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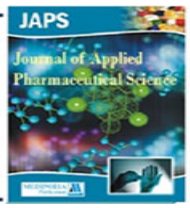

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Erythrocytes as Carrier for Drugs, Enzymes and Peptides

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ABSTRACT

Erythrocytes are potential biocompatible carriers for different drugs, peptide molecules and different enzymes. Now a day the method that is used for encapsulation of pharmaceuticals into the erythrocytes mainly based on the hypo-osmotic dialysis. Encapsulation of these drugs or enzymes or peptides into erythrocytes significantly changes the pharmacokinetic properties of drugs in both animals and humans, enhancing liver and spleen uptake and targeting the reticulo-endothelial system. By the encapsulation of these into erythrocytes can be applied as targeted drug delivery systems. Erythrocytes are successful as carrier systems for different drugs, enzymes and peptide molecules. The result after using drug encapsulated in erythrocytes is more compared to the free form of the drug.

Keywords: Drug loading methods; carrier erythrocytes; Applications; Drugs; Enzymes; Peptides

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INTRODUCTION

Erythrocytes or red blood cells constitute the largest population of blood cells and are the main carriers of oxygen to the body cells and tissues. Erythrocytes contain high concentrations of iron-rich haemoglobin; they can be easily isolated through centrifugation. In the human body, the mature form is normally a non-nucleated, yellowish, biconcave disk with a central pallor. The biconcave shape provides a large surface-to-volume ratio for oxygen delivery and better flexibility in narrow capillaries. The average content of erythrocytes in men and women is 5.4-106 and 4.8-106 per Al, respectively. The number of erythrocytes and their content in haemoglobin constitutes a determining factor in the supply of oxygen. As they mature, the erythrocytes lose their nuclei, become disk shaped, and begin to produce haemoglobin. Erythrocytes have a life of around 120 days, later they degenerate and are destroyed by the spleen. Although, potentially, all the cells of the reticule-endothelial system can destroy the erythrocytes, those in the spleen are more capable of detecting abnormalities in the red cell. Once in the reticulo-endothelial 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. 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 (Gutierrez Milan *et al.*, 2004). Erythrocytes constitute potential biocompatible vectors for different bioactive substances, including drugs, enzymes and proteins.

ERYTHROCYTES MAY BE EMPLOYED FOR TWO MAIN PURPOSES

- To act as a reservoir for the drug, providing the sustained release of the drug into the body.
- To selectively direct the drugs to the reticuloendothelial system of the liver, spleen and bone marrow, which constitute the usual sites for the destruction of erythrocytes.

ADVANTAGES AND DISADVANTAGES OF CARRIER ERYTHROCYTE

Advantages

- A remarkable degree of biocompatibility, particularly when the autologous cells are used for drug loading, and complete biodegradability and the lack of toxic product(s) resulting from the carrier biodegradation.
- The drug encapsulated in the erythrocytes does not show its pharmacological and toxicological function until it reaches the reticulo-endothelial system, which considerably diminishes the incidence of adverse effects. This is of considerable importance in the case of certain drugs with major toxicity, such as antineoplastics, amino glycoside antibiotics, etc. (Hamidi *et al.*, 2007).
- Erythrocytes are easy to handle and alteration may be made of the delivery mechanisms.
- Avoidance of any undesired immune responses against the encapsulated drug.
- Considerable protection of the organism against the toxic effects of the encapsulated drug, e.g., antineoplasms.
- Remarkably longer life-span of the carrier erythrocytes in circulation in comparison to the synthetic carriers. In the optimum condition of the loading procedure, the life-span of the resulting carrier cells may be comparable to that of the normal erythrocytes.
- 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.
- The substance encapsulated within the erythrocytes is protected from premature degradation and avoids immunological reactions.
- An easily controllable life-span within a wide range from minutes to months.
- Desirable size range and the considerably uniform size and shape.
- Protection of the loaded compound from inactivation by the endogenous factors.
- Possibility of targeted drug delivery to the reticulo-endothelial system organs.
- Relatively inert intracellular environment.
- Possibility of ideal zero-order kinetics of drug release.
- Wide variety of compounds with the capability of being entrapped within the erythrocytes.
- Possibility of loading a relatively high amount of drug in a small volume of erythrocytes.
- Modification of the pharmacokinetic and pharmacodynamic parameters of the drug.
- Considerable increase in drug dosing intervals with drug concentration in the safe and effective level for a relatively long time.
- Possibility of decreasing drug side effects.
- They permit the encapsulation of peptides of high molecular weight with significant biotechnological applications.

Disadvantages

The use of erythrocytes as carrier systems alsohas several disadvantages, they are as follows;

- 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 reticulo-endothelial cells. This seriously limits their useful life as drug carriers and in some cases may pose toxicological problems.
- The rapid leakage of certain encapsulated substances from the loaded erythrocytes.
- Several molecules may alter the physiology of the erythrocyte.
- 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.
- 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 immobilisation.
- 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

Different mammalians human, monkey, horse, sheep, goat, rabbit etc., are used for the collection of erythrocytes. 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 gm for 5 min at 4 ±1 0C. This helps in separation of plasma and Buffy coat. Erythrocytes so

obtained are washed with buffer solution. These erythrocytes are often stored in acid-citrate-dextrose buffer at 4 ^{0}C up to 48 hrs. Prior to use

METHODS OF DRUG LOADING

Several 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).

Hypotonic haemolysis

Hypotonic haemolysis is based on reversible swelling in a hypotonic solution. An increase in cell volume its shape changed from biconcave to the spherical. This change is leads to the absence of superfluous membrane; hence the surface area of the cell is fixed. The cells assume a spherical shape to accommodate additional volume while keeping the surface area constant. The volume gain is ~25–50%. The cells can maintain their integrity up to a tonicity of ~150 mosm/kg, above which the membrane ruptures, releasing the cellular contents. At this point (just before cell lysis), some transient pores of 200–500 A^{0}are generated on the membrane (Alvarez *et al.*, 1998). After cell lysis, cellular contents are released. The remnant is called an erythrocyte ghost. The principle of using these ruptured erythrocytes as drug carriers is based on the fact that ruptured membranes can be resealed by restoring isotonic conditions. Upon incubation, the cells resume their original biconcave shape and recover original impermeability.

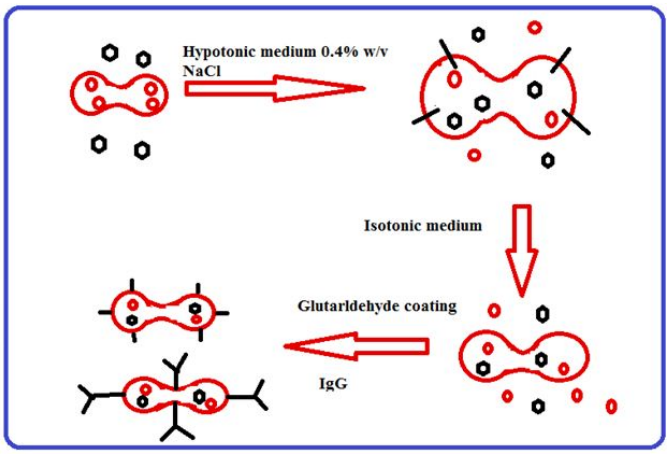

Fig. 1: Schematic diagram of hypotonic haemolysis and isotonic resealing method of loading erythrocytes.

Hypotonic dilution

Hypotonic dilution was the first method investigated for the encapsulation of chemicals into erythrocytes and is the simple and fast. 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 low entrapment efficiency and a considerable loss of haemoglobin and other cell components (Tajerzadeh *et al.*, 2000). This reduces the circulation half life of the loaded cells. These cells are readily phagocytised by RES macrophages and hence can be used for targeting RES organs. Hypotonic dilution is used for loading enzymes such as ß galactosidase and ß glycosidase, asparginase, and arginase, as well as bronchodilators such as salbutamol.

Hypotonic preswelling

L portions of an aqueous solution of the drug to be encapsulated. The mixture is centrifuged between the drug-addition steps. The lysis point is detected by the disappearance of a distinct boundary between the cell fraction and the supernatant upon centrifugation. The tonicity of a cell mixture is restored at the lysis point by adding a calculated amount of hypertonic buffer. Then, the cell suspension is incubated at 37^0 C to re anneal there sealed erythrocytes. Such cells have a circulation half life comparable to that of normal cells. This method is simpler and faster than other methods, causing minimum damage to cells. Drugs encapsulated in erythrocytes using this method include propranolol, methotrexate, insulin, metronidazole, levothyroxine, elaprnailat, and isoniazid (Alpar *et al.*, 1987). The technique is based upon initial controlled swelling in a hypotonic buffered solution. This mixture is centrifuged and the supernatant is discarded and the cell fraction is brought to the lysis point by adding 100–120.

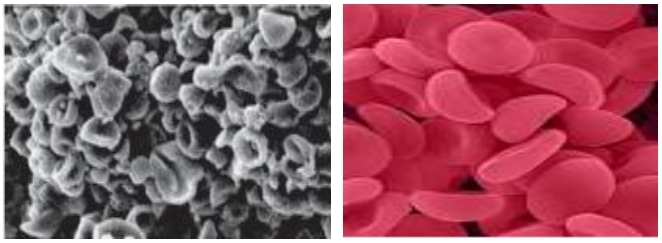

Fig. 2: SEM photograph of drug-encapsulated erythrocytes prepared by hypotonic pre swelling method.

Hypotonic dialysis

Several methods are based on the principle that semi permeable dialysis membrane maximizes the intra cellular: extracellular volume ratio for macromolecules during lysis and resealing. In the process, an isotonic, buffered suspension of erythrocytes with a hematocrit value of 70–80 is prepared and placed in a conventional dialysis tube immersed in 10–20 volumes of a hypotonic buffer. The medium is agitated slowly for 2h (Hamidi *et al.*, 2003). The tonicity of the dialysis tube is restored by directly adding a calculated amount of hypertonic buffer to the surrounding medium or by replacing the surrounding medium by isotonic buffer. The drug to be loaded can be added by either dissolving the drug in isotonic cell suspending buffer inside a dialysis bag at the beginning of the experiment or by adding the drug to a dialysis bag after the stirring is complete. In this method, the erythrocyte suspension and the drug to be loaded were placed in the blood compartment and the hypotonic buffer was placed in a receptor compartment. This led to the concept of “continuous flow dialysis,” which has been used by several other researchers. This method has been used for loading enzymes such as ß galactosidase,

glucose rebrosidase, asparginase, inositol hexa phosphatase, as well as drugs such as gentamicin, Adriamycin, pentamidine and furamycin, interlukin-2, desferroxamine, and human recombinant erythropoietin .

Isotonic osmotic lysis

This method, also known as the osmotic pulse method, involves isotonic haemolysis that is achieved by physical or chemical means. The isotonic solutions may or may not be iso ionic. If erythrocytes are incubated in solutions of a substance with high membrane permeability, the solute will diffuse into the cells because of the concentration gradient. This process is followed by an influx of water to maintain osmotic equilibrium. Chemicals such as urea solution, polyethylene glycol, and ammonium chloride have been used for isotonic haemolysis. However, this method also is not immune to changes in membrane structure composition (Jaitely *et al.*, 1996). The method that involved suspending erythrocytes in an isotonic solution of dimethyl sulfoxide was developed. The suspension was diluted with an isotonic-buffered drug solution. After the cells were separated, they were resealed at 37^0C.

Chemical perturbation of the membrane

This method is based on the increase in membrane permeability of erythrocytes when the cells are exposed to certain chemicals. The permeability of erythrocytic membrane increases upon exposure to polyene antibiotic such as amphotericin B. This method was used successfully to entrap the antineoplastic drug daunomycin in human and mouse erythrocytes. Halothane was also used for the same purpose .

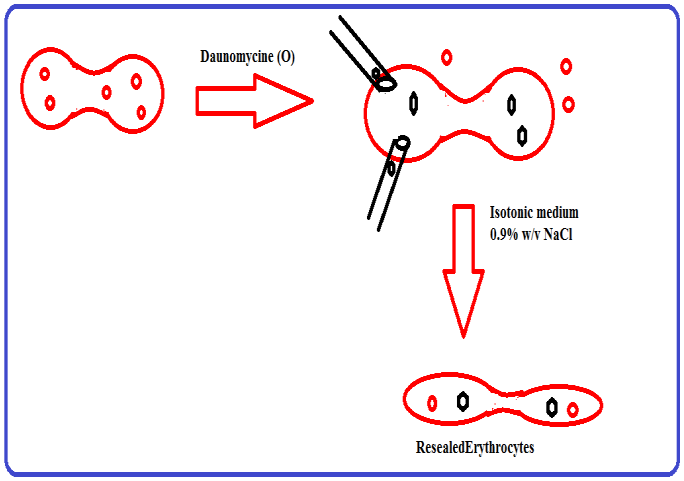

Fig.3: Schematic diagram of loading by chemical perturbation of Membrane.

Electro-insertion or electro encapsulation

The method is based on the observation that electrical shock brings about irreversible changes in an erythrocyte membrane. The erythrocyte membrane is opened by a dielectric breakdown. Subsequently, the pores can be resealed by incubation at 37 ^{o}s. An inverse relationship exists between the electric-field intensity and the discharge time (Li *et al.*, 1996). The compound to be entrapped is added to the medium in which the cells are suspended. 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 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 20–160m can been trapped within erythrocytes by this method. The main drawbacks are the need for special instrumentation and the sophistication of the process. Entrapment efficiency of this method is 35%. Various compounds such as sucrose urease, methotrexate, isoniazid, DNA fragments, and latex particles of diameter 0.2 can be encapsulated by this method.

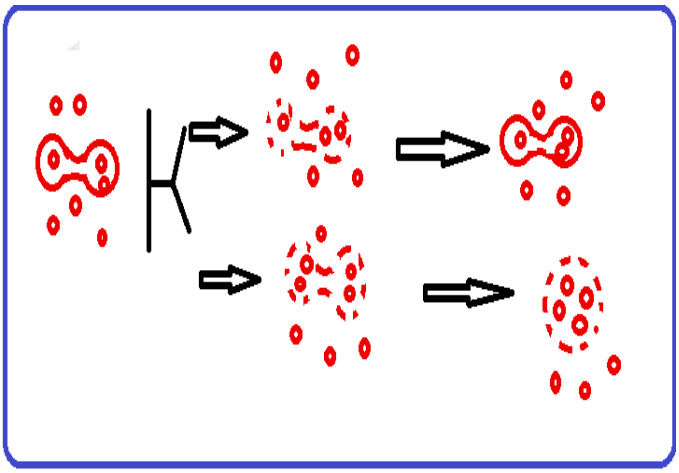

Fig. 4: Schematic diagram of electro–encapsulation.

Entrapment by endocytosis

Endocytosis involves the addition of one volume of washed erythrocytes to nine volumes of buffer containing 2.5 mM ATP, 2.5 mM$MgCl_2$, and 1mM $CaCl_2$, 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 37^0C for 2 min (Alvarez-Guerra *et al.*, 1998). 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 entrapped by this method include primaquine and related 8–amino–quinolines, vinblastine, chlorpromazine and related phenothiazines, hydrocortisone, propranolol, tetracaine, and vitamin A .

ENHANCINGTHE MACROPHAGE UPTAKE OF LOADEDERYTHROCYTES

The treatment of the carrier erythrocytes with certain substances gives alterations in the properties of the loaded erythrocytes to a greater receptiveness of the reticulo-endothelial system in the macrophages. The treatment of loaded erythrocytes with glutaraldehyde enhances its properties as a carrier system, given that it has been observed that the erythrocytes treated in this

way are more stable, which increases their osmotic resistance, as well as their resistance to turbulences. It also means that the output of the encapsulated substance from these erythrocytes into the circulatory flow is reduced. The treatment with glutaraldehyde increases the selectivity of the erythrocytes towards the resealed erythrocytes and specifically, towards certain organs such as the liver and the spleen (Gasparini *et al.,* 1992). The chemical alteration of the erythrocyte membrane with such substances as ascorbate, $Fe^{(2)}$, diamide or band 3-cross-linking reagents can induce increased phagocytosis of modified red cells by macrophages. The surface modification of erythrocytes has also been addressed using trypsin, phenyl hydrazine and N-hydroxysuccinimide ester of biotin, which increase the macrophage uptake of loaded erythrocytes both in vitro as *in vivo*.(Mishra *et al.,* and 2000).

IN-VITRO PROPERTIES OF LOADED ERYTHROCYTES

Cell counting and cell recovery

This involves counting the number of red blood cells per unit volume of whole blood, by automated counting. Red cell recovery may be calculated on the basis of the differences in the hematocrit and the volume of the suspension of erythrocytes before and after loading. The goal is to minimize the loss during the encapsulation procedure to maximize cell recovery.

Morphological aspect

The morphological examination of these ghost erythrocytes is undertaken by comparison with untreated erythrocytes using either transmission or scanning electron microscopy. By means of electron microscopy observation may be made of the morphological changes in the erythrocytes induced by osmosis-based encapsulation methods, when they are subjected to solutions of different osmolality (Luqueet *et al.,* 1992). Thus, when rat erythrocytes are subjected to isotonic solutions (300 mos (M/kg) they reveal the typical morphology of discocyte (biconcave). This evolves to a morphology of stomatocyte (uniconcave) when they are subjected to solutions of 200 mos (M/kg), attaining the spherocytic shape (the most fragile of the three) when the solution is of 150 mos (M/kg).

Turbulence shock

Turbulence shock enables an evaluation to be made of the stability of the loaded erythrocytes against the turbulence stress exerted by the cells against *in vivo* circulation turbulence. The test is performed by the method of whereby the suspension of cells is passed several times through a 22-gauge needle.

In-vitro drug or peptide release

The encapsulation of many drugs in erythrocytes can give rise to a sustained release of the drug that influences the pharmacokinetic behaviour *in vivo* of the loaded erythrocytes. *In vitro* leakage of the drug from loaded erythrocytes is tested using autologous plasma or an isoosmotic buffer at 37j with a hematocritadjusted between 0.5% and 50%. The supernatant is removed at the time intervals previously programmed and replaced by an equal volume of autologous plasma or buffer (Hamidi *et al.,* 2003). The molecular weight and lip solubility of the substance constitute two factors that have a decisive bearing on the release profile of the active principle from the loaded erythrocytes. Liposoluble drugs may be released from the red cells by a mechanism of passive diffusion. Other drugs may become attached to cell structures and are not released by the diffusion mechanism, requiring them lysis of the cell. Band 3 and glycophorinan are proteins present in high density on the extra-cellular surface of erythrocytes and which may act as potential targets for anchoring via covalent bond formation with different substances. Band 3 plays an important role as a carrier protein for anions.

Haemoglobin release

The content of haemoglobin of the erythrocytes may be diminished by the alterations in the permeability of the membrane of the red cells during the encapsulation procedure (Hamidi *et al.,* 2003). Furthermore, the relationship between the rate of haemoglobin and the rate of drug release contributes to interpreting the mechanisms involved in the release of the substance encapsulated from the erythrocytes. The haemoglobin leakage is tested using a red cell suspension by recording the absorbance of supernatant at 540 nm on a spectrophotometer.

In vitro stability

The stability of the loaded erythrocytes is assessed by means of the incubation of the cells in autologous plasma or in an iso-osmotic buffer, setting the hematocrit between 0.5% and 5% at temperatures of 4 and 37^0C.

PHARMACOKINETICS OF DRUGS OR PEPTIDES ADMINISTERED IN LOADED ERYTHROCYTES

Erythrocytes loaded with drugs and other substances have different release rates. *In vitro* studies on release performed with erythrocytes from various animal species loaded with different kinds of substances habitually reveal a slow release of the encapsulated substance. The *in-vitro* release of substances from loaded erythrocytes responds to a first-order kinetic process, revealing that the substance permeates the erythrocyte membrane by passive diffusion. However, human carrier erythrocytes containing enalaprilat have *in-vitro*, zero-order release kinetics (Lizanoet *et al.,* 2001). The treatment of loaded erythrocytes with glutaraldehyde and other substances produces a more delayed *in vitro* release of both the encapsulated substance and the haemoglobin from the loaded erythrocytes. The employment of prodrugs encapsulated in erythrocytes permits the use of the red cell as a bioreactor that controls the release rate of the drug. When the loaded erythrocytes are administered *in vivo,* circulating cells used as drug carriers may alter the pharmacokinetics of administered drugs. Encapsulation within erythrocytes affords the drug a clearance that depends on the biological half-life of the erythrocyte, allowing therapeutic levels to be maintained in the blood for long periods of time, together with the generation of a

sustained release of the therapeutic agent (Akrantz 1997). *In vivo* survival of human carrier erythrocytes labelled with 51Cr demonstrates a mean cell life and cell half-life of 89 to 131 days and 19 to 29 days, respectively. These changes in the pharmacokinetics when carrier erythrocytes are used involve the prolonged serum half-life of the encapsulated substance in comparison to the free substance, an increase in the area under the curve of serum concentrations and a greater accumulation of the drug in the liver and in the spleen (Alvarez *et al.*, 1996). Enhanced liver targeting *in vivo* of loaded erythrocytes may be achieved by means of surface treatment with glutaraldehyde and other substances.

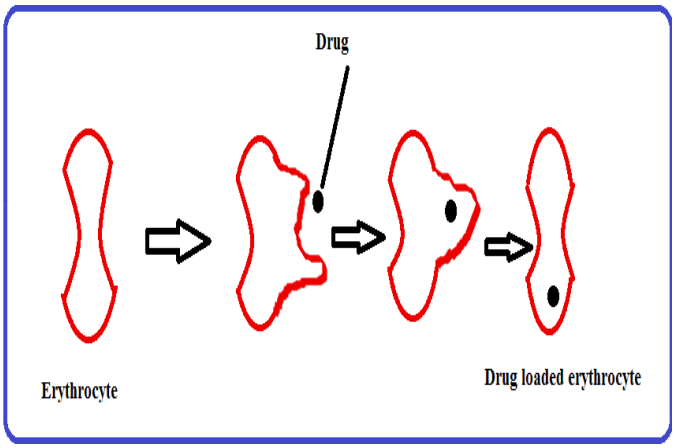

Fig. 5: Erythrocyte endocytosis produced by cations and trapping of molecules in the invagination or inside out endocytic vacuoles.

THERAPEUTIC APPLICATIONS OF CARRIER RED BLOOD CELLS

The potential use of red blood cells as a carrier system for the transport and delivery of pharmacological (drugs, enzymes, nucleic acids, genes) is well documented. One potential application is the delivery of these substances to cells responsible for or capable of erythrophagocytosis, which are located primarily in the liver and the spleen. A second potential application depends on the ability of loaded cells to survive for substantial periods of time in the circulation after reinfusion.

ERYTHROCYTES AS DRUG CARRIERS

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

Antineoplastic drugs

Antineoplasticsconstitute 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. 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 resealed erythrocytes, 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.

Actinomycin D

One of the first instances of research with antineoplastics encapsulated in erythrocytes to direct them selectively to the reticuloendothelialsystem 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 actinomycinD encapsulated in erythrocytes undergoes rapid leakage at 37 jC in an isotonic buffer. The rapid leakage of actinomycin D at 37 jC occurs through a diffusion process dependent on temperature (Lynch *et al.*, 1980). However, if the encapsulation of the actinomycinD-DNA is performed, the complex is retained in the cells for longer periods, with up to 50% of the encapsulated drug remaining attached to the membrane of the red cell.

Methotrexate

The pharmacological efficacy of methotrexate-loaded erythrocyte in treating mice bearing hepatoma ascites tumours was demonstrated in increases in average survival time. 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 proteins and is also reported to target them to the reticulo-endothelial system (Mishra *et al.*, 2002). The treated cells are rapidly cleared from circulation by liver and spleen recognition and are well tolerated by the liver. The glutaraldehyde treatment of methotrexate canine loaded erythrocytes selectively targeted the drug to the liver. Surface modified erythrocytes containing methotrexate by attachment with N-hydroxy succinimide ester of biotin is a new approach for liver specific delivery systems.

Daunomycin

The entrapment of daunomycin in erythrocytes was performed by means of treating the erythrocytes with amphotericin B, which is a polyene that binds to cholesterol in the plasma membrane of eukaryotic cells and perforates cell membranes (Kitao *et al.*, 1980). The erythrocytes with entrapped daunomycin may act as a time-release system so that cells are exposed to the drug as they enter DNA synthesis.

Etoposide

Etoposide is an inhibitor of the topoisomerase revealing a toxic action on tumour cells of macrophage origin. The encapsulation of etoposide in mouse erythrocytes by hypotonic dialysis a greater uptake of etoposide by the macrophages mainly by a process of phagocytises (Lotero *et al.*, 2003). The toxic effect, determined by the fragmentation of DNA in macrophages, is

greater with encapsulated etoposide than that shown by the free drug.

Carboplatin

The encapsulation of carboplatin has been studied in human erythrocytes *in vitro*. The incubation of the erythrocytes loaded in autologous plasma caused a very slow release of the drug from the cells. It has been suggested that haemoglobin and, specifically, divalent iron play a key role in this conversion (Tonetti *et al.,* 1992). The encapsulation of carboplatin in selectively targeted human erythrocytes may represent a therapeutic strategy for increasing the drug concentration in target organs, such as the liver.

Angiotensin-converting enzyme inhibitors

Enalaprilat

Enalaprilat is an angiotensinconverting enzyme inhibitor, widely used in the treatment of hypertension and congestive heart failure (Hamidi *et al.,* 2001). Human loaded erythrocytes with enalaprilatusing a hypotonic pre-swelling method release the drug in vitro according to zero-order kinetics.

Anti-infective agents

Gentamicin

The amino glycoside antibiotic gentamicin has been studied *in vivo* encapsulated in human erythrocytes as a selective carrier system to the reticulo-endothelial system (Eichler *et al.,* 1986) and as a potential slow release carrier

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 (Talwar *et al*., 1992). *In vitro* release of drug and haemoglobin was also retarded upon treatment of loaded erythrocytes with glutaraldehyde.

Metronidazole

Tests have also been performed involving erythrocytes treated with glutaraldehyde. As slow-release systems with other anti-parasite drugs, such as metronidazole (DeLoach 1985). The results of a study of release *in vitro* predict a sustained and targeted release following its possible administration *in vivo*.

Imizol

Babesiosis is an intra erythrocytic parasitic infection caused by protozoa of the genus Babesia. Erythrocytes have been used as slow release carriers for the delivery of anti-babesia agents for veterinary use (DeLoach *et al.,* 1989). Imizole was encapsulated in murine carrier erythrocytes and injected intraperitoneally in mice, resulting in higher drug levels than in blood in animals that had received the free drug and in a decrease in parasitemia.

Systemic corticosteroids

Dexamethasone

Loaded erythrocytes containing dexamethasone have been used *in vivo* in rabbits and humans. Dexamethasone entrapped within rabbit erythrocytes was slowly released from the loaded cells *in vivo*. The encapsulated drug had a much longer half-life than when free drug was administered intravenously. The rabbits were protected from inflammation caused by histamine for approximately 2 to 5 days after administration. The anti-inflammatory effect agreed reasonably well with the in vivo survival of loaded erythrocytes. Encapsulated dexamethasone 21-phosphate administered in patients with chronic obstructive pulmonary disease maintained detectable dexamethasone concentrations in blood for up to 7 days. Dexamethasone 21-phosphate loaded erythrocytes acted as circulating bioreactors, converting the non-diffusible drug into the diffusible dexamethasone (Rossi *et al.,* 2001). Erythrocytes are promising carriers for corticosteroid analogues and are an alternative to oral or inhaled drugs in patients with chronic obstructive pulmonary disease .

Antioxidant drugs

Encapsulation has also been made of action complexes such as copper (II) complexes in human erythrocytes to assess their possible use as anti-oxidant drugs. Encapsulation *in vitro* of copper (II) complexes causes slight oxidative stress, compared to the unloaded and native cells (Bonomo *et al.,* 1995). However, no significant differences were observed in the major metabolic properties of loaded erythrocytes, with the exception of methahemoglobin levels, which differ depending on the encapsulated complex. This suggests that meth haemoglobin formation can be affected by the type of complex encapsulated, depending on the direct interaction of the complex with the haemoglobin.

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 thalassemia and other forms of anaemia that require regular transfusions (Green *et al.,* 1981). 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.

Prodrugs

In certain cases, prodrugs have been encapsulated that are transformed into the active drug following its release from the carrier red cells for the purpose of resolving some of the problems stemming from the structure and behaviour of the drug or from the impossibility of encapsulating the drug as such. Erythrocytes have been used for the encapsulation of the new antiopioidprodrugs with increased duration of action (Noel-Hocquet *et al.,* 1992).

Naltrexone and naloxone are widely used narcotic antagonists but with very short-term effects. Their administrations encapsulated in erythrocytes allow for extending their active life, but they are not stable within the erythrocytes and are rapidly released. The administration of prodrugs of these narcotic antagonists seems to present undoubted advantages. These prodrugs are transformed into an active drug upon release, not within the erythrocytes.

ERYTHROCYTES AS ENZYME CARRIERS

The encapsulation of enzymes in erythrocytes provides unquestionable advantages as it permits resolving some of the problems inherent to the therapeutic use of enzymes, as are their short half-lives, their tissue toxicity in certain cases, immunological disorders and/or allergies associated with the treatments or the need for repeated administrations.

Alcohol dehydrogenase and aldehyde dehydrogenase

The encapsulation in erythrocytes of the alcohol dehydrogenase and the aldehyde dehydrogenase 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 haemolysis. 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 (Magnani *et al.,* 1990). The acetaldehyde only accumulates in toxicologically significant amounts following the ingestion of ethanol. It has been observed that following the administration of aldehyde dehydrogenase encapsulated in erythrocytes, the blood levels of acetaldehyde in mice previously treated with a high dose of ethanol were 35% lower than the level of acetaldehyde in control mice and no significant differences were found in the levels of acetone.

Glutamate dehydrogenase

Glutamate dehydrogenase is a mitochondrial enzyme that reversibly catalyses the oxidative desamination of the glutamate to a-ketoglutarate. It is involved in the breakdown of the amino acids. The encapsulation of glutamate dehydrogenase in erythrocytes has reduced the levels of ammonia in mice with induced hyperammonemia (Sanz *et al.,* 1999). Hyperammonemia is a major disorder caused by different serious and terminal illnesses, such as hepatic encephalopathy and certain neurological complaints. In view of its toxicity, levels of ammonia should be kept low, whereby encapsulation of this enzyme may constitute a potential means for reducing high levels of ammonia in the organism.

Uricase

Uricase responsible for the transformation of uric acid into alantoine.Uricase used as bioreactors for uric acid degradation. The concentration of the enzyme can be made high and the only rate-limiting step for the uric acid degradation is the rate of substrate entry, souricase-loaded erythrocytes are potentially capable of removing as much uric acid as the human kidney (Magnani *et al.,* 1992). By coupling the uricase to the biotin–avidin–biotin–enzyme bridge, maintain low plasma concentrations of uric acid for several days.

Urokinase

Urokinase is a plasminogen activator that facilitates its conversion to plasmin, which promotes thrombolysis (Ito *et al.,* 1987). Erythrocytes have been proposed as carriers of human urokinase, as a possible form of administration in the treatment of patients with thrombosis as an alternative to the use of high doses of urokinase.

Hexokinase and glucose oxidase

Hexokinase is an enzyme that catalyses the ATPdependent conversion and ketohexose sugars to the hexose-6-phosphate. Glucose oxidise is an enzyme for the catalysis of oxidation of h- D-glucose. Catabolising enzymes such as hexokinase and glucose oxidase encapsulated in erythrocytes may be employed as new therapeutic ways of reducing high levels of blood glucose. *In-vitro* studies with human erythrocytes encapsulating catabolising enzymes reveal a marked increase in the metabolisation of glucose in comparison to normal cells. *In vivo* studies show that a single intraperitoneal administration of hexoquinase/glucose oxidase encapsulated erythrocytes in diabetic mice was able to regulate blood glucose at physiological levels for 7 days (Rossi *et al.,* 1992) and injections repeated at intervals of 10 days were effective in the regulation of glucose levels for several weeks.

Lactate-catabolising enzymes

Enzymes that metabolise lactate in the presence of oxygen as lactate 2-mono-oxygenase and lactate oxidase were encapsulated in human and murine erythrocytes in both an isolated and a joint manner. The co encapsulation of both enzymes results in significant rates of lactate metabolism. The results obtained *in vitro* suggest that the encapsulation of lactate-catabolising enzymes may be useful in the treatment of hyperlactatemia (Garin *et al.,* 1995). *In vivo* studies to prove the efficacy of these enzyme-loaded erythrocytes in the removal of blood lactate in mice have failed because of the high aerobic capacity and high lactate metabolism of these animals.

Rhodanase

Rhodanase is a mitochondrial enzyme that converts cyanide into thiocyanate in the presence of sulphur donors.Rhodase encapsulated in erythrocytes used as an antidote in case of cyanide intoxication. The antidotes used have a short-term effect whereby the encapsulation of this enzyme may provide major advantages by maintaining its action for a sufficient period of time (Petrikovics *et al.,* 1994). Previous studies have indicated that resealed mouse erythrocytes containing rhodanase and sodium thiosulphates can rapidly metabolise cyanide, but the potential of this system is restricted because of the limited availability of thiosulphate, due to its poor permeability through erythrocyte membrane.

Recombinant phosphortriesterase

The encapsulation of recombinant phosphotriesterase has demonstrated *in vitro* its potential use for the treatment of intoxications by organophosphorates. This enzyme has been reported to hydrolyse many organophosphorus compounds, including paraoxon, a potent cholinesterase inhibitor. Paraoxon is rapidly hydrolysed by this enzyme to p-nitro phenol and Diethyl phosphate. The addition of paraoxon to reaction mixtures containing resealed murine erythrocytes loaded with phosphotriesterase resulted in the rapid hydrolysis of paraoxon (Pei *et al.,* 1994). Hydrolysis of paraoxon did not occur when these carrier erythrocytes did not contain the enzyme.

Urease

Urease is an enzyme that catalyses the hydrolysis of urea into carbon dioxide and ammonia. Alanindehydrogenase catalyses the reversible oxidative deamination of L-alanine to pyruvate and ammonium. Urease/Alanindehydrogenage has been encapsulated in human erythrocytes *in vitro*. With this encapsulated system, urea is broken down into ammonia and bicarbonate. The ammonia released is converted into alanine by reacting pyruvate under the catalytic action of AlaDH (HamaratBaysal *et al.,* 2002). The results *in vitro* suggest the potential application in the treatment of high blood levels of urea in patients with chronic renal failure.

Pegademase

Pegademase (polyethylene glycol-conjugated adenosine deaminase) is a modified enzyme used for enzyme replacement therapy in the treatment of severe combined immunodeficiency disease associated with a deficiency of adenosine deaminase (Bax *et al.,* 2000). 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.

Brinase

Brinase is a fibrinolytic enzyme produced by Aspergillusorzae. Brinase has been encapsulated in vitro in rabbit erythrocytes with potential use in thrombolytic therapy since the inclusion of this loaded into clotting blood revealed almost complete lysis of the clot (Flynn *et al.,* 1994).

Alglucerase

Gaucher's disease is a rare hereditary disorder in which the enzyme, h-glucocerebrosidase is deficient. This results in the accumulation of the lipid, glucocerebroside, within macrophages that become very enlarged and known as Gaucher's cells. This disease leads to a progressive haematological, skeletal and neurological dysfunction. Alglucerase is a modified form of the enzyme, h-glucocerebrosidase, and is a unique form of replacement therapy in patients with a Confirmed diagnosis of Gaucher's disease. Studies have been performed *in vitro* using different concentrations of alglucerase as a sustained delivery system to the macrophages of the resealed erythrocytes with human carrier erythrocytes using hypo-osmotic dialysis (Humphreys *et al.,* 1980). High concentrations of alglucerase in the formulation and an extended dialysis time favour the encapsulation of the enzyme.

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.

Anti-HIV peptides

The use of carrier erythrocytes containing anti-HIV peptides constitutes one of the most therapeutic applications of these biological carriers. We knew that the infectivity and replication of immunodeficiency viruses are inhibited by certain analogues of nucleosides following their intracellular transformation into triphosphate derivatives. The monocyte–macrophage system plays a main role in infection by HIV-1. These cells become infected immediately after exposure to HIV; they are relatively resistant to virus attack and constitute an important reservoir for the virus. Both AZT, which is an analogue of thymidine, and DDI, which is a nucleoside analogue, is reverse transcriptase inhibitors and both are prescribed as anti-human immunodeficiency virus drugs (Magnani *et al.,* 2002). Protecting the macrophages from infection by HIV-1 using AZT + DDI encapsulated in erythrocytes based on using a murine AIDS model. Mice treated with AZT + DDI GSH-loaded erythrocytes presented a pro-viral DNA content in macrophages that was significantly lower than in mice treated with AZT + DDI. Efficient protection of the macrophages was also obtained in a murine AIDS model, alternating the administering of AZT encapsulated in GSH-loaded erythrocytes to protect lymphocytes and macrophages not yet infected and the lymphocytic drug fludarabine to eliminate the infected cells. Carrier erythrocytes containing prodrugs for slow delivery of AZT and ethambutol have been successfully tested in *in vitro* studies. 2V, 3V-Dideoxycytidine 5V-triphosphate is the triphosphate of dideoxycytidine, a nucleoside analogue that targets the reverse transcriptase of the human immunodeficiency virus and is used to treat AIDS. The main characteristic of 9-(2-phosphonylmethoxyethyl) adenine is its activity upon retroviruses, including HIV and the herpes virus (including HSV-1) (Fraternale *et al.,* 1999). This double activity is of great importance in the treatment of infections by retrovirus, as they are frequently complicated by infections such as the herpes virus, which is the most common infection in these kinds of patients. Exposure of macrophages to PMEA-loaded erythrocytes inhibits the replication of both HIV and HSV-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

(DeFlora *et al.,* 1988). 5-Fluoro-2V-deoxyuridine 5V-monophosphate 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.

Erythropoietin

Recombinant human erythropoietin is used in the treatment of certain serious forms of anaemia 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 (Garin *et al.,* 1997). Furthermore, the encapsulated erythropoietin stimulates the erythropoietin of polycythaemic mice.

Heparin

A study has been made of the possible use of heparin encapsulated in human and canine erythrocytes for the prevention of thrombiembolism. Heparin has been encapsulated *in vitro* in human and canine erythrocytes through a hypo-osmotic method of dialysis (Eichler *et al.,* 1986). The *in vivo* pharmacokinetic behaviour of heparin carrier erythrocytes was biphasic with a prolonged half-life.

Interleukin 3

Interleukin-3 is a cytokine that regulates blood-cell production by controlling the production, differentiation and function of granulocytes and macrophages. *In vitro* studies have been performed on the encapsulation of interleukin 3 in mouse erythrocytes, as well as on the effect of chemical cross-linking with band 3 reagents in the incorporation of cytokine into peritoneal mouse erythrocytes. The performance of the encapsulation of interleukin 3 mouse carrier erythrocytes was higher when the erythrocytes were loaded hypotonically as opposed to the isotonic incubation method (Olmos *et al.,* 2000). The treatment of mouse erythrocytes containing interleukin 3 with band 3 cross-linking reagents increases the recognition of carrier erythrocytes by macrophages *in vitro.*

Vaccines

Erythrocytes may be of interest in the field of vaccines as natural carriers and adjuvant of antigens. Erythrocytes are encapsulated with three bacterial toxoids. A mutation of the diphtheria toxin, the tetanus toxoid and a double mutant of the pertussin toxin were encapsulated in murine erythrocytes by a hypotonic dialysis method. A comparative study was performed *in vivo* administering mice multiple intravenous injections of the different toxoids encapsulated in carrier erythrocytes compared to the same doses of toxoids administered in a saline solution (Polvani *et al.,* 1991). Titers in sera of specific antibodies against each antigen and neutralizing antibodies were tested. Titers of both antibodies against diphtheria toxin and tetanus toxoid were higher upon immunisation using toxoid-loaded erythrocytes in comparison with free toxoids.

CONCLUSION

This review addresses the use of erythrocytes as biological carriers of therapeutic agents, such as drugs, enzymes and peptides, as well as mainly used drug loading methods for erythrocytes encapsulation, andfurther potential applications of the erythrocyte carrier system. The various methods currently existing for encapsulating substances in erythrocytes mainly based on the osmosis-based methods and especially on hypo-osmotic dialysis, which is at present the method of encapsulation most widely used. The use of resealed erythrocytes helps in a safe and effective delivery of various drugs for passive and active targeting. The same concept also can be extended to the delivery of biopharmaceuticals. In near future, erythrocytes based delivery system with their ability to provide controlled and site specific drug delivery will revolutionize disease management. 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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