



DRUG, ENZYME AND PEPTIDE DELIVERY USING ERYTHROCYTES AS DRUG CARRIER

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ABSTRACT

Erythrocytes are potential biocompatible vectors for different bioactive and therapeutic agents. Erythrocytes are biodegradable, possess long circulation half-lives and can be loaded with a variety of biologically active compounds using various physical and chemical methods. Encapsulation of drug in erythrocytes significantly changes the pharmacokinetic properties of drugs in both animals and humans enhance liver and spleen uptake and target the reticulo-endothelial system (RES). Other advantages of erythrocyte as carrier system is that it prevents inactivation of loaded drug by endogenous chemicals attain steady state concentration of drug and decrease the side-effects of drug. Grasper is a new medicinal product consisting L-Asparaginase entrapped into human homologous red blood cells used in treatment of Acute Lymphoblastic Leukemia (ALL).

Keywords: Biocompatible vectors, Erythrocytes, Magnetic resealed erythrocyte, Targeting reticulo-endothelial system (RES).

INTRODUCTION

Current research is aim at development of drug delivery system with maximum therapeutic benefits for safe and effective management of disease(s). The development of drug delivery system in future will be aimed to maximize therapeutic performance, eliminate undesirable side effect of drug and increase patient compliance. The idea of a drug carrier system with target specificity has fascinated scientists and tremendous efforts have been made to achieve this goal. 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.¹

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, Microparticles, cells, cell ghosts, lipoproteins, liposomes, erythrocytes).²

Erythrocytes, also known as red blood cell (RBCs), 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 organisms of interest, separating erythrocytes from plasma, entrapping drug in the erythrocyte and resealing the resultant cellular carriers. Hence these carriers are called as resealed erythrocytes. Erythrocytes, the most abundant cells in the human body, have potential carrier capabilities for the delivery of drugs. Another important feature of these carriers is that they can be stored at 4°C for several hours to several days before re-entry to the

host body, depending on the storage medium and the entrapment method used. Upon re-injection to the same organism, these drug-loaded microspheres can serve as the intravenous slow-release carriers and/or targeted drug delivery systems especially to target the drug to the reticuloendothelial system (RES).³

BASIC FEATURES OF ERYTHROCYTES

RBCs have shapes like biconcave discs with a diameter of 7-8 μm and thickness near 2.0 μm . Mature RBCs have a simple structure Fig. (1). It is also elastic in nature.

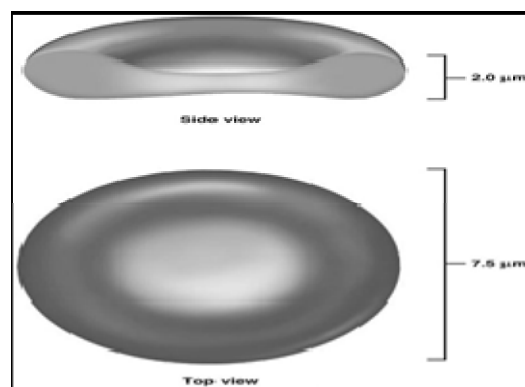


Figure 1: Shape of erythrocyte.

Their plasma membrane is both strong and flexible, which allows them to deform without rupturing as they squeeze through narrow capillaries. RBCs lack a nucleus and other organelles and can neither reproduce nor carry on extensive metabolic activities. RBCs are highly specialized for their oxygen transport function, because their mature RBCs have no nucleus, all their internal space is available for oxygen transport.

Each RBC contains about 280 million haemoglobin molecules. RBCs include water (63%), lipids (0.5%), glucose, (0.8%), mineral (0.7%), non-hemoglobin protein (0.9%), methemoglobin (0.5%), and hemoglobin



(33.67%). The average content of erythrocytes in men and women is 5.4×10^6 and 4.8×10^6 per μl , respectively. The number of erythrocytes and their content in hemoglobin constitutes a determining factor in the supply of oxygen. Erythrocytes are constantly developing from stem cells, the undifferentiated, self-regenerating cells that give rise to both erythrocytes and leukocytes in the bone marrow, with the production of red corpuscles being regulated by erythropoietin. As they mature, the erythrocytes lose their nuclei, become disk-shaped, and begin to produce hemoglobin. Erythrocytes have a life of around 120 ± 20 days, whereupon they degenerate and are destroyed by the spleen.³ As erythrocytes age, certain catabolic changes occur which lead to a loss of flexibility in the cell. These changes hinder the passage of the red cell through the microvasculature, which may lead to either lysis of the cell in circulation or phagocytosis in the reticulo-endothelial system (RES).¹ Once in the reticuloendothelial system, the erythrocyte is attacked by lysosomal enzymes that cause the breakage of the cellular membrane and the degradation of the haemoglobin by the heme-oxygenase enzyme.⁴ Although the greater part of the destruction of the old erythrocytes occurs in the reticulo-endothelial system, it is estimated that up to 10% of the loss of erythrocytes takes place in circulation, with this percentage increasing significantly in certain pathologies. Erythrocytes constitute potential biocompatible vectors for different bioactive substances, including drugs and proteins.⁵⁻⁷

Advantages of carrier erythrocytes

Erythrocytes feature certain advantages that enable them to be used in certain situations as an alternative drug delivery system to other widely used carrier systems, which can be summarized as follows:⁸⁻¹⁷

1. Biocompatible, particularly when autologous cells are used hence no possibility of triggered immune response.
2. Natural product of the body, which are biodegradable in nature.
3. Erythrocytes are easy to handle and alteration may be made of the delivery mechanisms.
4. Erythrocytes enable large amounts of a drug to be encapsulated in a small volume of cells, with a relatively high performance in the encapsulation.
5. Encapsulation within the erythrocytes provides the drug with a systemic clearance similar to the normal life of the erythrocytes, which enables therapeutic levels in the blood to be maintained for prolonged time spans, as well as to generate a sustained release of the active principle into the reticulo-endothelial system (RES).
6. The erythrocytes may act or be used as a reservoir for the drug, permitting a sustained release into the circulatory system.

7. The substance encapsulated within the erythrocytes is protected from premature degradation and avoids immunological reactions.
8. The drug encapsulated in the erythrocytes does not undertake its pharmacological and toxicological function until it reaches the reticulo-endothelial system (RES), which considerably diminishes the incidence of adverse effects. This is of considerable importance in the case of certain drugs with major toxicity, such as antineoplastic, aminoglycoside antibiotics, etc.
9. They permit the encapsulation of peptides of high molecular weight with significant biotechnological applications.

Disadvantages of carrier erythrocytes

The use of erythrocytes as carrier systems also presents several disadvantages, which can be summarized as follows:¹⁸⁻²³

1. The major problem encountered in the use of biodegradable materials or natural cells as drug carriers is that they are removed in vivo by the RES. This seriously limits their useful life as drug carriers and in some cases may pose toxicological problems.
2. The rapid leakage of certain encapsulated substances from the loaded erythrocytes.
3. Several molecules may alter the physiology of the erythrocyte.
4. The storage of the loaded erythrocytes is a further problem involving carrier erythrocytes for their possible use in therapeutics.
5. Liable to biological contamination due to the origin of the blood, the equipment and the environment, such as air. Rigorous controls are required accordingly for the collection and handling of the erythrocytes.

Source and isolation of erythrocytes

The cellular content is about 40-50% of the blood volume and contains erythrocytes (red blood cells, RBC), Leukocytes (white blood cells, WBC) and thrombocytes (platelets). The primarily water (90 to 92%) and protein (7%). 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 as mentioned in Table 1. To isolate erythrocytes, blood is collected in heparinized tubes by venipuncture. Fresh blood is typically used for loading purposes because the encapsulation efficiency of the erythrocytes isolated from fresh blood is higher than that of the aged blood. Freshly collected blood is immediately freed to 4°C and stored, it can be used for a period of two days. The whole blood is centrifuged at 2500 rpm for 5 min at $4 \pm 4^\circ\text{C}$ in a refrigerated centrifuge. The serum and Buffy coats are carefully removed and packed cells washed 3 times with phosphate buffer saline (PBS pH



7.4). The washed Erythrocyte are diluted with PBS and stored at 4°C until used.⁹

Table 1: Various condition and centrifugal force used for isolation of erythrocytes

Species	Washing buffer	Centrifugal force (g)
Rabbit	10mmol KH ₂ PO ₄ /NaHPO ₄	500-1000
Dog	15mmol KH ₂ PO ₄ /NaHPO ₄	500-1000
Human	154mmol NaCl	<500
Mouse	10mmol KH ₂ PO ₄ /NaHPO ₄	100-500
Cow	10-15mmol KH ₂ PO ₄ /NaHPO ₄	1000
Horse	2mmol MgCl ₂ , 10mmol glucose	1000
Sheep	10mmol KH ₂ PO ₄ /NaHPO ₄	500-1000
Pig	10mmol KH ₂ PO ₄ /NaHPO ₄	500-1000

REQUIREMENTS FOR ENCAPSULATION

Variety of biologically active substance (5000-60,000dalton) can be entrapped in erythrocytes. Non-polar molecule may be entrapped in erythrocytes in salts. Example: tetracycline HCl salt can be appreciably entrapped in bovine RBC. Generally, molecule should be polar.¹¹ Hydrophobic molecules can be entrapped in erythrocyte by absorbing over other molecules. Once encapsulated charged molecule are retained longer than uncharged molecule. The size of molecule entrapped is a significant factor when the molecule is smaller than sucrose and larger than β -galactosidase. Molecules which interact with the membrane and cause deleterious effects on membrane structure are not considered to be appropriate for encapsulation in erythrocyte.¹⁷⁻¹⁹

METHODS OF DRUG LOADING

There currently exist numerous procedures that permit the encapsulation of drugs, enzymes and peptides substances in the erythrocytes. The different methods of encapsulation existing seek an enhanced performance of the substance in the encapsulation, whilst ensuring that the erythrocytes undergo the fewest possible alterations, so that in functional terms it will as similar as possible to a normal erythrocyte. In order to achieve the encapsulation of the different molecules in erythrocytes, there are such methods as the following:

1. Hypo-osmotic lysis method:
 - a. Dilution method
 - b. Dialysis method
 - c. Preswell method
 - d. Isotonic osmotic lysis method
2. Electro-insertion/Electro encapsulation method
3. Endocytosis method
4. Membrane perturbation method
5. Lipid fusion method

1. Hypo-Osmotic Lysis Method

This method is based on capability of erythrocytes to retain their shape and morphology when placed in isotonic saline after being challenged with altered tonicity environment.⁹ The intracellular and extracellular solutes of erythrocytes are exchanged by osmotic lysis and resealing. The drug will be encapsulated within erythrocyte's membrane.¹¹

a) Dilution Method

Hypotonic dilution was the first method investigated for the encapsulation of chemicals into erythrocytes and is the simplest and fastest. In this method, a volume of packed erythrocytes is diluted with 2–20 volumes of aqueous solution of a drug. The solution tonicity is then restored by adding a hypertonic buffer. The resultant mixture is then centrifuged, the supernatant is discarded, and the pellet is washed with isotonic buffer solution. The major drawbacks of this method include a low entrapment efficiency¹¹⁻¹³ and a considerable loss of hemoglobin and other cell components^{4, 19}. This reduces the circulation half-life of the loaded cells. These cells are readily phagocytosed by RES macrophages and hence can be used for targeting RES organs. Hypotonic dilution is used for loading enzymes such as β -galactosidase and β -glucosidase¹⁸, asparaginase²⁰, and arginase²¹, as well as bronchodilators such as salbutamol²².

b) Dialysis Method

This method was first reported for encapsulation of proteins and other materials in the erythrocytes by Klibansky²³ and is modified by Jarde *et al.*²⁴ A desired hematocrit is achieved by mixing erythrocytes suspension and the drug solution. This mixture is placed into dialysis tubing and then both ends are tied with thread. The dialysis bag is inflated with an air bubble and sealed in such a way that erythrocytes suspension occupies not more than 75 % of internal volume. This bubble is critical for the procedure, in that during lysis and resealing, it traverses through the length of dialysis tubing and serves to blend the contents of the dialysis bag. The tube is placed in the bottle containing 100 ml of swelling solution. The bottle is placed at 4°C for desired lysis time. The content of dialysis tubing is mixed intermittently by shaking the tube using the tied strings. The dialysis tube is then placed in 100 ml resealing solution [isotonic phosphate buffer saline (PBS), pH 7.4] at room temperature (25-30°C) for resealing. The loaded erythrocytes thus obtained are then washed with cold PBS at 4°C. The cells are finally resuspended in PBS. The loaded cells exhibit the same circulation half-life as that of normal cells.^{6, 25} Also, this method has high entrapment efficiency in the order of 30–50%^{10, 16}, cell recovery of 70–80% and high-loading capacity²⁴. The drawbacks include a long processing time and the need for special equipment. This method has been used for loading enzymes such a β -galactosidase, glucoserebrosidase²⁷, inosito lhexaphosphatase²⁸, desferroxamine²⁹ and human recombinant erythropoietin³⁰.



c) Preswell Method

This method was first employed by Rechesteiner³¹ and was modified by Pitt *et al.*³² This method is 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 quicker and produces erythrocyte carriers having good *in-vivo* survival.¹¹ Drugs encapsulated in erythrocytes using this method include propranolol³³, asparaginase³⁴, cyclophosphamide, cortisol-21-phosphate³², methotrexate²⁷, insulin³⁵, metronidazole³⁶, Enalaprilat³⁸, isoniazide³⁹.

d) Isotonic Osmotic Lysis Method

Erythrocytes are incubated in the solution of a substance with high transerythrocytic membrane permeability. The solute will diffuse into the cells due to inwardly directed chemical potential gradient. This will be followed by water uptake until osmotic equilibrium is restored. Chemicals such as urea solution⁴⁰, polyethylene glycol⁴¹, and ammonium chloride have been used for isotonic hemolysis. This method utilizing PEG is based upon producing transient permeability in the erythrocyte membrane and this establishes equilibrium within the environment with resultant diffusion of the drugs/bioactive compounds. Incubation of the treated cells under isotonic condition can then reseal the equilibrated erythrocyte by diluting with glycol free buffer medium. This method is mainly used for small molecules⁹.

2. Electroinsertion/Electro Encapsulation Method

In 1973, Zimmermann tried an electrical pulse method to encapsulate bioactive molecules⁴². Also known as electroporation, the method is based on the observation that electrical shock brings about irreversible changes in an erythrocyte membrane. Tsong and Kinoshita⁴³ suggested the use of transient electrolysis to generate desirable membrane permeability for drug loading. The erythrocyte membrane is opened by a dielectric breakdown. Subsequently, pores can be resealed by incubation at 37°C in osmotically balanced medium. This method is based on creating electrically induced permeability changes at high membrane potential differences. The components can be entrapped when an electric pulse of greater than a threshold voltage of 2 kV/cm is applied for 20 μ s.⁴⁴ The potential difference across the membrane is built up either directly by inter- and intracellular electrodes or indirectly by applying internal electric field to the cells. The electrochemical compression of the membrane after breakdown leads to formation of pores. The extent of pore formation depends upon the electric field strength, pulse duration and ionic strength of the suspending medium.⁴⁵ 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 35%, and the life span of the resealed cells in circulation is comparable with that of normal cells. Various compounds such as sucrose,⁴⁵ urease⁴⁶ etc. can be entrapped within erythrocytes by this method.

Schrier *et al.*, described drug entrapment in erythrocyte ghosts by endocytosis. One volume of washed, packed erythrocytes is added to nine volumes of buffer containing 2.5 mM ATP, 2.5 mM MgCl₂ and 1mM CaCl₂, followed by incubation for 2 mins at room temperature. The pores created by this method are resealed by using 154 mM of NaCl and incubation at 37°C for 2 min.⁴⁷

The vesicle membrane separates endocytosed material from cytoplasm thus protecting it from the erythrocytes and vice-versa Fig. (2). The content of vesicles, however may release into erythrocytes cytoplasm depending on the nature of materials. The various compounds entrapped by this method include primaquine and related 8-amino-quinolines, vinblastine, and related phenothiazines, hydrocortisone, propranolol, tetracaine, and vitamin A.^{9, 48, 49}

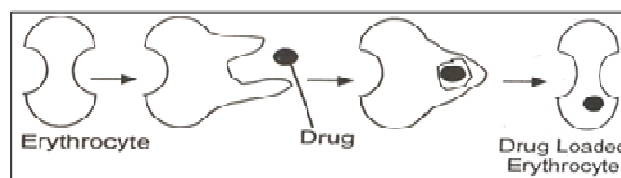


Figure 2: Entrapment by endocytosis method

3. Membrane Perturbation Method

Amphotericin B, a polyene antifungal drug, interacts with the cholesterol of the plasma membrane of eukaryotic cells causing change in permeability of the membrane to metabolites and ions.¹¹ Deuticke *et al.*⁵⁰ showed that the permeability of erythrocytic membrane increases upon exposure to Amphotericin B. Ketao and Hattori utilized this method to entrap the antineoplastic drug daunomycin in human and mouse erythrocytes.⁵¹ The *in vivo* survival of loaded erythrocytes was found to be poor as compared to that of normal erythrocytes hence this method is not very popular.

4. Lipid Fusion Method

Lipid vesicles containing a drug can be directly fused with human erythrocyte, which leads to an exchange of the entrapped drug. Nicholau and Gresonele,⁵² used this technique for loading inositol monophosphate to improve the oxygen carrying capacity of cells. However, the entrapment efficiency of this method is very low (1%).

IN-VITRO CHARACTERIZATION OF RESEALED ERYTHROCYTES

Resealed erythrocytes after loading are characterized for following parameters:^{5, 9, 11}



1. Physical Characterization

a) Shape and Surface Morphology

It decides their life span after administration. The morphological examination of these ghost erythrocytes is undertaken by comparison with untreated erythrocytes using transmission (TEM), scanning (SEM) electron microscopy or phase-contrast optical microscopy.

b) Drug Release

It is determined by Diffusion cell or dialysis.

c) Percentage Encapsulation or Drug Content

It is the percentage of amount encapsulated per amount added to RBC. The cell membrane of packed loaded erythrocytes are first deproteinized using methanol or acetonitrile and after centrifugation at 3000 rpm for a fixed time interval, the clear supernatant is assayed for drug content, radiolabeled markers or magnoresponsive markers such as magnetite.

d. Electrical Surface Potential and Surface pH

Electrical surface potential and surface pH is measured by Zeta potential measurements and pH sensitive probes respectively.

2. Cellular Characterization

a) In-Vitro Drug Release and Hemoglobin Content

In vitro release of drug(s) and hemoglobin are monitored periodically from drug loaded cells. The cell suspension (5% haematocrit in PBS) is stored at 4°C, in amber colored glass container. Periodically the supernatant are withdrawn using a hypodermic syringe, deproteinized using methanol and filtered through 0.45 µm filter. They are then estimated for drug or hemoglobin content. Another parameter used to evaluate hemoglobin disposition after the resealing, is mean corpuscular hemoglobin. It is the mean concentration of Hb per 100 ml of cells and is an index, which is independent of the red cell and therefore, it is true expression of their Hb content.

b) Percent Cell Recovery

Percent cell recovery can be determined by counting the number of intact cells per cubic mm of packed erythrocytes before and after loading the drug. Hematological analyzer and Neubaur's chamber are used for this purpose.

c) Osmotic Fragility

The osmotic fragility of resealed erythrocytes is an indicator of the possible changes in cell membrane integrity and the resistance of these cells to osmotic pressure of the suspension medium. The test is carried out by suspending cells in media of varying sodium chloride concentration and determining the haemoglobin released. In most cases, osmotic fragility of resealed cells is higher than that of the normal cells because of increased intracellular osmotic pressure.

d) Osmotic Shock

For osmotic shock study, erythrocytes suspension is diluted with distilled water and centrifuged at 3000 rpm for 15 min. The supernatant is estimated for drug and Hb spectrophotometrically.

e) Turbulence Shock

The turbulence fragility is yet another characteristic that depends upon changes in the integrity of cellular membrane and reflects resistance of loaded cells against hemolysis resulting from turbulent flow within circulation. It is determined by passing cell suspension through a 23-gauge hypodermic needle (10ml/min) and estimation of residual drug and Hb. The turbulent fragility of resealed cells is found to be higher than normal erythrocytes.

f) Erythrocyte Sedimentation Rate (ESR)

It is an estimate of the suspension stability of red blood cells in plasma and is related to the number and size of the red cells and to the relative concentration of plasma proteins. It is measured by ESR apparatus.

3. Biological Characterization

It includes Sterility testing, which is carried out by using aerobic and anaerobic cultures, pyrogenicity testing by rabbit fever response test or limulus amoebocyte lysate (LAL) test and Animal toxicity testing.

ROUTES OF ADMINISTRATION

The carrier cells are injected mainly intravenously or intraarterially, however, intraperitoneal and subcutaneous routes can also be utilized.^{5,11}

Release mechanisms

The various mechanisms proposed for drug release include:^{5,6,11,15,53}

- Passive diffusion
- Specialized membrane associated carrier transport
- Phagocytosis of resealed cells with senescent antigen by macrophages of RES, subsequent accumulation of drug into the macrophage interior, followed by slow release.
- Accumulation of erythrocytes in lymph nodes upon subcutaneous administration followed by hemolysis to release the drug.

IN-VITRO STORAGE

The major challenge in successful utilization of resealed erythrocytes as a drug delivery system is the availability of reliable and practical storage method. The most common storage media includes Hank's balanced salt solution^{8,10} and acid-citrate-dextrose²⁵ at 4°C. Cells remain viable in terms of their physiologic and carrier characteristics for at least 2 weeks at this temperature. The addition of calcium-chelating agents or the purine nucleosides³³ improve circulation survival time of cells upon reinjection.



Exposure of resealed erythrocytes to membrane stabilizing agents such as dimethyl sulfoxide, dimethyl-3,3-dithio-bispropionamide, glutaraldehyde, toluene-2,4-diisocyanate followed by lyophilization or sintered glass filtration has been reported to enhance their stability upon storage.^{6,22,36,53} The resultant powder was stable for at least one month without any detectable changes. But the major disadvantage of this method is the presence of appreciable amount of membrane stabilizers in bound form that remarkably reduces circulation survival time. Other reported methods for improving storage stability include encapsulation of a prodrug that undergoes conversion to the parent drug only at body temperature⁵³ and reversible immobilization in alginate or gelatin gels^{6,8}.

Therapeutic applications of carrier red cells

The potential use of red blood cells as a carrier system for the transport and delivery of pharmacological substances (drugs, enzymes, nucleic acids, genes) is well documented.

A. Erythrocytes as drug carriers

Erythrocytes may be used as drug-carriers for a wide range of drugs, such as antineoplastic, antiviral drugs, etc.

1. Antineoplastic drugs

Antineoplastic constitute the group of drugs involving the greatest number of studies both in vitro and in vivo using erythrocytes as carriers. The reticulo-endothelial system is the main site for the destruction of abnormal or old erythrocytes. In order to improve the recognition of these cells as carriers, many authors have subjected the loaded erythrocytes to treatments that imply some form of structural change that renders them more readily recognisable by the RES, basically by such organs as the liver and spleen, in a very short period of time. On this basis, the erythrocytes can be used as carriers of drugs to direct them selectively towards these organs, for example in the treatment of hepatic tumours.⁵⁴

Actinomycin D

One of the first instances of research with antineoplastic encapsulated in erythrocytes to direct them selectively to the reticuloendothelial system involved actinomycin D. Using a method of hypotonic exchange-loading reaction, a high yield encapsulation of actinomycin D has been achieved in packed human erythrocytes. The actinomycin D encapsulated in erythrocytes undergoes rapid leakage at 37°C in an isotonic buffer. The rapid leakage of actinomycin D at 37°C occurs through a diffusion process dependent on temperature.⁵⁵

Methotrexate

The pharmacological efficacy of methotrexate-loaded erythrocyte in treating mice bearing hepatoma ascites tumours was demonstrated in increases in average survival time. Numerous methods have been assessed to improve vectorisation of these carriers towards the RES. One of these is the exposure of the carrier erythrocytes to

agents that stabilise the membrane, basically glutaraldehyde, which acts by reducing the deformability of the erythrocytes. The glutaraldehyde treatment of erythrocytes results in the cross-linking of the membrane proteins⁵⁶ and is also reported to target them to the reticulo-endothelial system⁵⁴.

Adriamycin

The encapsulation of Adriamycin (doxorubicin) in human autologous erythrocytes has revealed a limited biotransformation of the drug at intracellular level, a slow release of the unmodified molecule to the medium and an absence of significant erythrocyte damage. The encapsulation in erythrocytes would permit a slow release that would increase its anti-tumour activity and reduce its side effects, largely cardiotoxicity.⁵⁷

2. Angiotensin-converting enzyme (ACE) inhibitors

Enalaprilat

Enalaprilat is an angiotensin converting enzyme (ACE) inhibitor, widely used as its esterified oral absorbable prodrug, enalapril, in the management of hypertension and congestive heart failure. Human loaded erythrocytes with enalaprilat using a hypotonic pre-swelling method release the drug in vitro according to zero-order kinetics. In a study involving rabbits, the area under the inhibition curve (ACE) versus time over the entire course of study, a measurement parameter of the extent of the inhibition within the sampled period of time, was significantly greater following the administration of the drug encapsulated in erythrocytes. Furthermore, the inter-individual variability in the values of this parameter was smaller, indicating a more reproducible behaviour.³⁸

3. Anti-infective agents

Gentamicin

The aminoglycoside antibiotic gentamicin has been studied in vivo encapsulated in human erythrocytes as a selective carrier system to the reticulo-endothelial system and as a potential slow release carrier. The antibiotic, given its nature of polar drug, remains inside the cells until their lysis and this property has been used for the kinetic study of the erythrocytes through the evolution of the levels of the drug. The use of anti-Rh antibody (IgG anti-D)-coated autologous human erythrocytes loaded with gentamicin makes the carrier erythrocytes more recognisable for the macrophages, which permits their uptake by the reticulo-endothelial system to be increased. It has also been shown that the injection volume is a basic parameter, with the use of small volumes being more efficient.⁵⁸

Primaquine

The glutaraldehyde treated erythrocytes appear to be promising carriers of the anti-malarial drug primaquine for its hepatic location and suggest the possible use of the drug as release system in intravenous administration for the prophylaxis and eradication of malaria.



Glutaraldehyde treatment stabilized the cells that are noted to be resistant to osmotic and turbulence shocks. In vitro release of drug and haemoglobin was also retarded upon treatment of loaded erythrocytes with glutaraldehyde.⁵⁹

4. Iron chelators

Carrier erythrocytes for the administration of desferrioxamine and other iron chelators may be useful for improving iron chelation efficiency. The use has been studied of iron chelation by desferrioxamine encapsulated in erythrocytes for the treatment of iron accumulation in patients with thalasemia and other forms of anemia that require regular transfusions. The RES is the main site of destruction of old erythrocytes and, consequently, of iron over-accumulation in these patients. Desferrioxamine is the most widely used chelator. However, the primary and major iron repositories in transfusional siderosis are reticulo-endothelial cells, and iron in these cells does not appear to be directly accessible to desferrioxamine. Accordingly, one alternative is encapsulation in erythrocytes to be able to release the desferrioxamine in the sites storing iron in the RES.⁶⁰

B. Erythrocytes as enzyme carriers

One of the more widely studied applications has been the encapsulation of enzymes in the erythrocytes. Enzymatic therapeutics is extensively used to redress complaints associated with enzymatic deficiencies (Gaucher's disease, galactosemia, hyperuricemia) breakdown of toxic compounds in intoxications or for the treatment of specific illnesses.

Alcohol dehydrogenase (ADH) and aldehyde dehydrogenase (AIDH)

The encapsulation in erythrocytes of the alcohol dehydrogenase (ADH) and the aldehyde dehydrogenase (AIDH) reduces excessively high levels of alcohol and acetaldehyde in blood due to chronic alcoholism or to genetic causes, through the release of these enzymes into the blood circulation following hemolysis.⁶¹ One of the more important paths for the metabolism of alcohol is its oxidation to acetaldehyde by the alcohol dehydrogenase and subsequently to acetate by means of aldehyde dehydrogenase. The acetaldehyde only accumulates in toxicologically significant amounts following the ingestion of ethanol.

Pegademase

Pegademase (polyethylene glycol-conjugated adenosine deaminase) is a modified enzyme used for enzyme replacement therapy in the treatment of severe combined immunodeficiency disease (SCID) associated with a deficiency of adenosine deaminase. The studies in humans using pegademase encapsulated in erythrocytes reveal a significant increase in the half life of this enzyme, whilst maintaining therapeutic blood levels, providing an

interesting outlook for the treatment of this enzymatic deficiency.⁶²

C. Erythrocytes as carriers of peptides and proteins

Erythrocytes are also potential vectors for releasing peptides, modified oligonucleotides and even genes, and direct them selectively to their site of action.¹¹

1. Antineoplastic peptides

2-Fluoro-ara-AMP (fludarabine) is a fluorinated analogue of adenine that is useful in the treatment of chronic lymphocytic leukaemia. The encapsulation of fludarabine in human erythrocytes in vitro permits a slow release that is prolonged for several days, whereby it is believed that it may be useful as a system for the slow release in the treatment of malignant lymphomas with fludarabine. 5-Fluoro-2'-deoxyuridine 5'-monophosphate (FdUMP) encapsulated in human erythrocytes may be used as bioreactors designed for time-programmed and liver-targeted delivery of the antineoplastic drug, 5-fluoro-2V-deoxyuridine (FdUrd).⁶³

2. Erythropoietin

Recombinant human erythropoietin is used in the treatment of certain serious forms of anemia linked to chronic IR, neoplasias, etc. In vitro studies have been performed using recombinant human erythropoietin encapsulated in human and mice erythrocytes to achieve greater stability and a slow release of the erythropoietin. Pharmacokinetic studies and those on biological activity in mice by administering erythropoietin encapsulated in erythrocytes by intravenous means reveal changes in the lower area of the curve of the protein when it is administered encapsulated.⁶⁴

3. Heparin

A study has been made of the possible use of heparin encapsulated in human and canine erythrocytes for the prevention of thromboembolism. Heparin has been encapsulated in vitro in human and canine erythrocytes through a hypo-osmotic method of dialysis. The in vivo pharmacokinetic behaviour of heparin carrier erythrocytes was biphasic with a prolonged half-life.⁶⁵

D. Magnetic resealed erythrocytes

Ferrofluid-containing red blood cells are the closest to the native components of organism and appear promising as potential drug carrier systems. Magnetically responsive ibuprofen-loaded erythrocytes were prepared and characterized *in-vitro*. The erythrocytes loaded with ibuprofen and magnetite (Ferro fluids) using the preswell technique. The loaded cell effectively responded to an external magnetic field. The loaded erythrocytes were characterized for in vitro drug efflux, haemoglobin release, morphology osmotic fragility, in vitro magnetic responsiveness and percent cell recovery. Local thrombosis in animal arteries was prevented by means of magnetic targeting of aspirin loaded red cell.⁶⁶



PRODUCT PIPELINE

- Graspera is a new medicinal product consisting in L-Asparaginase entrapped into human homologous red blood cells.
- Asparagine is a tumour growth factor. Tumour cells are unable to produce their own asparagine and depend of its presence in patient's serum. Asparaginase antitumoral activity is based on the degradation of circulating asparagine.⁶⁷
- Asparaginase enzyme is efficient and is part of numerous treatment protocols. It is used since decades in US, UK, Europe and Asian protocols for patients with Acute Lymphoblastic Leukemia (ALL).
- ALL is the first childhood cancer. It accounts for 80% of acute leukemias of childhood, with most cases occurring between ages 3 and 7. ALL also occurs in adults, where it accounts for 20% of all adult leukemias.
- L-asparaginase should be use more intensively and introduce in other indications but its administration is associated with high immunogenicity and great side effects such as liver toxicity and coagulation disorders.

➤ **Graspera in ALL.**⁶⁸

Graspera, an innovative form of L-Asparaginase, strongly reduces main side effects, and clearly fits with physicians expectations.

ERYtech Pharma has a first success with this lead compound which is currently in Phase III in ALL in Europe. Main results from Phase II clinical trials show that:

- Graspera achieved efficient asparagine depletion, longer than with other asparaginase formulations and with only one single injection.
- Better safety profile with great reduction in the occurrence of allergic reactions.

CONCLUSION

Human erythrocytes can provide an efficient way of delivering conventional and new drugs to selected target cells, in particular macrophages. The procedure has been validated by a number of in vitro studies, in animal models and recently in humans. The method can be easily adapted to deliver different amounts of the compound of interest and with different kinetics. The delivery of recombinant peptides and modified oligonucleotides suggest that the modulation of gene expression is possible by interfering with the activation of specific nuclear factors or with the selected mRNA. For the present, it is concluded that erythrocyte carriers are "golden eggs in novel drug delivery systems" considering their tremendous potential.

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