vesicle membrane gets separated out from the endocytosed material from cytoplasm thus protecting it from the erythrocytes and *vice versa*. The various chemicals entrapped by this method include primaquine and related 8-amino-quinolines, vinblastine, chlorpromazine and related phenothiazines, hydrocortisone, propanolol, tetracaine, and vitamin A.

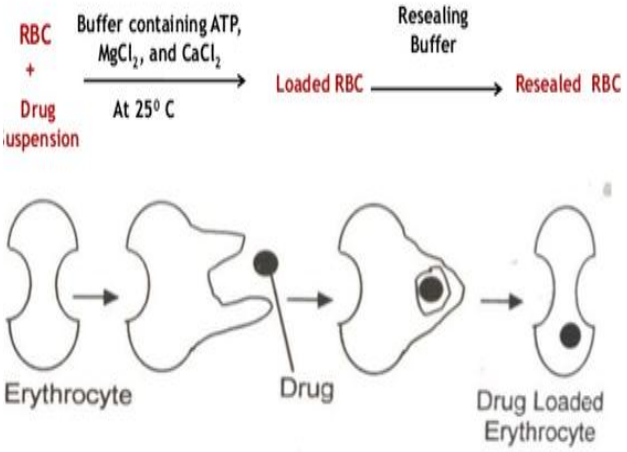

Fig 10: Schematic representation of Entrapment by endocytosis [22]

V. Loading by electric cell fusion:

Initially, loading of drug molecules into erythrocyte ghosts is followed by adhesion of these cells to target cells. The fusion is emphasized the application of an electric pulse, which causes the release of an entrapped molecule. Example of this method is loading a cell-specific monoclonal antibody into an erythrocyte ghost. Antibody against a specific surface protein of target cells can be chemically crossed linked with the drug loaded cells which would then direct these cells to once desired cells.

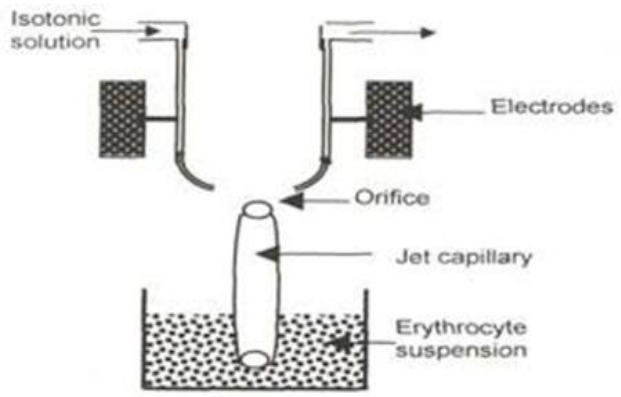

Fig. 11: Schematic representation of Loading by electric cell fusion [24]

VI. Loading by lipid fusion:

Direct fusion of drug in a lipid vesicle to human erythrocytes, which lead to exchange with a lipid-entrapped drug. To improve the oxygen carrying capacity of inositol monophosphate, this method was used. However, this method provides very low encapsulation efficiency (~1%).

6. Various factors affecting the loading of drugs in Erythrocytes include [11]:

I. Drug concentration: Drug concentration plays a vital role in loading drugs into erythrocytes to maintain its isotonic nature and integrity.

II. Haematocrit value: The ratio of the volume of red blood cells to the total volume of blood is an important aspect in loading of drugs in erythrocytes.

III. Lysing time: The time taken for cell lysis should be taken care of in the whole process.

IV. Resealing time: After loading drug into the erythrocytes, they are resealed under isotonic conditions.

V. Magnetite concentration: Some ferro-drugs loaded in erythrocytes can be directly delivered under the influence of externally applied magnetic field.

VI. Temperature: In to achieve high entrapment efficiency during lysis low temperature is preferred and higher or 37^{O} C is optimal for resealing in order to obtain discocyte cells.

7. Release Characteristics of Loaded Drugs [25, 26]:

These are significant to study so as to control efflux rate before constant release rate over prolonged period can be accomplished. There are mainly three ways for a drug to efflux out from erythrocyte carriers:

7.1. Phagocytosis: RBC's are isolated from circulation by process of phagocytosis subsequent heat treatment or antibody cross linking. The drug might be released from macrophages after phagocytosis if linkage is liable to lysosomal enzymes.

7.2. Diffusion through the cell membrane: The rate of diffusion depends upon the rate at which a particular molecule penetrates through a lipid bilayer. It is highest for a molecule with high lipid solubility. Hence extensive control over the rate of drug release is conceivable by introducing or eliminating polar or charged substituent.

7.3. Using a specific transport system: Many substances enter cells by specific membrane protein system since the carriers are proteins and are specific as that of enzymes. Moderate alteration in compound can often alter the rate of exit.

The release of drugs from erythrocytes rapidly follows sustained release profile and rate of exit is proportional to instantaneous intracellular drug concentration i.e. first order kinetics. However, erythrocyte's carriers have the potential of releasing encapsulated drug following zero order kinetics also release pattern can be modified by incorporating polymers. If the drug is encapsulated during a random population of erythrocytes, then constant fraction of cells are going to be be removed every day and constant amount of drug are going to be made available every day.

8. Routes of Administration: It had been projected that the survival of erythrocytes in circulation by intraperitoneal injection was comparable to their administration by intravenous injection. Subcutaneous route was also evaluated for slow release of entrapped agents. **[11]**

9. Storage of Erythrocytes [11]: Storage of Resealed Erythrocytes is one of the major challenges faced in their practical utilization as drug delivery system. During the storage special care should be taken care of in such a manner that there should be no loss of integrity. The following methods are followed for the storage of resealed erythrocytes:-

I. Suspending in Hank's balanced salt solution at $4^{O}C$ for two weeks.

II. After the process of encapsulation and suspending the cells in oxygenated Hank's balanced salt solution containing 1% soft bloom gelatine. The cells can be recovered by liquefying the gel by placing the tubes in water bath at $37^{O}C$ and centrifugation under clinical conditions.

III. Cryopreservation of erythrocytes at liquid nitrogen temperature.

10. EVALUATION METHODS OF RESEALED ERYTHROCYTES:

Table 2: *In-Vitro* Evaluation of Resealed Erythrocytes [2]

CHARACTERISTICS	**ANALYTICAL METHODS INSTRUMENTATION**
PHYSICAL CHARACTERIZATION	
Shape and surface morphology	Transmission electron microscopy (TEM), Scanning electron microscopy, Phase-contrast optical microscopy.
Vesicle size and size distribution	TEM, Optical microscopy.
Drug release	Diffusion cell / dialysis.
%Encapsulation	Deproteinization (using methanol or acetonitrile) of cell membrane and assay for released drug or radio-labelled markers.
Electrical surface potential and surface pH	Zeta potential measurements and pH sensitive probes.
CELL RELATED CHARACTERIZATION	
% Haemoglobin content / volume	Deproteinization (using methanol or acetonitrile) of cell membrane and assay for Hb; Laser light scattering for cell volume.
Mean corpuscular haemoglobin	Laser light scattering
% Cell recovery	Haematological analyzer; Neubeur's chamber.
Osmotic fragility	Stepwise incubation with isotonic to hypotonic saline solutions and estimation of drug and Hb.
Osmotic shock	Dilution with distilled water and estimation of drug and Hb.
Turbulent shock	Passing cell suspension through a 23 gauge needle, hypodermic needle (10 ml / min) and estimation of residual drug and Hb.
Erythrocyte sedimentation rate	ESR apparatus
BIOLOGICAL CHARACTERIZATION	
Sterility	Aerobic or anaerobic cultures
Pyrogenicity	Rabbit fever response test of LAL test
Animal toxicity	Toxicity tests

10.1. *In-vitro* Characterisation of Resealed Erythrocytes [27-29]

The below mentioned characterisation are vital for ensuring the in-vivo performance and therapeutic benefits of Resealed Erythrocytes:

I. Shape and surface morphology:

Examination of shape and surface morphology are very important and should be taken under consideration to help decide the shelf life and life span after administration. It is done by comparing treated and untreated erythrocytes using transmission electron microscopy, scanning electron microscopy or phase – contrast optical microscopy.

II. Drug content:

For the determination of entrapment efficiency this method is carried out by deproteinizing 0.5ml packed loaded erythrocytes with 2ml acetonitrile and then centrifuged at 2500 rpm for 10 minutes. The clear supernatant is analysed for drug content.

III. Deformability:

Deformation of erythrocytes i.e. changes in shape have a great effect on the life span of the loaded cells. The RES are evaluated for the ease of passage through capillaries by methods depending on the viscoelasticity of the cell membrane, viscosity of the cell contains and the cellular surface to volume ratio, helps determining rheological behaviour of the cells. A definite volume of cells were passed through capillary tube (4µm diameter) or polycarbonate filter (pore size 45 µm) and passage time to be noted.

IV. *In-vitro* drug release and Haemoglobin content [30-34]:

These properties are studied and monitored periodically from drug loaded cells. The cell suspension of 5% haematocrit in phosphate buffer saline is stored at 4^0 C in amber coloured glass containers. Occasionally the clear supernatant is withdrawn using a hypodermic syringe equipped with 0.45µ filter, deproteinized with methanol and then estimated for drug content. Then centrifuging the supernatant with sample is collected and assayed. Thereby the % Hb release is calculated.

$$\%\ Hb\ release = \frac{A540\ of\ sample - A540\ of\ background}{100\%Hb\ of\ A540}$$

OR

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

Where A540 stands for absorbance at 540nm.

V. Entrapped magnetite study:

To make resealed erythrocytes Magnoresponsive they are entrapped with magnetite. Both magnetite bearing erythrocytes and hydrochloric acid are heated at 600C for 2 hours. Then 20% w/v trichloroacetic acid is added and centrifuged after which the supernatant is examined under atomic absorption spectroscopy for Magnetite concentration.

VI. Stability and Cross-linking of resealed erythrocytes:

Osmotic fragility tests revealed that RBC which survives the cross linking process display a very comparable haemolysis curve as produced by normal RBC. The cells treated with dimethyl sulfoxide, dimethyl – 3,3 dithiobis propionamide, toluene 2,4- diisocynate and glutaraldehyde are even resistant to sonication, freezing and thawing. 0.2% glutaraldehyde treated erythrocytes in a sintered glass funnel by filtration and vacuum dried at 200mmHg for 10 hours are collected and filled into vials and lyophilized at -40^oC to 0.01 torr with the help of a laboratory lyophilizer. Then this dried powder form was filled into amber coloured vials and stored at 4^oC.

VII. Erythrocyte Sedimentation Rate (ESR):

ESR is done for the estimation of suspension stability of RBC in plasma and can be related to the following:-

(I) Number and size of red blood cells.

(II) The relative concentration of plasma proteins (alpha & beta globulins).

ESR (normal range 0-15 mm/hr) is performed in a standard tube of ESR apparatus.

VIII. Zeta Sedimentation Ratio:

This ratio is provides the measures of closeness with which RBC's will approach one another after standardised cycles of dispersion and compaction.

IX. Particle Size:

A drop of sample is diluted 10 times with distilled water in a dry accessory system and taken into cuvettes and was analysed for particle size.

X. Osmotic Fragility:

This method provides information on resistance of cells to haemolysis in decreasing concentration of hypotonic saline.

It is one of the reliable and most important parameter for the following:-

I. In-vitro evaluation of carrier erythrocytes with respect to its shelf life.
II. In-vivo survival of erythrocytes
III. To study the effect of encapsulated drug within it.
IV. For stimulating and mimicking the bio environmental conditions that would be encountered on *in-vivo* administration.

For the determination of above normal and drug loaded erythrocytes were incubated separately in a stepwise decreasing order of % of NaCl solution of 0.9% at 37^0C ± 2^0C for 10 minutes followed by centrifugation at 2000 rpm for 10 minutes after which the supernatant liquid is examined for drug and haemoglobin content.

XI. Osmotic Shock:

It is used to define a sudden exposure of drug loaded erythrocytes to an environment that is far from isotonic level so as to examine the ability of resealed erythrocytes to withstand the stress and maintain its integrity. This is done by taking 1 ml of 10% haematocrit erythrocyte suspension which was diluted 5ml distil water and centrifuged at 300 rpm for 15 minutes after which supernatant was examined for %Hb release spectrophotometrically.

XII. Turbulence shock:

This determines the effects of shear and pressure by which resealed erythrocytes formulations are injected on the integrity of the loaded cells. It is found that drug loaded erythrocytes appear to be less resistant to turbulence, the reason is resealing makes them much more sensitive against turbulence or mechanical agitation and thereby estimation of turbulence shock parameter gives their expected performance *in-vivo*. It is performed by taking 5ml of 10% haematocrit loaded erythrocytes which are passed through 23 gauge hypodermic needle at flow rate of 10ml/min (i.e. flow rate of blood inside body). An aliquot of suspension after every pass is withdrawn and then centrifuged at 2000rpm for 10-15min. After words the Hb contain is estimated spectrophotometrically.

XIII. Haemoglobin Release:

It can be estimated by altering the permeability of the erythrocytes. Also the red cell suspension haemoglobin leakage can be recorded by using an spectrophotometer at 540nm. The relationship between the rate of haemoglobin release and the rate of drug release provides data for interpreting the mechanism involved in the release of substances encapsulated inside the erythrocytes.

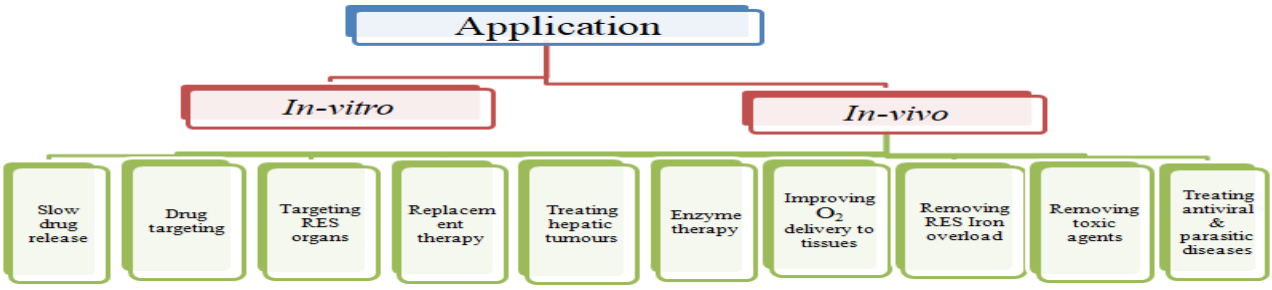

Chart 1: Applications of Resealed Erythrocytes in Drug Delivery

11.1. *In-vitro* Application [35-39]:

Phagocytosed cells have been used to aid the uptake of enzymes by phagolysosomes. RBC facilitated microinjection was the most recurrent *in vitro* application which is done by fusion process of a protein or nucleic acid to be injected into eukaryotic cells. In the same way, when antibody molecules are introduced using erythrocyte carrier system it is seen that they instantly disperse throughout the cytoplasm. Antibody RBC auto injected into living cells be predictable to the site of action of a fragment of diphtheria toxin. They can become useful tools in treating defects such as glucose-6-phosphate dehydrogenase (G6PD) deficiency.

11.2. *In-vivo* Application [40-53]:

I. Slow Drug Release: Erythrocytes have emerged as circulating depots for sustained delivery of antineoplastics, antiparasitics, veterinary antiamoebics, vitamins, steroids, antibiotics and cardiovascular drugs. The numerous mechanisms suggested 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 trailed by slow release, gathering of erythrocytes in lymph nodes upon subcutaneous administration followed by haemolysis to release the drug.

II. Drug Targeting: Generally, drug delivery is site-specific and target-oriented to demonstrate maximal therapeutic index with minimum adverse effects. Surface-modified erythrocytes are used to target organs of mononuclear phagocytic system/ reticuloendothelial system since the changes in the membrane are recognized by macrophage.

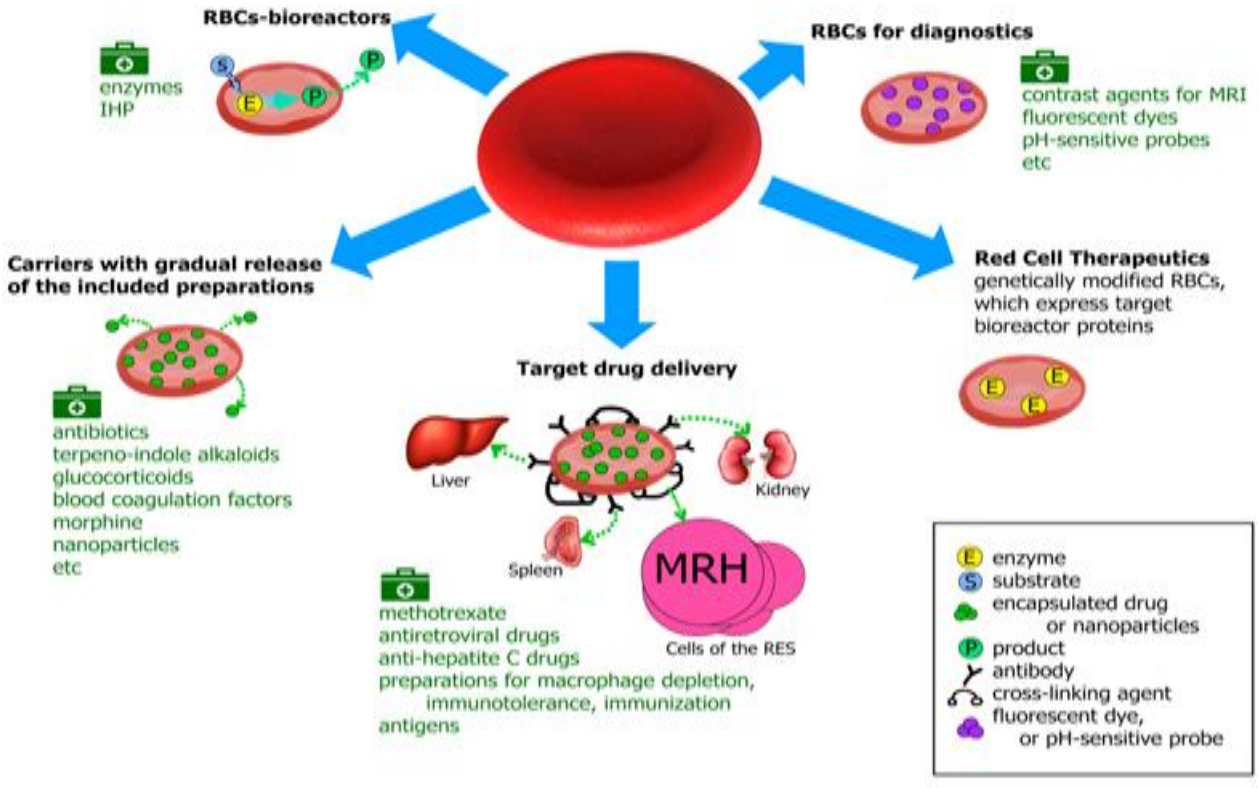

Fig. 15: *In-vivo* Application [54]

III. Targeting RES organs: Damaged erythrocytes are promptly excreted out from circulation by phagocytic Kupffer cells in liver and spleen thereby can be used to target these reticuloendothelial organs.

IV. Replacement Therapy: The main problem faced in treating enzyme deficiency by injecting these enzymes is shorter circulation half-life of enzymes, allergic reactions, and toxic manifestations. These problems can be positively overcome by administering the enzymes as resealed erythrocytes. The enzymes used include-glucosidase, glucoronidase, galactosidase. The disease caused by build-up of glucocerebrosides in the liver and spleen, which can be treated by glucocerebrosidase- loaded erythrocytes.

V. Treating of Hepatic Tumors: Hepatic tumors are one of the most prevalent types of cancer. Antineoplastic drugs such as methotrexate, bleomycin, asparginase, and adriamycin have been efficaciously delivered by erythrocytes. Drugs such as daunorubicin diffuse rapidly from the cells upon loading and thereby creating a problem which can be resolved by covalently linking daunorubicin to the erythrocytic membrane using gluteraldehyde or cis-aconitic acid as a spacer.

VI. Enzyme Therapy: Replacement therapies are seen to have scope in treating diseases related with deficiencies like Gaucher's disease, galactosuria and degradation of toxic compound that are secondary to some kind of poisoning like cyanide, organophosphorus. Enzymes are widely used in clinical trials of the resealed erythrocytes loaded with enzymes for replacement therapy in that of β-glucoserebrosidase for the treatment of Gaucher's disease. Enzyme loaded resealed erythrocytes release enzymes into circulation upon haemolytic action as a "circulating bioreactors" in which substrates enter into the cell, interact with enzymes, and generate products or accumulate enzymes in RES upon haemolytic for future catalysis.

VII. Improving Oxygen Delivery to tissues: Haemoglobin is responsible for the oxygen-carrying by erythrocytes. Normally 95% of haemoglobin is saturated with oxygen in the lungs, however in peripheral blood stream only 25% of oxygenated haemoglobin becomes deoxygenated under physiological conditions. Therefore, the major fraction of oxygen bound to haemoglobin is recirculated with venous blood to the lungs.2, 3-diphosphoglycerate (2, 3-DPG) as a natural effector of haemoglobin is used in the treatment of oxygen deficiency.

VIII. Removal of Reticuloendothelial System (RES) iron overload: Desferrioxamine-loaded erythrocytes have been used for treatment of over accumulation of iron in the thalassemic patients and different forms of anemia. RES is very beneficial for targeting this drug as the aged erythrocytes are destroyed in RES organs, resulting in an accumulation of iron in these organs.

IX. Removal of Toxic Agents: Reserve of cyanide intoxication with murine carrier erythrocyte containing bovine rhodanese and sodium thiosulfate. 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 phospho-diestrase also has been carried out.

X. Treating Antiviral and Parasitic Diseases: Antiviral agents entrapped in resealed erythrocytes for effective delivery and targeting because most antiviral drugs are nucleotides or nucleoside analogues, their entrapment and exit through the membrane needs cautiousness. Nucleosides are rapidly transported across the membrane whereas nucleotides are unable to do so and therefore exhibit prolonged release profiles. Parasitic diseases that involve harboring parasites in the RES organs can be successfully controlled by encapsulating drugs loaded erythrocytes for delivering antiparasitic agents. Results were complimentary in studies involving animal models for erythrocytes loaded with antimalarial, antileishmanial, and antiamoebic drugs.

12. NOVEL APPROACHES [55]:

12.1. Erythrosomes:

Erythrosomes are especially engineered vesicular systems in which chemically cross-linked human erythrocytes cytoskeletons are utilised as sport upon which a lipid bilayer is coated. This can be done by a modification procedure normally adopted for reverse phase evaporation. Erythrosomes are projected as useful encapsulation system for drug delivery particularly for macromolecular drugs. Large (3, tm diameter) mechanically stable proteoliposomes (erythrosomes) were prepared in decent yield by coating cross linked erythrocyte cytoskeletons by means of phosphatidyl choline.

12.1.1. Application:

1. Pentamidine primaquine phosphate and metronidazole have been efficaciously utilized in erythrosomes for the treatment of leshmaniasis, malaria on experimental basis.

2. It can be serveed as an ideal carrier for antineoplastic drugs delivery like bleomycin.

3. RBCs coated with recombinant interlukin-2 are testified to provide sustain release to allow low and nontoxic concentration of rlL-2 in circulation.

12.2. Nanoerythrosomes (nEs):

Nanoerythrosomes are vesicles with average diameter of 100 nm are prepared by the extrusion of RBC ghost. The process presented small vesicles with the size of liposomes. The drugs can be conjugated to the Nanoerythrosomes using definite cross linking agents such as gluteraldehyde have greater activity than when compared to free drug and they offer higher stability. The Nanoerythrosomes are characterized for several parameters like surface morphology, percentage of drug conjugation, centrifugal stress, in-vitro release etc. Thus nEs are very useful bioactive drug carriers or drug delivery system. These nEs are stable and maintain both the cytotoxic & antineoplastic activity of daunorubicin against mice leukaemia cells. In case of Gaucher's disease glucocortisone was encapsulated in erythrocytes and heparin was encapsulated in erythrocytes to inhibit thromboembolism.

12.2.1. Methods of Preparation of Nanoerythrosomes and drug loading:

I. Extrusion:

The erythrocyte ghost suspension (50% haematocrit) is extruded through the 25mm polycarbonate membrane filter (0.4μm) pore gained by 8-10 consecutive extrusions under nitrogen pressure. The ghosts obtained are then stained with uranyl acetate and they are observed under microscope. The extrusions are performed in a thermostatically controlled extrusion device at 37^{o}C and the final preparation is stored in a refrigerator at 40^{o}C. The extrusions are performed at 37°C in thermostatically controlled extrusion device.

II. Sonication:

Erythrocyte ghosts are transformed into small vesicles using a dismembrator.

III. Electrical breakdown method:

It is used to convert ghosts into small vesicles under the influence of electric potential.

12.2.2. Applications:

I. nEs are also utilised for delivery of mitomycinc, hydroxyurea and 6-mercaptopurine. Cytotoxicity is supplementary in the form of nE combinations than the free drug. They can also be used for delivering insulin.

II. Doxorubicin-E conjugates have greater antineoplastic activity than the free drug on CDF1 leukaemia tumours.

III. Nanoerythrosomes (nEs) were designed in such a way that with low dose they can maintain the optimum concentration of the drug for prolonged periods of time and they provide sustained action.

Table 3: Current research work done on the preparation of resealed erythrocytes using different techniques [56-65]

S.No.	Formulation	Active ingredient	Other ingredients	Preparatory method	Results	References
1.	Preparation of full length Ankyrin resealed erythrocytes	Full length Ankyrin binding domain of ß-Spectrin	Isopropyl ß-D-1-thiogalacto pyronoside, Urea, Sodium Chloride	Isotonic Lysis Method	Decrease in Barrier Properties and Change in Permeability properties	57
2.	Preparation of Salbutamol loaded resealed erythrocytes	Salbutamol	Adenosine 5-Triphosphate, Acetonitrile, Sodium Chloride	Endocytosis Method	Prolonged Release carrier for Salbutamol for the treatment of nocturnal asthma	58
3.	Preparation of Paclitaxel loaded resealed erythrocytes	Paclitaxel	Glutaraldehyde, Dimethyl Formamide, Methanol, Sodium Hydroxide	Preswell Dilution Method	Potential candidate for safe effective sustained drug release for the treatment of cancer	59
4.	Preparation of Prednisolone loaded resealed erythrocytes	Prednisolone	Glutaraldehyde, Dimethylsulphoxide	Preswell Dilution Method	Increased Therapeutic index and drug targeting to organs for prolonged release to treat arthritis and other skin conditions	60
5.	Preparation of Ribavirin loaded Resealed Erythrocytes	Ribavirin	Adenosine 5-Triphosphate, Disodium Hydrogen Phosphate, Calcium Chloride	Endocytosis Method	Drug Targeting to RES and thus improving Ribavirin effect for the treatment of Hepatitis C	61
6.	Preparation of combinatory paradigm using Resealed erythrocytes with Interferon alpha 2b, Ribavirin and Boceprevir	Interferon –α, Ribavirin, Boceprevir	Polyethylene Glycol	Hypnotic Preswell Dilution Method	Liver Targeted Triple combination therapy for HepatitisC	62
7.	Preparation of Resealed Murine Erythrocytes encapsulating Phosphodiesterase	Paraoxon	Dichloromethane, Sodium Chloride, Dextrose, Magnesium Chloride	Hypnotic Dilution Method	Beneficial & New Approach to antagonise toxic effects of Organo phosphorus compounds	63

8.	Preparation of Amikacin loaded Resealed Erythrocytes	Amikacin	Glutathione, Sodium Pyruvate, Adenine	Hypnotic Dilution Method	Elicits Sustained release effects of antibiotic and higher accumulation in RES organ like spleen and liver	64
9.	Preparation of Resealed Erythrocytes with Ascorbic acid	Ascorbic Acid	Dehydroascorbate, Tridecylamine	Hypnotic Preswell Dilution Method	Prevention of oxidation of cell membrane and limits the membrane lipid peroxidation	65

13. FUTURE PERSPECTIVES [66]:

I. The concept of utilising erythrocytes as drug or bioactive carrier still needs further optimization.

II. An enormous amount of valuable work is required so as to utilize the potentials of erythrocytes in passive as well as active targeting of drugs.

III. The resealed erythrocytes can be utilized for in humans as carriers for drugs delivering.

IV. Scientists and researchers have demonstrated and proven that such engineered red blood cells are suitable for blood transfusion.

V. Genetic engineering aspects can be coupled to give a newer aspect to the existing cellular drug carrier concept.

13.1. Future studies would concentrate on the following:

I. Employment of autologous properties of erythrocytes, improved understanding of the biology of the red blood cells and its membrane development of pulsatile and feedback control system, selective drug delivery to CNS and delivery peptide and protein drugs.

II. Technical advance in the procedure for preparing resealed erythrocytes, crosslinking of resealed erythrocytes, aseptic and sterile processing, optimization techniques, pilot- plant scale up studies and innovative ideas for the application of resealed erythrocytes. Either as carriers or as cellular bioreactors would pave the way for automation and commercialization of this novel drug delivery system.

III. With the accessibility of technology to clone human DNA prokaryotes and the potential to produce large quantity of human enzymes, the possibility of enzyme replacement therapy targeting and the use of RBC carrier reservoirs would become more of reality.

IV. In the future greater interest seems to be related to the targeting of immune-modulators on the phagocytic system anticancer drugs.

V. Drug loaded magnetite bearing cells aid as a promising carrier for delivering the drug to specific site.

Conclusion:

The review mainly focusses on the necessity, properties, isolation, preparation and applications of resealed erythrocytes. Resealed erythrocytes are the capable and promising drug carriers that can be utilised as emerging targeted drug delivery systems with none or minimum side effects of the conventional dosage form. The review also elaborates on different ways of preparation techniques. Due to the numerous potential advantages over other drug delivery system and also wide range of applications in areas of enzyme therapy, hepatic tumours, cancer, bacterial infections this drug loaded erythrocytes seems to be a hopeful delivery system. However more importance should be given on the novel approaches like nanoerythrosomes and erythrosomes so that it can be utilized in routine drug delivery system. Thus resealed erythrocyte is a promising and smarter approach for the delivery of drugs and biopharmaceuticals.

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