REVIEW ARTICLE

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Engineered Erythrocytes as a Drug Delivery System

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Engineered erythrocytes as a drug delivery system is a topic of interest to the pharmacists working in industry, research & development, education, drug control administration and profession. This review presents an overview of isolation, encapsulation methods, characterization, routes of administration, crosslinking, stability, survival *in vivo*, storage, mechanism of drug release, toxicity and immunological considerations, potential for targeting, recent advances and pharmaceutical application of engineered erythrocytes. This drug delivery system is endowed with several exclusive advantages and hence holds potential for further research and clinical application.

IN recent years considerable attention has been focused on the development of an ideal drug delivery system with desirable therapeutic response and capable of minimizing the "saw tooth" fluctuation of drug plasma level that accompany the periodic dosing. This phase of development was initiated by Paul Ehlrich in 1906, when he visualized the idea of drug targeting and foresaw the use of carriers to transport the drugs to the organs in question. Since then number of drug carriers have been devised and some new drugs and prodrugs have been synthesized to fulfill this objective. The approaches involve drug carriers and are now more popular in comparison to the generation of newer chemical entities, which involve lot of money and time to meet their screening protocol. Various drug carriers include the use of polymers, macromolecules, cellular components like erythrocytes, nanoparticles and various lipidic and non-lipidic organic molecules. Among these carriers the one which attracted researchers is the erythrocyte or red blood cell. The erythrocytes have been utilized as carrier for a wide variety of bioactive agents including drugs, enzymes, pesticides and DNA[1]. Ihler et al.[2] and Zimmerman[3] independently suggested that resealed erythrocytes could be useful as drug carriers. The term carrier RBC[4] was first introduced in 1979. When most of the hemoglobin of red blood cells and other cellular contents are retained, the cells on resealing lose some of the properties of normal erythrocytes and are referred to as "engineered erythrocytes (resealed erythrocytes)". Such erythrocytes which contain little or no hemoglobin are called "ghosts"[5].

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ADVANTAGES AND LIMITATIONS

They are the natural product of the body which are biodegradable and their isolation is easy and a large amount of drug can be encapsulated in a small volume of cells.

The entrapment of drugs does not require chemical modification of the substance to be entrapped.

They prolong the systemic activity of a drug while residing for a longer time in the body.

They protect the premature degradation, inactivation and excretion of proteins and enzymes and act as a carrier for a number of drugs.

They can target the drugs within the reticuloendothelial system and facilitate incorporation of proteins and nucleic acid in eukaryotic cells by cell infusion with RBC.

The erythrocytes being in common clinical usage in transfusions, the techniques for collecting and storing cells are very well known.

They have a limited potential as carrier to non-phagocytic target tissue and possibility of clumping of cells and dose dumping may exist[6].

Ideal requirements for encapsulation

A wide variety of biologically active substances (5000 - 600,000 daltons in size) can be entrapped in erythrocytes. Generally, the molecules should be polar or hydrophilic. Moreover, non-polar molecules have also been successfully entrapped e.g. adriamycin dipropionate salt[7]. Molecules which interact with the membrane and cause deleterious effects on membrane or interfere with membrane structure are not considered to be appropriate for encapsulation in erythrocytes, e.g. daunomycin[8].

Isolation of RBCs

Blood is withdrawn from cardiac/splenic vein puncture into a syringe containing a drop of anticoagulant. The whole blood is centrifuged at 1000 rpm for 5 min. at 4 ± 1^0 in a refrigerated centrifuge. The plasma and buffy coats are carefully removed and packed cells are washed and diluted with phosphate buffer saline and stored at 4^0 until used[9].

Encapsulation methods

The following methods have been used for drug entrapment in erythrocytes:

Hypo-osmotic lysis method

Dilution method: The erythrocytes have little capacity to resist volume. At an increase in volume above 50-75% of the initial volume and in hypotonic solution (corresponding to 0.6% sodium chloride), the erythrocyte membrane ruptures permitting escape of cellular contents and equilibrium is achieved within one minute, which results into cell swelling upto 1.6 times its original volume. The swelling results into the appearance of pores of 200 - 500 A^0 in size which allow equilibration of the intracellular and extracellular volume. Although this method[10] is the simplest and fastest yet the encapsulation efficiency is very low i.e. 1-8%.

Dialysis method: In this method a desired hematocrit [by dilution of packed RBCs with normal saline or phosphate buffer saline (PBS)] is achieved, then this mixture is placed into dialysis tubing and then both ends of the tube are tied with a thread. An air bubble of nearly 25% of the internal volume is left in the tube. The tube is placed in a bottle containing 100 ml of swelling solution. The bottle is placed at 4° for the desired lysis time. The contents of the dialysis tubing are mixed intermittently by shaking the tube using the tied strings. The dialysis tube is then placed in 100 ml of resealing solution (isotonic PBS, pH 7.4) at room temperature (25-30°) for resealing. The loaded erythrocytes thus obtained are then washed with cold PBS at 4°. The cells are finally resuspended in PBS. Good entrapment efficiency is obtained by this method. Later, a more refined procedure and apparatus were developed for dialysis of erythrocytes. The dialysis method is preferred over other methods when subsequent *in vivo* survival of erythrocytes is important[11].

Preswell dilution technique: It is based on the principle of first swelling the erythrocytes without lysis by placing them in slightly hypotonic solution. The swollen cells are recovered by centrifugation at low speed. Then, relatively small volumes of aqueous drug solution are added to the point of lysis. The slow swelling of cells results in good retention of the cytoplasmic constituents and hence good survival *in vivo*[12]. The method is simpler and quicker than the dialysis procedure and produces erythrocyte carriers which have good *in vivo* survival. Jain and Jain[13] reported modification and optimized preswell

technique which has maximum encapsulation efficiency ever reported.

Isotonic osmotic lysis technique: Billah et al.[14] have shown that transient permeability in erythrocyte wall could be produced using propylene glycol which also allows the drug to diffuse in. The lysed erythrocytes are resealed under isotonic condition by dilution with a glycol free medium.

Electrical breakdown:

The electrical hemolysis was pioneered by Zimmerman[15]. Electrical breakdown of a cell membrane is observed when the membrane is polarized very rapidly (in nano to microseconds) using a voltage of about one volt. At this potential difference, a dramatic increase in membrane conductance is observed. It was found that cell incorporated sucrose when pulse duration exceeded 20 micro sec at voltage of 1-2 KV/cm using mouse or human erythrocytes in ^{14}C sucrose solution[16]. Jain et al[17] reported loading of isoniazid and magnetite using this method. The method is very expensive and without any extra advantage over preswell and dialysis methods.

Endocytosis

Intracellular vesicles could be induced in erythrocytes containing small molecules, drugs or virus from external medium. This method is efficient for loading large particles such as viruses (upto 100 nm dia.), enzymes and small molecules[18].

Membrane perturbation

Antibiotics such as amphotericin-B damage microorganisms by increasing the permeability of their membrane to metabolites and ions. This property could be exploited for loading of drug into erythrocytes. Amphotericin-B was used to load erythrocytes with antileukaemic drug, daunomycin[19].

Lipid fusion method

Nicolau and Gresonde[20] fused lipid vesicle containing inositol hexaphosphate with human erythrocytes. The incorporated inositol hexaphosphate in erythrocytes provided a significant lowering of the O_2 affinity for hemoglobin in intact erythrocytes. The disadvantage of the method is low encapsulation efficiency.

The various factors affecting loading of drugs in erythrocytes include drug concentration, hematocrit value, lysing time, resealing time, mixing, magnetite concentration, sonication of magnetite temperature and pressure[21,22].

Characterization of Formulation

Drug-loaded cells can be characterized for drug content, *in vitro* drug release and hemoglobin content, morphology, osmotic fragility[23], osmotic shock[24], turbulence shock, erythrocyte sedimentation rate (ESR), zeta sedimentation ratio[25], miscellaneous (rheological and hematological) parameters[26].

Routes of Administration

Usually resealed erythrocytes during experimentation have been administered to the laboratory animals intravenously through caudal vein[27]. DeLoach and Drowleskey[28] studied the survival of resealed erythrocytes injected via intraperitonial injection in mice. DeLoach and Corrier[29] utilized subcutaneous route for slow release of entrapped agents. Recently Vyas *et al.*[30] have proposed erythrocyte-based nasal delivery of propanolol.

Crosslinking, stability and *in vivo* survival of resealed erythrocytes

Pinilla *et al.*[31] and Jain and Jain[32] reported *in vitro* stability of crosslinked red blood cells. The cells treated with dimethyl sulfoxide (DMSO). dimethyl-3-3' dithiobis propionamide (DTBP), toluene 2,4-diisocyante (TDI) and gluteraldehyde are even resistant to sonication, freezing and thawing. On the contrary, bis (sulfosuccinimidly) suberate (BS^3) and 3,3-dithiobis (sulfosuccinimidyl) propionate (DTSSP) crosslinked erythrocytes are less stable than control

erythrocytes. Chemical crosslinking of rat erythrocytes renders an yield of 55-97% of non-lysed cells. Extent of chemical modification was analyzed measuring free amino groups before and after crosslinking. An attempt was made to get drug loaded cells in lyophilized form. The dried powder was filled in amber color glass vials and stored at 4° for a month. Improvement in shelf life of the carrier erythrocytes was achieved by storing the cells in powder form, ready for reconstitution at 4°. This is important in the large scale manufacturing of drug loaded resealed erythrocytes[33].

According to Kinosita and Tsong[34] carrier RBC prepared from hypotonic dialysis, hypotonic dilution, hollow filter dialysis and the electrical hemolysis method appear to survive normally in circulation. Carrier RBC from chickens[35], rats and rabbits[36] have relatively less survival times than the carrier RBC from dog[37], cattle[38], sheep[39], pig, goat[40], mice[41] and monkeys[42]. The life span of RBC ranges from 60-140 days for mammals. A bimodal survival curve has been shown by host of carrier red blood cells. Initially, a rapid loss of cells in first 24 h observed which is followed by slow loss. The first 15% cells lost were of those cells which are damaged during *in vitro* handling. The second phase has a half-life of the orders of weeks. Murciano et al[43] reported biotinylation on erythrocytes to prepare circulation stable immunoerythrocytes capable of recognizing the antigen.

Storage

Considerable effort has been devoted to determine optimum storage conditions for erythrocytes. Most of the workers have used their loaded preparation in circulation immediately after encapsulation. Encapsulated preparation can be stored without loss of integrity when suspended in Hank's balanced salt solution [HBSS] at 4° for two weeks. Standard blood bag may be used for both encapsulation and storage. The other method could be to use Group'O' [Universal donor] cells and by using the preswell or dialysis technique, batches of the preparation could be prepared in advance and stored under conditions used for the normal storage of blood for transfusion[44]. Cryopreservation of erythrocytes at liquid temperature has also been reported[45].

Mechanism of Release

Drug may efflux out from erythrocyte carriers by [a] phagocytosis, [b] diffusion through the membrane of the cell, and [c] using a specific transport system[46]. The rate of diffusion depends upon the rate at which a particular molecule penetrates through a lipid bilayer. 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 e.g. nucleotides and nucleosides. Release of drugs from erythrocytes rapidly following sustained release profile and rate of exit is proportional to the instantaneous intracellular drug concentration [first order kinetics]. By incorporating polymer to erythrocytes, the release pattern may be modified. The drug, however, could be released from macrophages after phagocytosis if the linkage is susceptible to lysosomal enzymes[47].

Toxicity and Immunological considerations

The use of erythrocyte as circulatory blood carriers have shown no toxic effect as evident from animal studies. No side effects were observed when patients were infused with glucocerebrosidase encapsulated in erythrocytes as a replacement therapy in Gaucher's disease[48]. Green *et al*[50] reported no untoward effects in patients receiving desferrioxamine encapsulated in erythrocyte ghosts. Patients gave the normal value when tested for liver function and coagulation factors. Gluteraldehyde - treated erythrocytes have also been reported to be tolerated well in the liver, no damage results from their extensive and prolonged uptake. The autologous erythrocytes are not immunogenic. However, there is concern that the lysis procedure utilized for drug entrapment might elicit some cryptic antigens. Fiddler *et al*[51] and Desnick *et al*[52] examined this

phenomenon and found no immunological response against resealed erythrocytes.

Potential for Targeting

Some workers[5,53,54] selectively targeted drug-loaded erythrocytes after gluteraldehyde treatment to the liver or spleen. Attempts have been made to target drugs elsewhere, *in vivo* apart from uptake of carrier erythrocytes by RES. Kitao and Hittori *et al*[55] reported the use of a lectin [wheat germ agglutinin] to target daunomycin-loaded erythrocytes to tumor cells. Another novel approach is to magnetise the carrier so that the carrier cells can be retained at or guided to the target site by the application of an external magnetic field of an appropriate strength[56]. Retention of magnetic carrier at target site will delay reticuloendothelial clearance and prolong the action of drug. Recently Jain and Jain[57] have reported erythrocytes based lung targeting of rifampicin.

Recent Advances

Nanoerythrosomes are vesicles prepared by the extrusion of RBC ghosts, the average diameter of these vesicles being 100 nm. These spheroid particles were named 'nanoerythrosomes' and appear to be stable and maintain both the cytotoxic and antineoplastic activity of daunorubicin against mice leukemia P338-D- cells[58]. Jain and Jain[59] has reported nanoerythrosomes based delivery of 6-mercaptopurine. Significant advances have been made with the use of erythrocyte for specific targeting to cells of the immune system. Antiviral drugs can be pretreated to deliver drug directly to macrophages[60]. Several laboratory techniques have been developed for the encapsulation of allosteric effector of hemoglobin, inositol hexaphosphate, which are effective at oxygen delivery, much more effective than normal erythrocytes[61,62].

Pharmaceutical Applications

Carrier erythrocytes have proved to be useful for human and non- human applications. These are important tools of *in vitro* tests[63] and *in vivo* targeting to RES [for treatment of lysosomal storage diseases (B-glucuronidase)[64], treatment of Gaucher's disease (glucocerebrosidase)[65], treatment of liver tumors (adriamycin)[66], treatment of parasitic disease (primaquine phosphate)[67], removal of RES iron overload (desferrioxamine)[68], targeting of mycotoxin to livers[69], erythrocytes as circulating carriers (cytosine arabinosidase)[70], erythrocytes as circulatory bioreactors (arginase)[71], in enzyme delivery (L-asparaginase)[72], prevention of thromboembolism (aspirin)[73], targeting to lungs (rifampicin)[13].

CONCLUSION

During the past decade, numerous applications have been proposed for the use of resealed erythrocytes as carrier for drugs, enzyme replacement therapy and so on. The commercial medical applications of carrier erythrocytes are currently being tested in Europe by a recently formed company that is developing products for human use. In near future, erythrocytes based delivery system with their ability to provide controlled and site specific drug delivery will revolutionize disease management. The International Society for the Use of Resealed Erythrocytes [ISURE] through its biannual meetings provides an excellent forum for exchange of information to the scientists in this exiting and rewarding field of research. Success of the system is obvious as more than 200 bioactive agents have been incorporated successfully, some of which are in clinical use e.g. L- asparaginase[74].

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