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# Cellular Drug Delivery Systems

**Swetha Pothula[1], Ravinder Kumar Sarepalli[1], Yadagiri Phalguna*[2], G. Sandhya Rani[2]**

**[1]Research scientist, Granules Pharmaceuticals Inc., 3701 Concorde pkwy, Chantilly, VA, 20151, USA.**

**[2] Bharat Institute of Technology,Mangalpalli(V),Ibrahimpatnam(M),RR-Dist.**

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***Corresponding Author Email:** yphalgun@gmail.com

## Abstract

Delivery vehicles are often used to deliver low molecular weight (< 600 Daltons) therapeutic drugs, enzymes, proteins, nucleic acids and other biological agents to target organs or tissues in pathological conditions. Liposomes, Micelles, Hydrogels, Polymers, Vesicles, Nanospheres etc. are most commonly used drug carriers. Scientists explored new methods to improve circulation properties of drug and its carriers. In this approach, cellular based drug delivery systems may represent another alternative in which biological cells such as erythrocytes and lymphocytes are used as drug carriers. The natural characteristic properties such as biocompatibility, biodegradability, stability, specificity, longevity, capacity of holding high volumes of drug and efficiency in delivering drugs made them one of the incredible drug carriers. Therefore, this technique is named as ***"Cellular Drug Delivery Systems."*** As of today, erythrocytes are most widely used carriers but use of lymphocytes (especially cytotoxic T-lymphocytes) as drug carriers is still in progress. The main goal of this paper is to provide basic and important information about the various topics related to cellular drug delivery systems. It discusses about the properties of carrier cells, their advantages, and disadvantages, methods of drug encapsulation, methods to determine in vitro and in vivo characteristics of drug-loaded cells, storage methods, and few therapeutic applications. For better understanding and to show how efficient these drug carriers, experimental results of individual carrier cells are discussed later.

**Keywords**

CDDS, Liposomes, Erythrocytes, Lymphocytes

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## INTRODUCTION

Drug inactivation, drug interactions, rapid elimination from the body, toxicological effects, multiple administrations, reduced plasma half-life, insufficient concentration at target site and many more are the disadvantages of a drug when it is administered in its free form or in other conventional dosage forms such as tablets, capsules, injections etc(1). These and other disadvantages are eliminated or minimized by administering the drug after it is loaded into natural drug carriers such as erythrocytes and lymphocytes(2). These cells are chosen as drug carriers because of the natural properties they possess such as biodegradability, specificity, biocompatibility, long life span, capacity to hold high volumes etc. As this technique is highly sensitive, all the processes must be performed under sterile conditions. Before administration, cells are isolated,

processed, and loaded with therapeutic drug. Remarkable and well-differentiated results are observed when applied therapeutically with this cellular drug delivery technique (1).

**Erythrocytes**

Erythrocytes, also called as Red Blood Corpuscles (R.B. C's). Dutch scientist called Lee Van Hock in 1674 first discovered them (3). The primary function of erythrocytes in animals is to transport oxygen from lungs to various organs and in turn carries carbon dioxide from various organs to lungs for expiration. They are the most common type of present in the human body (5, 200, 000 +/- 3, 00,000 cells/$mm^3$) and are biconcave discs in shape with an average diameter of 7.8 µmeter, thickness of 2.5 µmeter in around, 1 µmeter in the centre and hold a volume of 85-91 fl (µ$mm^3$). The life span of healthy red blood cell is 120 days and in its entire life span, it travels hundreds of kilometers in the vasculature. Shape of the erythrocytes becomes cup-shaped and finally spherical when osmolarity of the surrounding medium is decreased. This characteristic swelling behavior of erythrocytes enables to efficiently load drugs (3, 4).

The plasma membrane of erythrocytes consists of lipids, proteins, carbohydrates and two special proteins called spectrin and actin (3) . These proteins provide mechanical strength and maintain the structure and shape of erythrocytes when exposed to high shear rates during drug loading process as well as in blood circulation (4, 5) . Organs of the reticulo endothelial system (RES) such as Kupfer cells of liver and spleen remove damaged and aged erythrocytes from the circulation (3, 6). Alveolar macrophages of the lung, peripheral blood monocytes and vascular endothelial cells also aid in removing abnormal, old, and dead erythrocytes. This mechanism enhances the drug-loaded erythrocytes for targeting the drugs to specific pathological tissues. The main advantage of this mechanism is that required concentrations of drug can be delivered and maintained at the specific site.

In 1953, Gardos for the first time tried to load chemicals into erythrocyte ghosts (3, 7). Later in 1959, Marsden and Ostling reported the entrapment of dextrans of molecular weight 10 to 250 kD into erythrocyte ghosts (3, 8). After a gap of fourteen years, Ihler et.al (3, 8) and Zimmerman independently published encapsulation of therapeutic drugs into erythrocytes. In the year 1979, term ***"carrier erythrocytes"*** was used for the first time (3).

In one of the approaches, it was experimentally proved that therapeutic index (TI) value was increased by three times when anti-tumor drug, doxorubicin, is administered intravenously both in its free state and encapsulated form. Toxicity values ($LD_{10}$ and $LD_{50}$) are observed to be less in the animals that are treated with drug encapsulated erythrocytes. It is also observed that hepatic and pulmonary tumor growth was significantly inhibited with the encapsulated form (9).

**Advantages of erythrocytes as drug delivery carriers(10)**

1) Compared to other biodegradable carrier systems erythrocytes are comprised of high and natural biocompatibility.

2) Large amounts of drug can be encapsulated in a small volume of cells with a high rate of encapsulation.

3) Erythrocytes can act as bioreactors, where they enable the encapsulation of prodrug and due to the enzymatic reactions; it will be converted to an active drug.

4) As drug is encapsulated within the erythrocytes, signs of any pharmacological and toxicological actions of the drug will not be seen until they reach the specific organ or tissue.

5) They act as reservoir for the drug, enabling the sustained release of drug into circulation after hemolysis.

6) Erythrocytes allow the encapsulation of high molecular weight peptides that have therapeutic value.

7) Drug encapsulated within the erythrocytes is protected from premature degradation and avoids immunological reactions.

8) Drug will always be eliminated through systemic circulation similar to the erythrocyte elimination process by RES organs; thereby it enables the drug to remain in the blood for prolonged period.

**Disadvantages**

1. One of the main disadvantages of biodegradable materials and natural cells is that they will be rapidly removed by reticulo endothelial organs such as liver and spleen. Thereby, limits the usefulness of drug carriers, and even sometimes may cause toxicological problems (10).
2. Sometimes encapsulated substance leaks out of the erythrocytes at high rate by simple diffusion process.
3. The physiological functions of erythrocyte will be altered by most of the macromolecules or drugs.
4. Storage of drug-loaded erythrocytes is another major problem. Tests revealed that encapsulated erythrocytes have to be

suspended in isotonic buffers containing essential nutrients that are required in maintaining the efficacy and life span until they are administered.

5. As erythrocytes are taken off from the blood, they are very much susceptible to contamination due to surrounding environment such as air, equipment, chemicals etc.; therefore, thorough examination of the material for cleanliness is necessary before collecting and handling erythrocytes.

**Lymphocytes**

Lymphocytes are one of the five types of white blood cells. Three major types of lymphocytes present in the body; Natural Killer cells (NK), T-lymphocytes, and B-lymphocytes. NK cells are part of cell-mediated immunity (CMI), acts only during the innate immune response. They attack host cells displaying foreign (e.g. viral) peptide belonging to MHC class 1 molecules on particular cell surface. Once they determine the cell is infected, NK cells release cell killing (cytotoxic) granules that destroy the infected cell. NK cells do not require prior activation in order to perform their cytotoxic effect (11).

Lymphocytes that are produced in bone marrow but mature in thymus gland are called T-lymphocytes and the lymphocytes that are produced and mature in bone marrow are called B-lymphocytes. Major role of lymphocytes is to provide cell-based immunity in humans and are designed in such a way that they will specifically bind with the antigens entering into the body. Scientists took advantage of this characteristic feature of lymphocytes and utilized them as targeted and controlled drug delivery carriers.

**T-Lymphocytes and Tumor antigens**

T-lymphocytes have their unique way of exerting immune response different to B-lymphocytes. Regulatory T-cells are differentiated from cytotoxic T-cells by the presence of CD4+ receptor on their surface, which help in activating B-cells and other T-cells. Whereas, cytotoxic T-Lymphocytes (CTL's) carry CD8+ receptor on their surface helping in direct attack of body cells that are infected or malignant (abnormal).

Use of T-lymphocytes as drug carriers is in its starting stage of scientific research in the treatment of cancer. The CD8+ receptor on the surface of CTL's allow them to specifically bind with the antigens that are displayed on the surface of cancer cell. These antigens will be expressed only when a normal cell transforms into a cancer cell and these cell surface antigens are called ***Tumor antigens*** (12). These antigens are rarely, if ever, will be displayed on the surface of normal cells. If the immune system recognizes tumor antigens as non-self, it destroys the cancer cells carrying specific type of antigens. Such an immune response is called Immunological surveillance and is carried out by the T-lymphocytes, macrophages, and natural killer cells. This makes T-cells an attractive targeted drug carrier. They can be autologously engineered to contain chemotherapeutic drug before directing them to tumor antigens to achieve the complementary effect of chemotherapeutic agent in addition to the immune response. The non-specific effect can be minimized maximally as, since the antigens are tumor specific.

In one of the studies of cancer treatment, antigen specific T-lymphocytes are used as carriers of doxorubicin coated negatively charged nanoparticles. They showed remarkable results (13), which are explained later in the paper.

**Advantages of T-lymphocytes as drug carriers**

1) Drug can be localized: - Presence of CD8+ receptor makes them to specifically bind with the diseased (Abnormal) cells, which helps in the localization of drug at the target site.
2) Complementation and amplification of cytotoxic T-cell effect when drug is loaded: - With the combined effect of complementation and cytotoxic effect of T-cell, anti-tumor drug exerts its action and destroys more number of tumor cells
3) Detects and attacks metastasis that is not yet diagnosed (in tumor conditions):- Metastasis (Spreading of cancer to other organs) will be detected by the T-cells and will help in diagnosing cancer when it is still in its starting stage.
4) Drug will be protected from RES and is not eliminated rapidly from the body: - As the drug is entrapped within the cell and is not exposed to the external environment such as various pH conditions, enzymes, RES organs etc. it will not be eliminated rapidly from the circulation.
5) Work autonomously: - Once drug loaded carrier T cells are re-infused into the blood circulation, they detect abnormal cells by itself and the drug exert its therapeutic action.
6) High specificity: - Presence of receptor on the surface of CTL's directs them to act only on the specific organ and limits the pharmacological and toxicological effects on the other tissues.

**Disadvantages**

1) Sterile conditions have to be maintained throughout the experimental processes because this technique deals with the blood and its components which are very much prone to

contamination. Even chemicals, equipments and surrounding environment have to be maintained aseptic.

2) As they have very limited life span of about 30 minutes in blood circulation, it limits the amount of drug delivered to the target site.

**METHODS OF DRUG ENCAPSULATION**

Electrical pulse method, Osmotic pulse method, Hypotonic Hemolysis, Hypotonic dilution method, Hypotonic Dialysis, and Chemical perturbations are most commonly used methods for loading which the therapeutic drugs and other bioactive agents into carrier cells. Drugs that have to be encapsulated must possess high degree of water solubility, resistance against inactivation within the cells, devoid of physical and chemical interaction with membrane or other cell constituents and also should possess well-defined pharmacokinetic and pharmacodynamic properties (3).

**Hypotonic Hemolysis**

Hypotonic Hemolysis is a drug entrapment method. In this method cells are first placed in a hypotonic solution. Due to swelling, volume of the cell increases by 25% to its original volume and is allowed to swell up to a definite degree of tonicity (about 150 mOsm). At this point, before the cell lyses, transient pores present on the surface of the membrane will open to a diameter of 200-500 A° and the pores are immediately closed by keeping the cells in an isotonic medium. If this step is not performed on time especially at the lyses point, some irreversible ruptures and openings occur in the cell membrane through which all the cell contents will deplete. Finally, it results in the cell death. The remainder erythrocyte is called **"*erythrocyte ghost*"** (14), which is considered as the main disadvantage of this method (3, 7, 14).

**Hypotonic Dilution**

It is a simple and rapid encapsulation method. In the first step definite volume of packed erythrocytes are diluted with 2 to 20 volumes of aqueous drug solution. Then, tonicity of the suspension. was restored by adding hypertonic buffer and centrifuged. After centrifugation, supernatant liquid was discarded, and pellet is washed at least three times with isotonic buffered solution. The end microsphere product of this method is called ***"white ghost."*** (3, 14).

Low entrapment efficiency, and remarkable loss of hemoglobin and other cell constituents are the main drawbacks associated with this encapsulation method. Due to the destructive conditions of the method, when loaded cells are re-injected, they will have short life span because the RES organs will rapidly degrade them. Therefore, they are very much useful for targeting drugs to the RES organs.

**Hypotonic Dialysis**

In 1977, Deloach and Ihler first used this method to load enzymes and lipids into erythrocytes (15). In this method, buffered cell suspension with hematocrit value of 70 to 80 was prepared and placed in a conventional dialysis tube. The tube was immersed in 10 to 20 volumes of hypotonic buffer. After the medium is gently stirred for about 2 hours, tonicity of the cell suspension was restored, either directly by adding hypertonic buffer or by replacing the surrounding medium with isotonic buffer. The resulting erythrocytes are called as ***"pink ghosts"*** (3, 15).

During this process drug is loaded into the cells at two steps.

1) Either it is dissolved in the isotonic buffered cell suspension present inside the dialysis sac at the beginning of the procedure or.
2) It is added to the dialysis sac after the first stirring period.

As this encapsulation method involves the gentle conditions, it results in the production of viable cells that has the natural life span upon re-injection. In addition, other advantages of this method are high entrapment efficiency (30 to 50%), high rate of cell recovery (70 to 80%), probability of loading high volumes of erythrocytes as a single batch and possibility of automating and on-line monitoring of the process variables. However, it also has some disadvantages such as requires more time for processing and requires special equipment especially for semi-automated methods.

Enzymes such as glucoserebrosidase, beta-galactosidase, and asparaginase; inositol-hexa-phosphate; desferrioxamine (an iron-chelating agent); gentamicin; adriamicine; human recombinant erythropoietin; Interleukine-2 etc., are encapsulated into the intact erythrocytes with this method (3).

**Hypotonic Pre-swelling**

This is relatively simple and rapid method of loading drugs into cells. It was first developed by Rechsteiner in 1975 (3). In this method, first packed erythrocytes are suspended in a hypotonic buffer solution and centrifuged, and then supernatant liquid is discarded. Later, cells are brought to lysis point with stepwise addition of 100-200 portions of drug solution and centrifugating after each addition, at one moment lysis point is detected by the disappearance of distinct boundary between cell fraction and supernatant portion. With the addition

of hypertonic buffer at lysis point tonicity of the cell mixture is restored. Finally, cell suspension is incubated at 37°C to reanneal the resealed erythrocytes. This gentle loading procedure results in high entrapment efficiency with reduced destruction of cells that increases the life span of cells and is considered as the main advantage of this method (3, 16).

**Osmotic Pulse Method**

The scientist Franco et al. first introduced this drug incorporation method in 1987 (17). In this method, packed erythrocytes are treated with isotonic solution of dimethyl sulfoxide (DMSO), cell suspension is then diluted with isotonic drug solution. Finally, cells are separated and resealed at 37°C (18).

Mechanism: When cells are exposed to DMSO, it rapidly diffuses into the cytoplasm through the cell membrane until the concentration of DMSO reaches equilibrium on both sides of the cell membrane. The transient concentration of DMSO across the membrane results in the osmotic pulse and moves the water intracellularly. This causes the opening of some transient pores in the cell membrane through which drug enters the erythrocytes. The rapid diffusion of DMSO results in closing of pores entrapping the drug within erythrocytes.

Electric Breakdown

This method is also known as electroporation (19-21), which is used to load drugs in to carrier cells. In this method, cells are suspended in an isotonic buffer solution present in an electric discharge chamber. Optimal electric pulse of 1-10KW/cm for a period of 20-160 microseconds is discharged through the suspension chamber. The pore diameter depends on the intensity of the electrical field, discharge time, and the ionic strength of the suspension medium. The drug that has to be entrapped is dissolved in the suspension medium at the beginning of the procedure. Uniform distribution of drug into the cells, lowered degree of chances of damage to membrane structure, more uniform size distribution of the resulting carrier cells, 35% entrapment efficiency, retention of natural life span in circulation are some of the advantages with this procedure (22, 23). The only drawback is more advanced equipment and sophisticated procedure is required (3).

**Chemical Perturbation**

In 1973, Deutike et al. (14, 24) observed that permeability of the cell increases when exposed to polyenic antibiotics (antibiotics that bind to sterols and perforates cell membranes) such as amphotericin-B (25, 26). Scientists made use of this property and successfully loaded antineoplastic drug daunomycin into human erythrocytes by exposing the erythrocytes to amphotericin-B. Other chemicals such as urea, ethylene glycol, ammonium chloride, and halothane can also be used. Due to some irreversible destruction occurs in the cell membrane, this method is no longer used for entrapment (3, 26).

## DETERMINING *IN VITRO* AND *IN VIVO* CHARACTERISTICS

The ability to maintain cell morphology of carrier cells is an important consideration. Morphology relates to the life span of the cells upon reinfusion. The in vitro evaluation of loaded cells is determined by several tests such as Osmotic fragility, Turbulence fragility and Deformability tests. On the other hand, efficacy of in vivo drug delivery is influenced by the biological properties of the carrier cells, which can be monitored by the labeling method. Therefore, in vitro and in vivo evaluation of loaded carrier cells is important and described below.

### *IN VITRO* EVALUATION

**1) Osmotic fragility Test**: After loading procedure, this simple test determines the integrity of cell membrane and resistance of cells to the changes in osmotic pressure (3, 24, 27, 28). In this test, 0.1 ml aliquots of packed samples of each type of erythrocytes i.e. native and loaded, are suspended in NaCl aqueous solution or in 1.5 ml of K+-reversed HBSS having the osmolarities of 0 to 300 mosm/liter. After gentle shaking at 37°C for 15 min, suspensions are centrifuged at 500 g for 5 min, and the absorbance of the supernatants is determined spectrophotometrically at 540 nm. In each case, release of hemoglobin is determined by comparing the percentage of absorbance of individual sample to that of the completely lysed sample. Samples are prepared by diluting 0.1 ml of packed cells of each type with 1.5 ml of distilled water. For comparative studies, osmotic fragility index was defined as, the NaCl concentration required to produce 50% hemoglobin release (28, 29).

**2) Turbulence fragility Test**: This test is performed to determine the integrity of cell membrane after the loading process. This test also reflects the resistance of loaded cells to hemolysis against the turbulent flow of circulation (3, 28, 30). In this test, either isotonic cell suspension is passed through needles bearing very small internal diameter e.g.30G or it is shaken vigorously. In both the cases percentage of hemoglobin release is measured (29).

In detail, turbulence fragility test for carrier erythrocytes is evaluated as follows (56). In the first step, 0.5 ml samples of packed cells of individual type

are suspended in 10ml of eutonic $K^+$-reversed HBSS and are shaken vigorously for 4 hrs at high speed of 2000 rpm. Hemoglobin release from erythrocytes is measured by withdrawing 0.5 ml portions from each cell suspension at 0, 0.5, 1, 2, 3, and 4 hr and after centrifuged at 500 g for 5 min. Absorbance of the supernatant was determined spectrophotometrically at 540 nm. The percent of hemoglobin release will be determined in reference to a completely lysed cell suspension with the same cell fraction.

In order to determine the turbulence fragilities of individual cell suspensions, turbulence fragility index (TFI) is defined as, shaking time required to produce 20% release of hemoglobin from erythrocytes (3, 28).

3) **Deformability Test**: This test helps in determining the life span of carrier cells in circulation. Dehpour et al.(3, 28) developed this test. This test determines the cell survival while passing through narrow pathways in RES. Therefore, in this test transit time is measured, when a definite volume of cell suspension is allowed to pass between two points in a capillary tube with a diameter of 4 micrometer or it is measured as, time taken by the solution is measured when passed through a polycarbonate filter with an average pore size of 45 micrometer.

Turbidimetrically deformability test for erythrocytes is measured by inducing the cell suspension with different concentrations of chlorpromazine HCl. (28, 29). In this test, 10 micro liters of chlorpromazine HCl solutions in saline is added to 1 ml of erythrocyte suspensions in eutonic K+-reversed HBSS with a hematocrit value of about 1% in order to produce the final concentrations of 5, 10, 15, 20, 30, 40, and 50 µM. In each case using a two-channel pen recorder, change in intensity of transmitted light beam was analyzed.

Other tests such as Mean corpuscular Volume (MCV), Mean Corpuscular Hemoglobin (MCH), Mean Corpuscular Hemoglobin Content (MCHC) can also be used to evaluate and estimate the biological state of the cells in vitro (3).

***IN VIVO* EVALUATION**

*In vivo* life span of the drug loaded carrier cells is determined using radioactive labeling method. The life span of the cells depends on the size, shape, surface electric charge, and extent of loss of hemoglobin and other cell constituents during loading procedure. In this method, loaded carrier cells are labeled with radioactive material. In most of the studies $Cr^{51}$, $I^{125}$ or florescent markers such as florescin isothiocyanate (FITC) or encapsulating C-14 containing sucrose or gentamicin is used (31-34). The life span of carrier cells is determined by observing the rate of disappearance of cells from circulation. Initially there will be high rate of disappearance of cells because of the abnormal sized and shaped cells will be trapped and eliminated by RES. Disappearance of carrier cells follows bi-exponential kinetics (3, 18, 31, 32, 35).

***IN VITRO* STORAGE**

Drug loaded carrier cells are very susceptible to changes in the surrounding environment. To prevent them from getting damaged by all means of sources they has to be stored in a suitable and stable environment until they are clinically used.

One of the most convenient methods of storing carrier cells is by suspending them in isotonic buffered solution containing all nutrients that are essential for the cells. Hank's balanced salt solution (HBSS) (32, 36, 37) or acid citrate dextrose (ACD) are used in maintaining the cell suspensions at low temperatures. By this method cell suspensions are stored for at least two weeks without any significant changes in their physiologic and carrier characteristics cells.

Another method of storing carrier cells is by exposing the cells to membrane stabilizing agents such as glutaraldehyde, DMSO, Di-methyl-3,3-di-thio-bis propionamide (DTBP) and toluene-2,4-di-isocyanate (TDI) etc., Later cell suspension is subjected to dehydration either by lyophilization or by sintered glass filtration. The resultant product obtained in the form of powder can be stored for about 1 month without any significant changes in carrier characteristics. The main drawback with this method is decreased cell survival time in circulation because considerable amount of membrane stabilizing agent will still remain bound to the carrier cells.

Freezing drug-loaded cells using "high glycerol freezing technique," reversible immobilization of carrier cells in gels such as alginates and soft gelatin, encapsulation of a stable prodrug, addition of purine nucleosides or calcium chelating agents to the suspending medium also improves the cell survival time in circulation upon reinjection (3, 32, 34).

It was observed that on prolonged storage of drug loaded erythrocytes; internal osmolarity of erythrocytes was increased and resulted in the damage of membrane. The reason is due to the production of lactate. With increase in the time of storage, concentration of lactate production also increased that resulted in the increase in osmolarity (38).

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