REVIEW ARTICLE

ENCAPSULATED ERYTHROCYTES FOR NOVEL DRUG DELIVERY SYSYTEM

[*]Ravindra Kumar[1], Shubhendra Jha[1], Mukesh Kumar[1], Chauhan PS[1], Singh SK[2], Sagar BPS[1]

[1] Department of Pharmacy, IEC Group of Institution, Plot No. 4, Knowledge Park 1, Greater Noida, U.P,

[2] Apco Pharma Ltd. Haridwar (UK)

[*]*Correspondening Author's Email: ravindrarawal@hotmail.com*

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ABSTRACT

The current Pharmaceutical scenario focuses only on the development of targeted and sustained drug delivery systems to achieve required therapeutic concentration with less amount of dose. Among the various carriers used for targeting of the drugs to various body tissues, the encapsulated erythrocytes meet several criteria desirable in clinical applications, among the most important being biocompatibility as a carrier and its degradation products. Erythrocytes loaded with drugs and other substances have different release rates. Encapsulated erythrocytes permit controlled release of drug and increase specificity of delivery to the target cells or organs and use of novel routes of drug into cells. Using this technique with a drug present in the extra cellular solution, it is possible to entrap up to 40% of the drug inside the resealed erythrocytes. A variety of bio-macromolecules ranging from 5000 to 600,000 Da can be entrapped into in erythrocytes. The study focused on various methods of Encapsulation of erythrocytes, various techniques of drug loading such as hypo-osmotic-lysis, Chemical perturbation of the membrane, Electro-insertion, Entrapment by endocytosis etc. and it's applications in various clinical purposes .

Key words: Ghost cell, Encapsulated erythrocytes, Phagolysosomes, Reticulo-endothelial system(RES)

INTRODUCTION

Now a day's The Pharmaceutical scientists and academicians emphasize on the development in newer drug delivery system which focuses on the drug targeting to achieve high therapeutic effects and minimize adverse effects[1].The NDDS includes various carriers such as natural and synthetic polymers, polysaccharides, monoclonal antibodies and other biodegradable polymers. It also enlists multi-component structure like liposome, microcapsules, lipoproteins, microparticles, ghost cells and cells.

The cellular carrier for drug delivery includes fibroblasts and erythrocytes, leukocytes, platelets and hepatocytes. Resealed erythrocytes are one of the most reviewed systems for targeted delivery because they have one of the most interesting novelties in the development of cell-carrier drug delivery systems. Erythrocytes cells can be loaded with drugs and these can then be released during circulation or at targeted sites. Among the cells studied are erythrocytes as these cells offer many advantages including prolonged delivery times and an elevated biocompatibility[2].

CHARACTERIZATION OF ERYTHROCYTES:

Erythrocytes (RBCs) have the basic function to transport oxygen in the blood. Erythrocytes appear to be circular from the top and side view shows that they are actually biconcave discs with diameter of 7.8 µm and thickness aprx 2.2 µm. This shape increases the surface area-to-volume ratio of the cell, thus increasing the efficiency of diffusion of O_2 and CO_2 into and out of the cell. Erythrocytes also have a flexible plasma membrane which is very useful during drug loading process.[5,6,7]

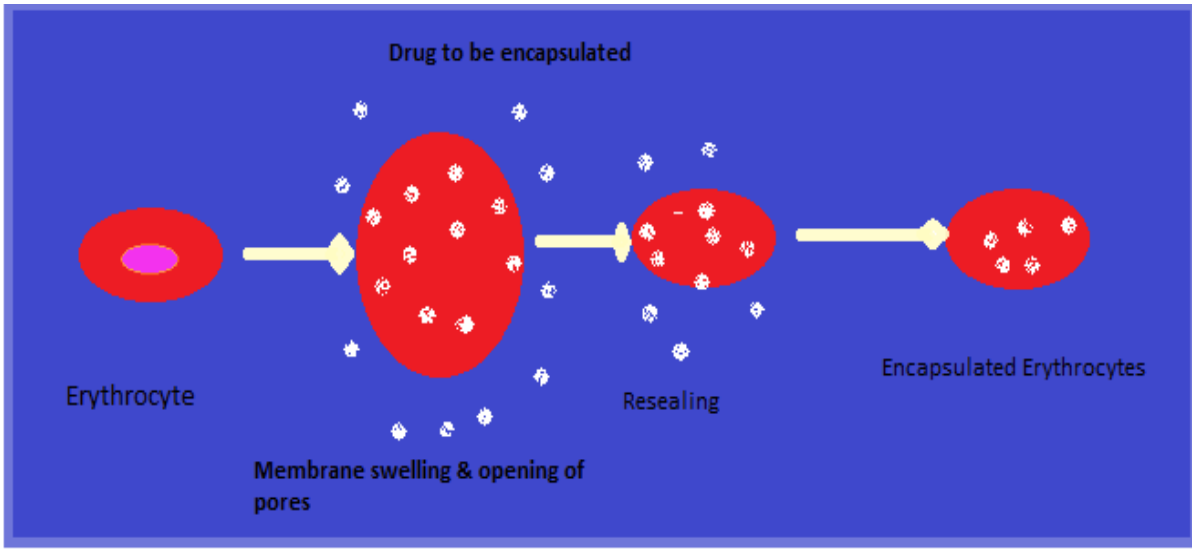

Figure 1: Erythrocytes as a Carrier

SUITABILITY OF ERYTHROCYTES AS DRUG CARRIER

Erythrocytes as a Carrier are one of the innovative drug delivery systems used for years. These are elastic, biconcave shape which enables them to squeeze through narrow capillaries and are selectively removed from circulation by the macrophages in the Reticulo Endothelial System (RES), hence be able to used in targeting of drugs to RES. The average life span of RBCs is 100-120 days in the circulation so can be used for sustained delivery of therapeutic agents.[8]

They posses various characterstics like easy isolation, biocompatibility, specific physico-chemical properties, High drug loading efficiency , Low leaching or leakage of drug and have enough volumes for loading drug. Eryhrocytes modify the pharmacokinetic and pharmacodynamic parameter of drug.It also minimizes the toxic effect of drug due to fluctuation in concentration to protect the organism. The breakdown products are recycled; hemoglobin is breakdown into globin and heme. Globin degraded to amino acids for amino acid pools in the body, while iron reused in hemoglobin synthesis; therefore, they are well suited to be used as drug carriers.[9]

ADVANTAGES OF ENCAPSULATED ERYTHROCYTES

Erythrocytes can be used as carriers in two ways; one is that Targeting particular tissue or organ using only the erythrocyte membrane and other is for continuous or sustained release of drugs which provide prolonged drug action. They remain in the circulation for prolonged periods of time (apprx. 120 days) and release the entrapped drug at a slow and steady rate[3,4].

The entrapment of drug does not require the chemical modification of drugs. They are non immunogenic in action and can be targeted to diseased tissue/organ. They facilitate incorporation of protein and nucleic acid in eukaryotic cells by cell infusion with RBC. In addition, they can be used as substitute carriers instead of liposomes, Ethosomes, Niosomes, nanoparticles, microparticles or microspheres that have been used for the controlled Drug delivery.

DISADVANTAGES [10,11,12,13]

- Limited potential as carrier to nonphagocytic target tissue.
- Given that they are carriers of biological origin, encapsulated erythrocytes may present greater variability and lesser standardisation in their preparation, compared to other carrier systems.
- Possibility of clumping of cells and Dose dumping may also be there.
- The leakage of certain encapsulated substances may be there from the loaded erythrocytes.
- Several molecules may alter the physiology of the erythrocyte.
- Liable to biological contamination due to the origin from the blood, the equipment used and the loading environment. Rigorous controls are required accordingly for the collection and handling of the erythrocytes.
- The storage of the loaded erythrocytes is a problem involving carrier erythrocytes for their possible use in therapeutics. Tests have been performed on their conditioning in suspension in isotonic buffers containing all essential nutrients, as well as in low temperatures, with the addition of nucleosides or chelators, lyophilisation, freezing with glycerol or gel immobilization.

SOURCE AND ISOLATION OF ERYTHROCYTES

Different mammalians like humen, monkies, horses, sheeps, goats, rabbits etc., are used for the collection of erythrocytes[14]. Usually to isolate erythrocytes, blood is collected into heparinised tubes by venipuncture. EDTA or heparin can be used as an anticoagulant. Whole blood from horse, sheep, goat, dog and rabbit is easily collected through venipuncture. Fresh whole blood is defined as any blood collected and immediately chilled to 4 0C and stored for not more than 2 days. Red blood cells are harvested and washed by centrifugation. After recovering the blood from vain puncture and mix with heparin, it is centrifuged at 2000 rpm for 5 min at 4 ±1 ^{0}C. This helps in separation of plasma and Buffy coat. Erythrocytes so obtained are washed three times with buffer solution. These erythrocytes are diluted with phosphate buffer and often stored in acid-citrate-dextrose buffer at 4 0C up to 48 hrs prior to use.

Table 1: Source and Isolation of erythrocytes [14-17]

Species	Washing buffer	Centrifugal force(g)
Rabbit	10mmol KH2P04 /NaHPO4	500-1000
Dog	15mmol KH2P04 /NaHPO4	500-1000
Human	154mmolNaCl	<500
Mouse	10mmol KH2P04 /NaHPO4	100-500
Cow	10-15mmol KH2P04 /NaHPO4	1000
Horse	2 mmolMgCl2, 10 glucose	1000
Sheep	10 mmol KH2P04 / NaHPO4	500-1000
Pig	10 mmolKH2P04 /NaHPO4	500-1000

IDEAL PROPERTIES OF DRUGS [10, 11]

- The Drug should be polar and hydrophilic.
- The drug should resist degradation within erythrocytes.
- It should not have any physical or chemical interaction because drug molecules (non polar and hydrophobic drugs) which interact with the membrane and cause deleterious effects on membrane structure so these are not considered to be appropriate for encapsulation in

erythrocyte.

- It should have well defined pharmacokinetic and pharmacodynamic properties.
- Variety of biologically active substance i.e. mol wt ranges from 5000-60,000 Dalton can be entrapped in erythrocytes.

DRUG LOADING METHODS

Various methods can be used to load drugs or other bioactive compounds in erythrocytes, including physical (electrical pulse method) osmosis-based systems, and chemical methods (chemical perturbation of the erythrocytes membrane).

1. Hypo-osmotic lysis method for drug loading

Various methods for Hypo-osmotic lysis are discussed in table below:

Table 2: Hypo-osmotic lysis method for drug loading in erythrocyte[26-27]

Method	Procedure	% Loading	Advantages	Disadvantages
Dilution method	Based upon hypotonic lysis of cells in a solution containing the 1-8% Fastest and simplest, Entrapment capacity low. Drug/enzyme to be entrapped followed by restoration of tonicity especially for low to reseal them and also ability of erythrocytes to undergo molecular weight drug. Reversible swelling in a hypotonic solution.	1-8 %	Fastest and simplest, especially for low molecular weight drug.	Entrapment capacity low
Dialysis	It can be carrying out lysis and resealing within a dialysis tube using hypotonic and isotonic solution	30-40 %	Better *in vivo* survival.	Time consuming
Preswell dilution	The technique is based upon initial controlled swelling of erythrocytes without lysis by placing them in slightly followed by centrifugation at low 'g' to take them up to and good survival *in* point of lysis. Finally, the addition of small volume of drug solution to *vivo*, attain drug loaded resealed erythrocytes.	20-70 %	Good retention of cytoplasm constituents and good survival *in-vivo*.	
Isotonic osmotic lysis[23]	Resealed erythrocytes were prepared under isotonic conditions. Haemolysis in isotonic solutions can be achieved both by chemical agent and physical methods.	20-70 %	Better *in vivo* Surveillance Consuming.	Impermeable only to large molecules, process is time

2. Chemical perturbation of the membrane[28-29]

According to this method the membrane permeability of erythrocytes increase when the cells are exposed to certain chemicals. The permeability of erythrocytic membrane increases upon exposure to polyene antibiotic such as amphotericin B. The antineoplastic drug daunomycin was introduced successfully in human and mouse erythrocytes. This method has been established by Lin et al for same purpose using halothane.[30] However; this method is not very popular because it induces irreversible destructive changes in the cell membrane.

3. Electro-insertion or electroencapsulation[31-35]

This method is based on the observation that electrical shock brings about irreversible changes in an erythrocyte membrane[31.] The erythrocyte membrane is opened by a dielectric breakdown. Subsequently, the pores can be resealed by incubation at 37 ^{0}C in an isotonic medium.

The procedure involves suspending erythrocytes in an isotonic buffer in an electrical discharge chamber. A capacitor in an external circuit is charged to a definite voltage and then discharged

Within a definite time interval through cell suspension to produce a square-wave potential . The optimum intensity of an electric field is between 1–10 kW/cm and optimal discharge time is between 20–160 μs. An inverse relationship exists between the electric-field intensity and the discharge time. The compound to be entrapped is added to the medium in which the cells are suspended from the commencement of the experiment. The characteristic pore diameter created in the membrane depends upon the intensity of electric field, the discharge time, and the ionic strength of suspending medium. The colloidal macromolecules contents of the cell may lead to cell lysis because of the increase in osmotic pressure. This process can be prevented by adding large molecules (e.g., tetrasaccharide stachyose and bovine serum albumin) and ribonucleose[64]. One advantage of this method is a more uniform distribution of loaded cells in comparison with osmotic methods. The main drawbacks are the need for special instrumentation and the sophistication of the process. Entrapment efficiency of this method is close to 35%. Various compounds such as sucrose[36], urease[39], methotrexate[47], isoniazid[43], human glycophorin[44], DNA fragments, and latex particles of diameter 0.2 μm can be entrapped within erythrocytes[33].

4. Entrapment by endocytosis[46-48]

In 1975 Schrier et al reported this method by Endocytosis involves the additionof one volume of washed packed erythrocytes to nine volumes of buffer containing 2.5 mM ATP, 2.5 mM MgCl2, and 1mM CaCl2, followed by incubation for 2 min at room temperature. The pores created by this method are resealed by using 154 mM of NaCl and incubation at 37 ^{0}C for 2 min. The entrapment of material occurs by endocytosis. The vesicle membrane separates endocytosed material from cytoplasm thus protecting it from the erythrocytes and vice-versa. The various candidates for example primaquine and related 8–amino–quinolines, vinblastine, chlorpromazine and related

phenothiazines, hydrocortisone, propranolol, tetracaine, and vitamin are entrapped by this method[57].

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 entrapped drug. The methods are useful for entrapping inositol monophasphte to improve the oxygen carrying capacity of cells and entrapment efficiency of this method is very low (~1%). [49]

Use of Red Cell Loader [50]

A method for entrapment of non diffusible drugs into erythrocytes with 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 hrs. at room temperature under blood banking conditions. The process is based on two sequential hypotonic dilutions of washed erythrocytes followed by concentration with a hemofilter and an isotonic resealing of the cells. There was 30% drug loading with 35–50% cell recovery. The processed erythrocytes had normal survival in vivo. The same cells could be used for targeting by improving their recognition by tissue macrophages.

CHARACTERIZATIONS OF ENCAPSULATED ERTYTHROCYTE

Various characterization parameters are given in table below:

Table 3: Characterization parameters and their determination methods for encapsulated Erythrocytes[36-45]

1. Physical parameter

PARAMETER	METHOD / INSTRUMENT USED
Shape and surface morphology	Transmission electron microscopy, scanning electron microscopy, phase contrast microscopy, optical microscopy
Vesicle size and size distribution	Transmission electron microscopy, optical microscopy
Drug release	Diffusion cell, dialysis
Drug content	Deproteinization of cell membrane followed by assay of resealed drug, radio-labelling
Surface electrical potential	Zeta potential measurement
Surface Ph	pH-sensitive probes
Deformability	Capillary method

II. Cellular characterization

% Hb content	Deproteinization of cell membrane followed by hemoglobin assay
Cell volume	Laser light scattering
% Cell recovery	Neubaur‟s chamber, hematological analyzer
Osmotic fragility	Stepwise incubation with isotonic to hypotonic saline solutions and determination of drug and hemoglobin assay
Osmotic shock	Dilution with distilled water and estimation of drug and hemoglobin
Turbulent shock	Passage of cell suspension through 30-gauge hypodermic needle at 10 ml/min flow rate and estimation of residual drug and hemoglobin, vigorous shaking followed by hemoglobin estimation
Erythrocyte sedimentation rate	ESR methods

III. Biological Characterization

Sterility	Sterility test
Pyrogenicity	Rabbit method, LAL test
Animal toxicity	Toxicity tests

IV. Safety Considerations

STEPS TO BE CONSIDERED	SAFETY ISSUE
Different blood types	Blood clotting
Possible risk of contamination	HIV,HBV etc.
Changing on physical and possible of clotting	Rigidity of membrane modification on erythrocytes membranes biochemical characteristics proteins lead to lysis. Extensive biotynilated leads to rapid elimination and kidney problems
Changing in pharmacokinetic and pharmacodynamic behaviors of loaded drug	Increase the production of unfavorable metabolites

IN-VITRO STORAGE[32, 34]

The most common storage media include Hank's balanced salt solution and acid–citrate-dextrose at 4 ^{0}C. Cells remain viable in terms of their physiologic and carrier characteristics for at least 2 weeks at this temperature.

ROUTE OF ADMINISTRATION [60,61]

Encapsulated erythrocytes are commonly administered by IV Route[60]. Intraperitoneal and subcutaneous routes are other means of administration for targeting cells into peritoneal macrophages and for sustaining drug release respectively.

MECHANISM OF RELEASE OF LOADED DRUGS [62,63]

There are mainly three ways for a drug release from the erythrocyte carriers

- **Phagocytosis**: By the process of phagocytosis normally erythrocyte cells removed from the blood circulation. The degree of cross linking determines whether liver or spleen will preferentially remove the cells.
- **Diffusion through the membrane of the cells:** Diffusion through the membrane depends on the drug molecule penetrate through a lipid bilayer i.e. bioactive compound have lipid solubility.
- **Using a specific transport system**: Most of the drug molecules enter cells by a specific membrane protein system because the carriers are proteins with many properties analogous to that of enzymes.

APPLICATIONS OF ENCAPSULATED ERYTHROCYTES [58,59]

Encapsulated erythrocytes have been used as targeted Drug delivery systems for a variety of applications in human and veterinary medicine. In vivo application of the drugs loaded erythrocytes are used in two ways as for prolonged drug released and for drug targeting to RES or non RES. The applications of encapsulated erythrocytes are as follows:

[I] In vitro

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 diphtheria toxin. Antibodies introduced using RBC mediated microinjection is recorded not to enter the nucleus, thus limiting the studies to the cytoplasmic level. Encapsulated erythrocytes have been used to facilitate the uptake of enzymes by phagolysosomes for *In vitro* phagocytosis. Enzymes content within carrier RBC could be visualized with the help of cytochemical technique. The biochemical defects such as the glucose- 6-phosphate dehydrogenase (G6PD) deficiency can be useful tool for discerning the mechanism that eventually causes these effects. Other *in vitro* tests include utilization of erythrocytes carrier to introduce ribosome inactivating proteins into cells by fusion technique.[51]

[II] In vivo

A. Targeting of bioactive agents to RES

After drug release, remnants of erythrocytes are rapidly cleared from circulation by phagocytosis. Targeting of the drug minimizes its side effects and the dose to be administered as well as drug utilization. Modifications of erythrocytes membranes by treating them with antibodies, gluteraldehyde, sialic acid, ascorbate, ferrous ion, biotin and sulfhydryl containing substances accelerate their targeting to the liver as well as spleen[18].

B. Targeting to sites other than RES-rich Organs

Encapsulated Erythrocytes have the ability to deliver a drug or enzyme/proteins to the macrophage-rich organs and these have been used to target non RES organs.

Table 4: Encapsulated erythrocytes used in other than RES organ targeting [21-25]

Approaches	Type of Drugs	Application
Ultrasound Mediated Delivery of Encapsulated Erythrocytes	Erythrocytes colloidal particles and RBC	Delivery to tissue through micro vessel ruptures created by targeted micro bubble destruction with ultrasound.
Photosensitized Erythrocytes	Methotrexate and photosensitized by subsequent exposure to a haematoporphyrin derivative	Useful in the treatment of tumors of body located at site other than RES predominant organs or as a phototriggered carrier/delivery system for methotrexate in tumor therapy.
Magnet-responsive Erythrocyte Ghosts	encapsulation of small paramagnetic particles into erythrocytes	Localization to a particular location under the influence of external magnetic field.
Antibody Anchored Erythrocytes (Immunoerythrocytes):	Antibody coating of resealed drug carrier	Drug targeting to the RES.

C. Carrier erythrocytes as slow drug release system

Sustained release dosage forms are designed to produce a prolonged therapeutic effect by continuous drug release over an extended period of time after administration of single dose.[26]

Carrier erythrocytes have long life span in the circulation, so that they can be used as circulating depots for antitumor, antiparasitics, antibiotics as well as cardiovascular drugs. This happened only when the drug and the selected method for the drug loading don't change the morphological and physiological parameters of erythrocytes. Encapculated erythrocytes serve as an ideal carrier for antineoplastic agents, antimicrobial drugs, vitamins and steroids.[10]

D. Erythrocytes as Carriers for Drugs, Enzymes , Proteins, Macromolecules and as a circulating bioreactors

Several drugs like Actinomycin-D, Methotrexate,Daunomycin,Etoposide Gentamycin, Primaquine and few enzymes L-Asparaginase, Aminolevulinate dehydratase have been encapsulated in Erythrocytes. Other than these, proteins like Insulin, micotoxin and recombinant human erythropoietin (rHuEpo) have been encapsulated. Various application of encapsulated are given in table below:

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Table 5: Various application of resealed erythrocytes[19,20]

Application	Drug/Enzyme/Macromolecule
Enzyme deficiency replacement therapy	β -galactosidase,β-fructofuronodase, urease, glucose 6-phosphatedehydrogenase
Thrombolytic activity	Brinase, aspirin, heparin
Iron overload	Desferroxamine
Chemotherapy	Rubomycin, methotrexate, daunomycin, cytosine
Immunotherapy	Human recombinant interleukin-2
Circulating bioreactor	Arginase, uriease, luciferase

NOVEL APPROACHES

Erythrosomes are specially designed vesicular systems that are chemically cross-linked to human erythrocytes, support upon which a lipid bilayer is coated. This is achieved by modifying a reverse-phase evaporation technique. These vesicles have been proposed as useful encapsulation systems for macromolecular drugs.[52-54]

Nanoerythrosomes are prepared by extrusion of erythrocyte ghosts to produce small vesicles with an average diameter of 100 nm. Daunorubicin was covalently conjugated to nanoerythrosomes using gluteraldehyde spacer. This complex was more active than free daunorubicin alone, both in vitro and in vivo.[55, 56]

CONCLUSION

This review focuses on the suitability of erythrocytes as biological carriers for therapeutic agents, such as drugs, enzymes and peptides. Encapsulated erythrocytes are effective and safe for targeted and sustained Drug delivery systems with less or no toxicity. Through unexpected twist and turns, encapsulation of erythrocytes became a game changing technology for pharmaceutical sciences. However, the concept needs further optimization to become a routine drug delivery system.

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