RESEALED ERYTHROCYTES AS NOVEL DRUG CARRIERS

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Introduction

Erythrocytes, also known as red blood cells, 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 organism of interest, separating erythrocytes from plasma, entrapping drug in the erythrocytes, and resealing the resultant cellular carriers. Hence, these carriers are called resealed erythrocytes.
Basic features of erythrocytes:

A. Composition of erythrocytes:

- Heme
- Hemoglobin
- Erythrocyte
A. Composition of Erythrocytes

- Blood contains about 55% of fluid portion (plasma) and 45% of corpuscles or formed elements.
- Normal blood cells have extensile, elastic, biconcave and non-nucleated configuration with a diameter ranging from 6–9 µ and the thickness is nearly 1–2 µ.
- Erythrocytes have a solid content of about 35% most of which is Hb and rest 65% being water.
- Lipid content of erythrocytes includes cholesterol, lecithin and cephaelins.
B. Electrolyte composition of erythrocytes:

- Although qualitatively similar to that of plasma however, quantitatively it differs from that of plasma.
  - The concentration of $K^+$ is more in erythrocytes and $Na^+$ in plasma.
- The osmotic pressure of the interior of the erythrocytes is equal to that of the plasma and termed as isotonic (0.9% NaCl or normal physiological saline.)
- Changes in the osmotic pressure of the medium surrounding the red blood cells changes the morphology of the cells.
• If the medium is Hypotonic water diffuses into the cells and they get swelled and eventually loose all their hemoglobin content and may burst.

• And if the medium is hypertonic,(i.e. higher osmotic pressure than 0.9% NaCl) they will shrink and become irregular in shape.

• Balanced ion solutions like Ringer’s and Tyrode’s soln. which are not only isotonic but also contains ions in proper quantity are used in erythrocyte related experiments.
C. Haematocrit value

- If blood is placed into a tube and centrifuged, the cells and the plasma will separate.
- The erythrocytes, which are heavy, will settle down to the bottom of the tube, while the plasma rises up to the top and the leukocytes and platelets will form a thin layer (buffy coat) between the erythrocytes and the plasma.
- The **haematocrit** is defined as the percentage of whole blood made up of erythrocytes.

  Males.......... 40–50%
  Females....... 38–45%
D. Source, Fractionation and Isolation of Erythrocytes

- Different mammalian erythrocytes have been used for drug loading, resealing and subsequent use in drug and enzyme delivery.
- E.g. mice, cattle, pigs, dogs, sheep, goats, monkeys, chicken, rats, and rabbits etc.
- EDTA or heparin can be used as anticoagulants agents.
Isolation of erythrocytes:

- Blood is collected into heparinized tubes by venipuncture.
- Blood is withdrawn from cardiac/splenic puncture (in small animals) and through veins (in large animals) in a syringe containing a drop of anti coagulant.
- The whole blood is centrifuged at 2500 rpm for 5 min at 4 ± 1 °C in a refrigerated centrifuge.
- The serum coats are carefully removed and packed cells washed three times with phosphate buffer saline (pH=7.4).
- The washed erythrocytes are diluted with PBS and stored at 4°C until used.
Advantages Of Erythrocytes as Drug Carriers:

- Their biocompatibility, particularly when autologous cells are used, hence no possibility of triggered immune response.
- Their biodegradability with no generation of toxic products.
- The considerably uniform size and shape of the carrier.
- Relatively inert intracellular environment.
- Prevention of degradation of the loaded drug from inactivation by endogenous chemicals.
- The wide variety of chemicals that can be entrapped.
- The modification of pharmacokinetic and pharmacodynamic parameters of drug attainment of steady-state plasma concentration decreases.
- Fluctuations in concentration protection of the organism against toxic effects of drugs.
METHODS OF DRUG LOADING
Drug loading in Resealed erythrocytes

- Membrane perturbation
- Electro encapsulation
- Hypo-osmotic lysis
- Lipid fusion endocytosis

- Dilution method
- Preswell method
- Osmotic lysis
Hypotonic Haemolysis and Isotonic Resealing Methods:

Loading of drugs is done by two steps:

1. Hypotonic lysis of cells in a solution containing the drug/enzyme to be entrapped.

2. This step involves restoration of isotonicity to reseal the erythrocytes.
Loading by “Red Cell Loader”

- Magnani and coworkers, 1998 developed a novel method for the entrapment of non-diffusible drugs into human erythrocytes.

- The method requires as little as 50ml of blood.

- The method is based on two sequential and controlled hypotonic dilutions of washed red blood cells.

- Subsequent isotonic resealing of erythrocytes allows a 35–50% cell recovery and approximately 30% entrapment of added drug.
Dilutional Haemolysis

e.g. Enzymes such as ß-galactosides, ß-glucosides, asparaginase and arginase.

RBC → Membrane ruptured RBC

0.4% NaCl → Hypotonic

Drug → Loaded RBC

Loading buffer → Incubation at 25°C

Resealing buffer → Resealed Loaded RBC

Efficiency → 1-8%

Enzymes delivery

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Isotonic Osmotic Lysis

RBC

Physical rupturing

Chemical rupturing

Isotonically ruptured RBC

Drug

Loaded RBC

Incubation at 25°C

Resealed RBC

Chemicals – urea, polyethylene, polypropylene, and NH₄Cl
Preswell Dilutional Haemolysis

e.g. Propranolol, insulin, asparagine, methotrexate,

RBC → 0.6% w/v NaCl → Swelled RBC

5 min incubation at 0 °C → Drug + Loading buffer → Loaded RBC

Incubation at 25 °C → Resealing Buffer → Resealed RBC

Efficiency → 72%

Fig: - Preswell Method
<table>
<thead>
<tr>
<th>Method</th>
<th>%Loading</th>
<th>Advantages</th>
<th>Disadvantages</th>
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<tbody>
<tr>
<td>Dilution method</td>
<td>1–8%</td>
<td>Fastest and simplest for low mol. Wt.</td>
<td>Less Entrapment efficiency</td>
</tr>
<tr>
<td>Preswell dilution</td>
<td>20–70%</td>
<td>Good retention of cytoplasm constituents and good survival invivo</td>
<td>---</td>
</tr>
<tr>
<td>Isotonic osmotic lysis</td>
<td>---</td>
<td>Better in vivo surveillance</td>
<td>Time consuming, Impermeable to large molecules</td>
</tr>
</tbody>
</table>
Dialysis

80% Haematocrit value

RBC + Phosphate buffer

Placed in dialysis bag with air bubble

Dialysis bag placed in 200ml of lysis buffer with mechanical rotator 2hrs.

Drug

Resealed RBC

Dialysis bag placed in Resealing buffer with mechanical rotator 30 min

Loaded RBC

Efficiency → 30-45%

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Electro-insertion or Electro-encapsulation

RBC + 2.2 Kv Current for 20 micro sec
At 25°C
Pulsation medium

Drug + Loading suspension

3.7 Kv Current for 20 micro sec
Isotonic NaCl

Loaded RBC
Resealing Buffer
Resealed RBC

Isotonic solution
Electrodes
Orifice
Jet capillary
Erythrocyte suspension

Fig: Electro-encapsulation Method
Entrapment By Endocytosis

RBC + Drug Suspension

Buffer containing ATP, MgCl₂, and CaCl₂
At 25°C

Loaded RBC

Resealing Buffer

Resealed RBC
Membrane perturbation method

- RBC
  - Amphotericin B
  - e.g. Chemical agents
  - Increased permeability of RBC

- Drug
  - Resealing Buffer
  - Resealed RBC

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Lipid fusion

- Lipid vesicles containing drug can be directly fused with human erythrocytes leading to exchange of lipid entrapped drug.
- Nicolau and gresonele, 1979 used this technique for loading of inositol hexaphosphate into resealed erythrocytes for increased oxygen carrying capacity.
- The encapsulation efficiency by this method is however very low i.e. (1 %).
# In–vitro characterization

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<th>Method/instrument used</th>
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<td>Shape and surface morphology</td>
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<td>Vesicle size and size distribution</td>
<td>Transmission electron microscopy, optical microscopy.</td>
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<td>Drug release</td>
<td>Diffusion cell, dialysis</td>
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<td>Deproteinization of cell membrane followed by assay of resealed drug, radiolabelling</td>
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<tr>
<td>Surface electrical potential</td>
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<tr>
<td>Surface pH</td>
<td>pH-sensitive probes</td>
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<tr>
<td>Deformability</td>
<td>Capillary method</td>
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<tr>
<td>Parameter</td>
<td>Method/instrument used</td>
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<tr>
<td>---------------------------------</td>
<td>----------------------------------------------------------------------------------------</td>
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<tr>
<td>% Hb content</td>
<td>Deproteinization of cell membrane followed by hemoglobin assay</td>
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<tr>
<td>Cell volume</td>
<td>Laser light scattering</td>
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<td>% Cell recovery</td>
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<td>Osmotic fragility</td>
<td>Stepwise incubation with isotonic to hypotonic saline solutions and determination of drug and hemoglobin assay</td>
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<tr>
<td>Osmotic shock</td>
<td>Dilution with distilled water and estimation of drug and hemoglobin</td>
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<tr>
<td>Turbulent shock</td>
<td>Passage of cell suspension through 30-gauge hypodermic needle at 10 mL/min flow rate and estimation of residual drug and hemoglobin, vigorous shaking followed by hemoglobin estimation</td>
</tr>
<tr>
<td>Erythrocyte sedimentation rate</td>
<td>ESR methods</td>
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</table>
Drug Content

Packed loaded erythrocytes (0.5 ml) are first deproteinized with acetonitrile (2.0 ml).

Centrifugation at 2500 rpm for 10 min

Clear supernatant liquid is analyzed for the drug content using specific estimation methodology for the entrapped drug.
In vitro Drug and Haemoglobin release

Normal and loaded erythrocytes incubated at $37\pm2^\circ\text{C}$ in PBS(7.4) at 50% haematocrit in a metabolic rotating wheel incubator bath.

Samples withdrawn with hypodermic syringe fitted to 0.8µ Spectropore membrane filter.

Samples deproteinized with acetonitrile and can be estimated for amount of drug released.

Percent Hb calculated at various time intervals at 540nm spectrophotometrically.

Cumulative percent release profile as function of time and % haemolysis is calculated.
• **Laser light scattering** – To evaluate haemoglobin content of individual resealed erythrocytes.

• **MEAN CORPUSCULAR HAEMOGLOBIN**: Mean concentration of haemoglobin per 100ml of cells, independent of the size of the red cell.

Mean corpuscular haemoglobin =

\[ \text{[Haemoglobin(g/100 ml)x10} \times \text{Erythrocyte count (per mm}^3) \]

Percent cellular recovery (after loading) determined by assessing the number of intact erythrocytes remaining per cu.mm with help of haemocytometer.
**Osmotic fragility:**

It is used to determine the effect of loaded contents on the survival rates of the erythrocytes.

Drug loaded erythrocytes are incubated with saline solutions of different tonicities (0.9% w/v to 0.1% w/v) at 37°C for 10 min, centrifuged and assayed for drug/ Hb. Content.

**Osmotic shock:**

A sudden exposure of drug loaded erythrocytes to an environment, which is far from isotonic to evaluate the ability of resealed erythrocytes to withstand the stress and maintain their integrity as well as appearance.
Turbulence shock

- Indicates the effects of shear force and pressure by which resealed erythrocytes formulations are injected.
- Loaded erythrocytes (10% haematocrit, 5 ml) passed through a 23 gauge hypodermic needle at flow rate of 10 ml/min.

- After every pass, aliquot of the suspension is withdrawn and centrifuged at 300G for 15 min, and haemoglobin content, leached out are estimated spectrophotometrically.
Shelf Life And Storage Stability

- Encapsulated product and carrier both show satisfactory self-stability when stored in Hank’s balanced salt solution (HBSS) at 4°C for two weeks (Lewis and Alpar, 1984).
- Suspending cells in oxygenated HBSS with 1% soft bloom gelatin. Cells recovered after liquefying the gel by placing tubes in water bath at 37°C followed by centrifugation.
- Cryopreservation of Erythrocytes in liquid nitrogen temp (Brearly et al., 1988; 1990).
Different forms of drug loaded RBCs

- Normally, more than 80% erythrocyte ghosts loaded with drug or enzyme appear as biconcave under electron microscope.

- Less than 20% cells show abnormal morphology, and the rest appear as stomatocytes or spherocytes or other destroyed forms.

- Erythrocytes on haemolysis and washing with large volumes of hypotonic medium, looses nearly all their haemoglobin and on resealing the resultant cells appear as pale or transparent and are referred to as “Erythrocytes ghosts”.

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A rapid loss of cells during first 24 hrs followed by much slower loss afterwards.

The first phase represents the cells that are severally damaged during the drug loading procedures.

The second phase has a half life of the orders of weeks for different mammalian erythrocytes.

Resealed erythrocytes prepared from RBCs of chicken, rats and rabbits exhibited relatively poor circulation profile as compared against unloaded normal erythrocytes.
Release characteristics of loaded drugs

- There are mainly three ways for a drug to efflux out from the erythrocyte carriers:
  - **Phagocytosis**
  - Diffusion through the membrane of the cells
  - Using a specific transport system.

- RBCs are normally removed from circulation by the process of phagocytosis.

- The degree of cross-linking determines whether liver or spleen will preferentially remove the cells.
Carrier erythrocytes following heat treatment or antibody cross-linking are quickly removed from the circulation by phagocytic cells located mainly in liver and spleen.

The rate of diffusion depends upon the rate at which a particular molecule penetrates through a lipid bilayer. It is greatest for a molecule with high lipid solubility.

Many substances enter cells by a specific membrane protein system because the carriers are proteins with many properties analogous to that of enzymes, including specificity.
Applications of released erythrocytes

Erythrocytes as drug/enzyme carriers:
Erythrocytes as carriers for enzymes.
Erythrocytes as carriers for drugs.
Erythrocytes as carriers for proteins and macromolecules.

Drug targeting:
Drug targeting to RES organs
Surface modification with antibodies.
Surface modification with Glutaraldehyde.
Surface modification involving sulphhydryls.
Drug targeting to Liver
Enzyme deficiency/replacement therapy
Treatment of liver tumors
Treatment of parasitic diseases
Removal of RES Iron Overload

Targeting to sites other than RES– rich organs

Erythrocytes as Circulating Bioreactors
<table>
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<th>Application</th>
<th>Drug / Enzyme / Macromolecule</th>
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<tr>
<td>Enzyme deficiency, replacement therapy</td>
<td>β-galactosidase, β-fructofuranodase, urease</td>
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<tr>
<td>Thrombolytic activity</td>
<td>Brinase, Aspirin, Heparin</td>
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<td>Immuno therapy</td>
<td>Human recombinant Interleukin-2</td>
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<tr>
<td>Circulating carriers</td>
<td>Albumin, Salbutamol, Tyrosine kinase, Prednisolone</td>
</tr>
<tr>
<td>Targeting to RES</td>
<td>Pentamididine, Mycotoxine, Homidium bromide</td>
</tr>
<tr>
<td>Application</td>
<td>Drug / Enzyme / Macromolecule</td>
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<tr>
<td>Iron overload</td>
<td>Desferroxamine</td>
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<td>Chemotherapy</td>
<td>Rubomucin, Methotrexate, Doxorubicin, Daunomycin, Cytosine, Adriamycin</td>
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<tr>
<td>Circulating Bioreactor</td>
<td>Arginase, Acetaldehyde dehydrogenase</td>
</tr>
<tr>
<td>Targeting to sites other than RES</td>
<td>Daunomycin, Diclofenac sodium, Methotrexate</td>
</tr>
</tbody>
</table>
Nanoerythrosomes:

- An erythrocyte-based new drug carrier, named nanoerythrosome has been developed which is prepared by extrusion of erythrocyte ghosts to produce small vesicles having an average diameter of 100 nm.

- Daunorubicin (DNR) was covalently conjugated to the nEryt (nEryt–DNR) using glutaraldehyde as homobifunctional linking arm. This led to a complex that is more active than free DNR both *in vitro* and *in vivo*.

- Daunorubicin (DNR) conjugated to these nanoerythrosomes has a higher antineoplastic index than the free drug.
Erythrosomes:

Erythrosomes are specially engineered vesicular systems in which chemically cross-linked human erythrocyte cytoskeletons are used as a support upon which a lipid bilayer (phosphatidyl choline) is coated.

These vesicles have been proposed as useful encapsulation systems for macromolecular drugs.
FUTURE PERSPECTIVES

- A large amount of valuable work is needed so as to utilize the potentials of erythrocytes in passive as well as active targeting of drugs.
- Diseases like cancer could surely find its cure.
- Genetic engineering aspects can be coupled to give a newer dimension to the existing cellular drug carrier concept.


