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# ERYTHROCYTES AS A POTENTIAL CARRIER FOR CHRONIC SYSTEMIC DISEASES

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**ABSTRACT:** Chronic systemic diseases are diseases which require long term drug therapy. Parenteral controlled drug delivery system is often used to treat these diseases. Vesicular bodies like liposome are commonly used as Parenteral controlled depot formulation. It possess disadvantage that they are rapidly removed from body by phagocytosis because they are considered as hapten. So formulating a slow release depot formulation is a challenge for pharmaceutical scientist. Erythrocytes as they are derived from human body, they serves as a potential carrier for loading drugs intended for chronic systemic diseases that require long term therapy. Erythrocytes unlike other carrier possess biocompatibility, biodegradability and have long circulation half-lives. They act as slow release depot formulation by following a zero order kinetic drug release profile and without being attack by the complement system, provided the method adopted for loading should not cause any structural deformity or disturbance in lipid packing. This review will give an insight to those researchers who wish to work with erythrocyte for using it as a slow release depot formulation.

**INTRODUCTION:** Systemic diseases affect different parts of human body, sometimes the whole body. Chronic systemic diseases include cardiovascular diseases like hypertension, autoimmune diseases, diabetic mellitus which require long term drug therapy [1]. Some of the drugs used for these chronic diseases have relatively shorter half-life. In order to maintain the therapeutic plasma level concentration, multiple dosage regimens is needed. Continued multiple dosage regimens may lead to toxicity due to accumulation of drug in systemic fluid [2, 3].

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Most often chronic systemic diseases are treated with oral drugs. Oral route requires high dosage in order to maintain steady plasma drug concentration. Novel drug delivery system includes encapsulation of drugs into vesicular bodies like liposome, niosomes, transfersomes etc.

Administration of these drugs as long lasting parenteral depot is often followed in chronic systemic diseases [4]. But the problem is that the vesicular bodies are rapidly cleared off by phagocytosis and RES (Reticulo-Endothelial System, now known as Mononuclear Phagocytic System) as they are treated to be haptens.

To avoid this problem, the cellular based drug delivery is adopted. As the most abundant cells in all the vertebrates are erythrocytes or Red Blood Cell (RBC) they are used for this purpose.

The concept of encapsulating drug in erythrocyte first came in early 1970. The term carrier RBC was first introduced in 1979 by DeLoach` [5]. From that onwards many researchers encapsulate drug in carrier erythrocyte for their suitability as parental controlled drug delivery by both *in vitro* and *in vivo* methods, unfortunately yet now no drug-loaded RBC is available in market probably because of the stability problem associated. However they are widely used for encapsulating drug and its efficiency as carrier for depot formulation is proven even by clinical trials [6].

RBC cell membrane is composed of phospholipids bilayer like liposome. The outer layer is uncharged while inner layer is charged. The former is composed of phosphatidyl choline (30%) and sphingomylein (25%) while latter is composed of lipids like phosphatidyl ethanol amine (28%) and phosphatidyl serine (14%) [7]. Between the lipoid bilayer is the cholesterol. Both hydrophilic and lipophilic drug can be loaded successfully in erythrocytes.

These cells are not recognized by the macrophages system because RBC membrane contains many proteins like CD47, C8bp, HRP, MCP, CR1 etc. which protect RBC from being attack by the complement system [8, 9].

**Methods used for loading drug into Erythrocytes:** Blood is collected from suitable human candidate or suitable animal in a heparinized vaccute to prevent coagulation. It is centrifuged to separate erythrocytes from plasma and leucocytes buffy coat. Separated upper plasma and buffy coat of leucocytes are removed by aspiration and replaced with the same volume of phosphate buffer saline. Then it is washed 4 times and resuspended in phosphate buffer saline to get a concentration of $2\times10^9$ cell/ ml [10].

There are various methods for loading drug in erythrocytes. But all the methods are not equally suitable for loading drugs to treat chronic systemic diseases. The method we adopt should be suitable for not to cause any structural change during the treatment. This can be confirmed by viewing under an electron microscope. Any plasma membrane deformity occurs during the loading processes may cause it recognized by the macrophages and they will be cleared off from the circulation.

Some of the methods adopted for the purpose are discussed below:

1. **Hypotonic Dilution method:** The first investigated method for the encapsulation of chemicals into the erythrocytes. In this method, a volume of packed erythrocytes is diluted in 2-20 volumes of aqueous solution of drug in hypotonic solution. The solution tonicity is then adjusted by placing in hypertonic buffer. The resultant mixture is then centrifuged the supernatant was discarded and the pellet is washed with isotonic buffer solution. This method produces erythrocytes with reduced circulation half-life. They are easily recognized by the macrophage and cleared from the circulation. Hence, this method is not used as a carrier for treating chronic systemic disease. But it is used for targeting reticuloendothelial system [11, 12].

2. **Endocytosis:** This method was reported by Schrier *et al*, in 1975. Endocytosis involves the addition of one volume of washed packed erythrocytes to 9 volumes of buffer containing 2.5 mM $MgCl_2$, 2.5 mM ATP and 1 mM $CaCl_2$, followed by incubation at $37^oC$ for 2 min. The entrapment occurs by the phenomenon of endocytosis. The various drug candidates that can be entrapped by this method include primaquine and related 8 aminoquinoline, vinblastine, chlorpromazine, propanalol, tetracaine, and vitamin A [13, 14].

3. **Normal Transport mechanism***:* This method involves the loading of erythrocytes with drug without disrupting the erythrocyte membrane in any way by incubating the drug and erythrocytes for varying period of time. The drug will enter the RBC and exit to the circulation in the same kinetic order [15].

4. **Membrane Perturbation method:** In this method, Amphotericin B, a polyene antifungal drug is used to load drugs and metabolites into erythrocyte. Amphotericin B has a special property that it interacts with the cholesterol of the plasma membrane of the eukaryotic cells and causes change in the permeability of the membrane to allow the drugs and ions to enter the cells.

Deukie *et al*, noticed for the first time that the permeability of erythrocyte membrane is increased by the exposure of amphotericin B. Ketao and Hattori utilized this method to entrap the antineoplastic drug daunomycin in human and mouse erythrocytes [16].

5. **Preswell dilution Hemolysis:** In 1975, Rechsteiner initially utilized this technique and in 1983 the method was modified by Pitt and co-workers. Hemolysis and subsequent resealing occur in the following 3 step process;

A. At first, one volume of washed cell is suspended in 5 volumes of slightly hypotonic buffer (0.6%w/v NaCl). Under this condition, only a small percentage of cells which are the most fragile population of the suspended erythrocytes are lysed. The remaining ones are intact and the cell volume increases to an average of 150% of the normal cell volume.
B. The second step is to incubate the RBC suspension at $0^oC$ for 5 min. Then the cells are to be recovered by gentle centrifugation.
C. Finally the swelling process is further extended by the adding half the volume of suspended hypotonic buffer. As a result, more swollen cells become lyses. This hypotonic buffer medium also contains the material to be loaded and the cells are allowed to remain lyses for 10 min at $0^oC$. This is followed by restoration of tonicity and resealing of the membrane [17-19]. Enalaprilat, an ACE inhibitor is successfully loaded in human erythrocytes by the method of preswelling technique. The resultant loaded erythrocytes have acceptable loading parameters, and they release drug according to zero order kinetics [20].

6. **Electroporation:** This method is based on the observation that electrical shock brings about irreversible change in an erythrocyte membrane. The erythrocytes membrane is opened by a dielectric break down. Subsequently the pore can be resealed by incubation at $37^oC$ in an isotonic medium. The procedure involves suspending erythrocytes in an electrical discharge chamber which contains a capacitor. A capacitor is an external circuit which is charged to a definite voltage and then discharged. Murine interleukin 2 expression of plasmid DNA was efficiently loaded to erythrocyte ghost (EG) by electroporation in hypotonic condition. At 21 min after IV administration into mice, the level of plasmid DNA in the blood was 92000 – fold higher following EG – mediated delivery as compared to the injection of naked form. EG – mediated gene delivery revealed higher and more prolonged mRNA expression of levels of plasmid DNA in the blood until 9 days after the single intravenous injection [21-22].

7. **Dialysis Method:** Jarde *et al*, in 1987 described this method for the encapsulation of drug into the erythrocytes. A desired hematocrit is achieved by mixing erythrocyte suspension with the drug solution. The mixture so produced is taken in a dialysis tube and both the ends of this tube are tied with thread. An air bubble of nearly 25% of the internal volume is left in the tube. The tube is then placed in 100 ml of swelling solution. The bottle is placed at $4^oC$ for desired lyses time. The content of the dialysis tube is mixed by shaking the tube. The dialysis tube is then placed on a resealing solution.

The loaded erythrocytes thus obtained are then washed with cold PBS (Phosphate Buffer Saline) at $4^oC$. The cells are then resuspended in PBS. This method is important because the drug loaded to the erythrocyte by this method yields greater *in vivo* survival than other methods. Hence, used as a carrier for treating chronic systemic diseases, the various therapeutic agents which are encapsulated by this method include phosphotriesterase in murine erythrocytes [23].

After loading drug in erythrocyte, it should store properly to maintain the cell viability for its future use. Storage of loaded erythrocyte is a challenge faced by many researchers. Since it is a biological product, the *in vitro* stability is less. The most common storage media include Hanks Balanced salt solution and acid-citrate dextrose at $4^oC$; cells remain viable in terms of their physiologic and carrier characteristics for at least 2 weeks at this temperature [24]. Many scientists use cross linking agents like glutraldehyde to stabilize the loaded erythrocyte [14, 15].

The addition of $Ca^{2+}$ chelating agents or purine nucleosides improves circulation survival time of cells up on reinjection. It have been proved that RBC can be stored up to 11 weeks in alkaline additive solution (alkaline CPD pH = 8.7 which is made with trisodium citrate, dextrose and disodium phosphate [25, 26]. Freeze drying (lyophilization) of loaded erythrocyte is now widely accepted. Powdered form so obtained after freeze drying is reconstitute using suitable vehicle before injection [11].

**Evaluation of Loaded Erythrocytes:**

**Cell Counting and Cell Recovery**: Test involves counting the number of cells before and after loading. By a less destructive loading procedure, the number of cells recovered after loading of drug should be more than 75%. The cell counting is usually done with an optical microscope [5].

**Drug content determination**: Drug content is determined by deproteinized the loaded cells using methanol or acetonitrile followed by centrifugation. Then 1ml of the supernatant is pipette out (extract with methanol if necessary to reduce the interference by the proteins) and the drug content is estimated by suitable spectrophotometric and chromatographic method [5, 22].

***In vitro* Drug release and Hemoglobin content**: The in vitro release of drugs and hemoglobin are monitored periodically from drug loaded erythrocytes. The cell suspension is stored at $4^0$C in amber colored glass bottles. Periodically the supernatant are withdrawn using a hypodermic syringe equipped with 0.45 µm filter, deproteined using methanol or acetonitrile and the drug content were estimated. The supernatant of each sample after centrifugation is collected and assayed; % hemoglobin released is calculated by noting the absorbance at 540 nm [27- 30].

**Osmotic Fragility test***:* In this test, both the loaded and unloaded erythrocytes are incubated with series of concentration of NaCl from hypotonic to isotonic. Then the percentage hemolysis is determined. Usually the resealed erythrocyte has slightly higher osmotic fragility than the normal cell. The % hemolysis is calculated by the formula;

$$\% \text{ Hemolysis} = \frac{\text{Absorbance of Sample} - \text{Absorbance of Blank}}{\text{Absorbance of Positive Control}}$$

Absorbance is measured UV Spectrophoto-metrically at 540 nm [15].

**Mean Corpuscular Volume**: Conventionally it is calculated by the formula

Mean corpuscular volume (MCV) =

$$\frac{10 \times \text{Hematocrit (\%)}}{\text{RBC count (millions/µl)}}$$

Now the mean corpuscular volume is measured by means of a continuous mean corpuscular volume analyzer.

**Turbulence Shock***:* It is the measure of simulating destruction of loaded erythrocytes during injection. It is measured by passing erythrocyte suspension through a 23 gauge needle at flow rate of 10ml/min which is comparable to the flow rate of blood. The number of passes varies as a function of turbulence. The drug leakage is measured analytically. Drug loaded erythrocytes appears to be less resistant to turbulence indicating destruction of cells during injection [5].

**Electrical Surface Potential***:* Electric surface potential is determined by measuring the zeta potential. Here surface charge is measured, similar charge tends to repel. For RBC the overall surface charge is negative. This negative charge prevents from clumping of cells. The instrument used to measure zeta potential is Zetasizer.

**Shape and Surface Morphology**: It fairly gives information about the life span of erythrocytes after administration. The morphological examination of the loaded erythrocytes and the unloaded erythrocyte is compared using transmission electron microscopy (TEM), Scanning Electron microscopy (SEM). If there is significant difference in morphology long cell half-life would become a problem. The morphologic change occurs during loading produce RBC with biconcave (discocyte), uniconcave (stomatocyte), and spherocytic shape. The most fragile one is with spherocytic shape (**fig. 1**) [31].

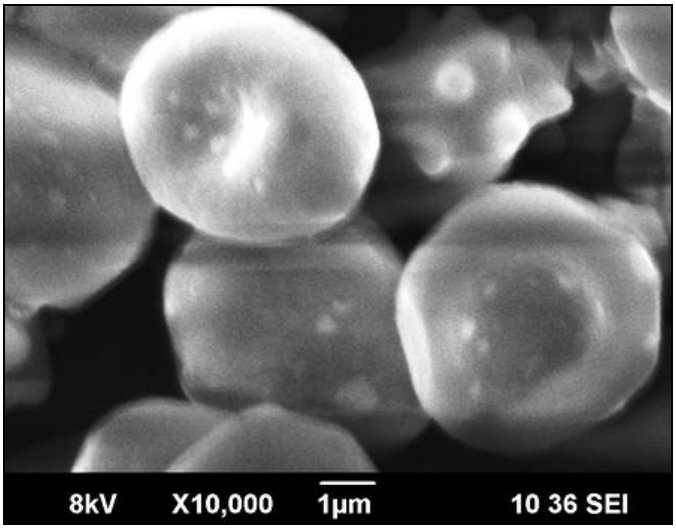
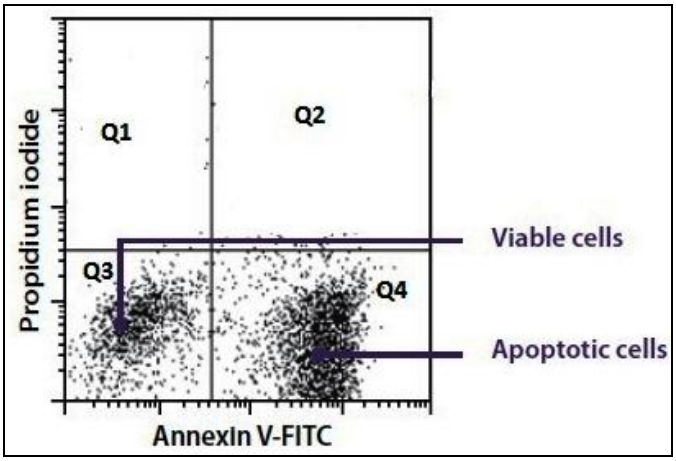



**FIG. 1 SHOWS THE SEM IMAGE OF ERYTHROCYTE**

**Detection of Phospholipid Asymmetry*:*** As we know, erythrocytes meant for chronic systemic disease should not be recognized by macrophages. Detection of phospholipid asymmetry provides a tool for identify whether the RBC is recognized by the macrophages. Phospholipid asymmetry means the conservation of phosphotidyl choline in the outer layer and phosphotidyl serine in the inner layer of the RBC membrane. Phosphotidyl serine expose on outer layer in case of apoptosis (cell death). Phosphotidyl serine on outer layer is a marker for rapid clearance by macrophages. Exposure of phosphotidyl serine is detected by binding with Annexin V.

Fluorescent labeled annexin V-phosphotidyl serine complex is quantified by flow cytometer. Annexin V is a 35-36 kDa Ca2+ dependent phospholipid-binding protein that has a high affinity for PS, and binds to cells with exposed PS. The binding assay result will be provided by flow cytometry in a four quadrant table (**Fig. 2**).




Quadrant 3(Q3): This quadrant shows the no: of cells that do not have affinity for Annexin, which means all cells are live and no exposure of PS.

Quadrant 4(Q4): Annexin V positive cell is quantified in this table. That means it gives information about the apoptotic cell or PS exposed erythrocytes.

Q1And Q2 have no role in determining the phospholipid asymmetry of RBC as these quadrants are meant for nucleus containing cells .This quadrants give positive results for propidium iodide dye which stains the nucleus. When phosphotidyl serine is exposed, the erythrocytes could not be used as a carrier for chronic systemic diseases [32, 33].

***In vivo* studies:** *In vivo* studies mainly focused on the uptake of loaded erythrocytes by the RES system than any pharmacologic efficacy studies. Normally uptake by RES is not recommended for those carriers intended to treat chronic systemic disease. However after long journey through the circulation, the transbilayer orientation of phospholipid in RBC is disrupted and dummy ghost is cleared from circulation by this Reticulo endothelial system [17].

**Recent development:** Nanoerythrosomes, patented nanovesicles derived from red blood cell through extrusion, sonification or electrical break down method. In extrusion method erythrocyte ghosts are passed through polycarbonate membrane filter, which breaks down into smaller vesicles, having a diameter of about 100nm [34].

Recently, a RBC membrane derived vesicles with polymeric nanoparticles made from poly lactic-co-glycolic acid (PLGA), an FDA approved polymer, and the resulting nanoparticle has a core shell structure that consists of a PLGA core surrounded by a RBC formed shell.

In combining the advantages of RBC-based carrier with that of polymeric nanoparticles, this technology has the potential to enable the delivery of slow releasing drug which has long *in vivo* circulation half-life than that coated with PEG [35].

**CONCLUSION:** Even though stability problems and donor-recipient incompatibility is still a challenging problem faced when using erythrocytes as carrier, they offer potential advantage its peculiar zero order kinetics drug release and long circulation half-life compared to other novel vesicular drug delivery system. However, autologous erythrocyte loading and administration helps to reduce the chance of immunogenicity.

Recently, synthetic RBC was developed to overcome the challenges of stability problems and immunogenicity reaction when using erythrocyte as carrier. Hence erythrocytes can be used as carrier for treating diseases which require daily multiple dosing. Thus we can minimize many dose related side effects of majority of drugs used for chronic disease.

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