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Carrier Erythrocytes (Red Blood Cells) for Delivery of Biopharmaceuticals

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Abstract

Application of erythrocytes as promising slow drug release or site-targeted delivery systems for a variety of bioactive agents from different fields of therapy has gained a remarkable degree of interest in recent years. The term biopharmaceutical is most commonly used to refer to all therapeutic, prophylactics, and in vivo diagnostic agents produced using live organisms or their functional components. At least in the US, biopharmaceuticals are often considered to include products manufactured using both 'new' technologies (recombinant DNA and monoclonal antibody/hybridoma) and 'old' technologies (fermentation, non-recombinant cell culture derived proteins, vaccines, and other products from live organisms including blood/plasma products). Thus, a biopharmaceutical results from bio-processing and can, therefore, be defined as the intersection of pharmaceutical technology and biotechnology. This, in turn, has evoked plenty of research projects with the ultimate goal of using the potential capabilities of these carriers in different clinical situations. Biopharmaceuticals are among the most widely exploited candidates for being delivered to the host body using these cellular carriers. Biopharmaceuticals, therapeutically significant peptides and proteins, nucleic acid-based biologicals, antigens and vaccines, are among the recently focused pharmaceuticals for being delivered using carrier erythrocytes. Here there is, the potential applications of erythrocytes in drug delivery have been reviewed with a particular stress on the studies and laboratory experiences on successful erythrocyte loading and characterization of the different classes of biopharmaceuticals.

Keywords: monoclonal antibody, biopharmaceuticals, characterization, therapeutic, prophylactics

Introduction

Carrier erythrocytes have been evaluated in thousands of drug administration in humans proving safety and efficacy of the treatments[1-3]. Erythrocyte -based delivery of new and conventional drugs is thus experiencing increasing interests in drug delivery and in

managing complex pathologies especially when side effects could become serious issues. Carrier erythrocytes, resealed erythrocytes loaded by a drug or other therapeutic agents, have been exploited extensively in recent years for both temporally and spatially controlled delivery of a wide variety of drugs and other bioactive agents owing to their remarkable degree of biocompatibility, biodegradability and a series of other potential advantages.

Drugs of the future:

It is thus synonymous with biotechnology pharmaceuticals and pharmaceutical biotechnology products[4]. Some 160 biopharmaceuticals have now gained medical approval and several hundred are in the pipeline. Most biopharmaceuticals are protein-based, although two nucleic acid-based products are now on the US/European market. An increasing proportion of approvals are engineered in some way and advances in alternative production systems and delivery methods will also likely impact upon the approvals profile over the remainder of this decade [5]. Development of biopharmaceutical products is a broad and multidisciplinary field. Science and technology are combined with new manufacturing, regulatory and commercial challenges [6]. Effective formulation development and appropriate delivery strategies for biopharmaceuticals are critical and timely issues. From DNA-based products to antibodies, vaccines to therapeutics, administered by every route known to medicine, formulation and delivery system play a critical role in the ultimate success of biopharmaceutical products [7].

Carrier erythrocytes:

Cellular carriers, including erythrocytes, leukocytes, platelets, islets, hepatocytes, and fibroblasts have all been suggested as potential carriers for drugs and other bioactive agents. They can be used to provide slow release of entrapped drugs in the circulatory system, to deliver drugs to a specific site in the body, as cellular transplants to provide missing enzymes and hormones (in enzyme or hormone replacement therapy), or as endogenous cells to synthesize and secrete molecules capable of affecting the metabolism and function of other cells. Since these carriers are actual endogenous cells, they produce little or no antigenic response, and upon aging or being damaged can be removed from the circulation by macrophages as a complete natural process. Another important feature of these carriers is that they can be stored at 4 °C for several hours to several days before re-entry to the host body, depending on the storage medium and the entrapment method used [8]. Since erythrocytes have been receiving the greatest attention for their potential applications as drug delivering microspheres [9–11].

The discussion that follows will be limited to these carriers. These carriers offer some unique characteristics such as excellent biocompatibility and biodegradability as well as considerably long life-span in circulation [9, 12, 13]. Upon re-injection to the same organism, these drug-loaded microspheres can serve as the intravenous slow-release carriers and/or targeted drug delivery systems especially to target the drug to the reticuloendothelial system (RES)[10–12,14].

Advantages and disadvantages of erythrocytes in drug delivery

Some of the most important advantages encouraging the use of erythrocytes in drug delivery include:

1. A remarkable degree of biocompatibility, particularly when the autologous cells are used for drug loading [11, 13].
2. Complete biodegradability and the lack of toxic product(s) resulting from the carrier biodegradation [11, 13].
3. Avoidance of any undesired immune responses against the encapsulated drug [15]
4. Considerable protection of the organism against the toxic effects of the encapsulated drug, e.g., antineoplasms [16]
5. Remarkably longer life-span of the carrier erythrocytes in circulation in comparison to the synthetic carriers [12, 13]. 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 [17, 18].
6. An easily controllable life-span within a wide range from minutes to months;
7. Desirable size range and the considerably uniform size and shape;
8. Protection of the loaded compound from inactivation by the endogenous factors [15, 20].
9. Possibility of targeted drug delivery to the RES organs [11, 13].
10. Relatively inert intracellular environment [15].
11. Availability of knowledge, techniques, and facilities for handling, transfusion, and working with erythrocytes [11, 13].
12. Possibility of an ideal zero-order kinetics of drug release [21].
13. Wide variety of compounds with the capability of being entrapped within the erythrocytes [13, 16, 22–24]
14. Possibility of loading a relatively high amount of drug in a small volume of erythrocytes, which, in turn, assures the dose sufficiency in clinical as well as animal studies using a limited volume of erythrocyte samples [13]
15. Modification of the pharmacokinetic and pharmacodynamic parameters of the drug [14].
16. Remarkable decrease in concentration fluctuations in steady state in comparison to the conventional methods of drug administration [11, 14, 25, 26], which is a common advantage for most of the novel drug delivery systems;
17. Considerable increase in drug dosing intervals with drug concentration in the safe and effective level for a relatively long time [11].
18. Possibility of decreasing drug side effects [11]

Drawbacks:

The use of erythrocytes as carrier systems also presents some disadvantages, which can be summarized as follows [14, 27–30]

1. 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 RES as result of modification that occurred during loading procedure in cells. This, although expands the capability to drug targeting to RES, seriously limits their life-span as long-circulating drug carriers in circulation and, in some cases, may pose toxicological problems.
2. The rapid leakage of certain encapsulated substances from the loaded erythrocytes.
3. Several molecules may alter the physiology of the erythrocyte.
4. Given that they are carriers of biological origin, encapsulated erythrocytes may present some inherent variations in their loading and characteristics compared to other carrier systems.
5. The storage of the loaded erythrocytes is a further problem provided that there are viable cells and need to survive in circulation for a long time upon re-entry to the host body. Conditioning carrier cells in isotonic buffers containing all essential nutrients, as well as in

low temperatures, the addition of nucleosides or chelators, lyophilization with glycerol or gel immobilization have all been exploited to overcome this problem.

6. Possible contamination due to the origin of the blood, the equipment used and the loading environment. Rigorous controls are required accordingly for the collection and handling of the erythrocytes.

Delivery Strategies:

As mentioned earlier, there are two major strategies in the delivery of drugs using erythrocytes as carriers which include intravenous slow drug release strategy and target gene delivery. Intravenous slow drug release strategy the normal life-span of an erythrocyte in systemic circulation is about 120 days [19]. As mentioned as an advantage, in the optimum conditions of the loading procedure (using more gentle methods for loading), the life-span of the resulting carrier cells may be comparable to that of the normal erythrocytes [17, 18].

Erythrocytes have been used as circulating intravenous slow-release carriers for the delivery of antineoplasms, [20, 31–38], antiparasitic, antiretroviral agents, vitamins, steroids, antibiotics and cardiovascular drugs among others. A series of mechanisms have been proposed for drug release in circulation from carrier erythrocytes, including passive diffusion out of the loaded cells into circulation, specialized membrane-associated carriers, phagocytosis of the carrier cells by the macrophages of RES and, then, depletion of the drug into circulation, accumulation of the drug in RES upon lysis of the carrier and slow release from this system into circulation [13] accumulation of the carrier erythrocytes in lymphatic nodes following subcutaneous injection of the cells and drug release upon haemolysis in this sites, and, finally, haemolysis in the injection sites (in cases of the injection routes other than IV).

Targeted drug delivery:

RES or non-RES ‘targeting’ is another important strategy using erythrocytes as carriers.

RES targeting

It is a well-known fact that, in physiologic conditions, as a result of the gradual inactivation of the metabolic pathways of the erythrocyte by aging, the cell membrane loses its natural integrity, flexibility and chemical composition. These changes, in turn, finally result in the destruction of these cells upon passage through the spleen trabecules [19]. The other effective site for the destruction of the aged or abnormal erythrocytes is the macrophages of the RES including peritoneal macrophages, hepatic Kupffer cells, and alveolar macrophages of the lung, peripheral blood monocytes, and vascular endothelial cells [48]. We know that aging and a series of other factors (e.g., stress during non-gentle loading methods) make the erythrocytes recognizable by the phagocytosing macrophages via changing the chemical composition of the erythrocyte membrane, i.e., the phospholipids component [47, 48].

Therefore, a considerable fraction of carrier erythrocytes that have undergone some degrees of structural changes during the loading procedure will be trapped by the RES organs, mainly the liver and spleen, within a short time period after re-injection. Consequently, a part of the encapsulated drug is depleted rapidly in RES. It has been shown that up to a definite limit of cell damage during the encapsulation procedure, the spleen is the preferred site for accumulation of carrier erythrocytes [50]. Non-RES targeting. Recently, carrier erythrocytes have been used to target organs outside the RES. The various approaches include:

- Co-encapsulation of paramagnetic particles or photosensitive agents in erythrocytes along with the drug to be targeted;

Application of Ultrasound Waves:

Site-specific antibody attachment to erythrocyte membrane. The magnetic erythrocytes, resulting from the co-encapsulation of the drugs with some Ferro fluids such as cobalt–ferrite and magnetite, have been reported to direct the encapsulated drug predominantly to the desired sites of the body by means of an external magnetic field [12, 16]. In addition to drug targeting, this method has been evaluated for induction of local ischemia in tumours which may consequently help in the tumour remission because of reduced local blood flow [16]. The magnetically guided erythrocytes have been tested successfully for targeting ibuprofen and diclofenac to inflamed tissues [13]. Photosensitized erythrocytes have been studied as a photo triggered carrier and delivery system for methotrexate in cancer treatment [51-52]. Price et al. reported the delivery of colloidal particles and erythrocytes to tissue through microvessel ruptures created by targeted microbubble destruction with ultrasound [53]. IV fluorescent erythrocytes were delivered to the interstitium of rat skeletal muscle through microvessel ruptures by insonifying micro bubbles in vivo.

This technique provides a non-invasive means for delivering resealed erythrocytes across the endothelial carrier to the target tissue. In another study, the differential response of photosensitized young and old erythrocytes to photodynamic activation has been studied by Rollan and McHale [54]. Among the other reports on drug targeting to non-RES organs, preparing the carrier erythrocytes fused to the thermo responsive liposomes, and their localization using the external thermal source [12], intraperitoneal injection of the carrier erythrocytes for drug targeting to the peritoneal macrophages [56], and pretreatment of the erythrocytes loaded by the antineoplastic drugs with the lectin extracted from the wheat to improve the targeting to the neoplastic cells [37] all have been associated with improvements in targeting index of the encapsulated drug.

Drug–erythrocyte associations:

There are two major approaches for the association between pharmaceuticals and erythrocyte carriers. The most widely used approach is drug encapsulation in erythrocytes using several encapsulation methods which are described below. The second approach is reversible or irreversible attachment of the ligand (e.g., drug) to red blood cell membrane. The most widely used strategy in the second approach is the so called ‘avidin–biotin’ technology.

Encapsulation:

Several methods have been reported for encapsulation of drug or other bioactive agents in erythrocytes [9]. Some of these methods such as electrical pulse methods and osmosis-based methods have a physical nature whereas the other methods such as the chemical perturbation of the membrane are chemically based. Regardless of the method used, the optimal characteristics for a compound to be encapsulated successfully in erythrocytes include a considerable degree of water solubility, resistance against inactivation within the erythrocytes, the lack of physical and/or chemical interaction with erythrocyte membrane or the other cell constituents, and well-defined pharmacokinetic as well as pharmacodynamic properties [12, 37]. Hypotonic haemolysis hypotonic dilution hypotonic dialysis hypotonic pre swelling and

osmotic pulse methods are categorized as osmosis-based methods. Chemical perturbation of the membrane [12], electrical breakdown or 'electroporation' [11–13] endocytosis [29] lipid fusion [12,13] laser loading, and intrinsic uptake of substances by erythrocytes [44] are other Membrane binding Avidin–biotin technology has reached several applications in biological sciences [56–58] including drug delivery [57– 59] during the last two decades. Membrane association of pharmaceuticals, especially biopharmaceuticals, by means of avidin–biotin bridges is the most widely used strategy for nonencapsulation loading of erythrocyte carriers with bioactive agents.

Biotinylation of intact mammalian erythrocytes could be performed either by attachment to the amino groups by means of biotin N-hydrosuccinimide ester (NHS–biotin) or by oxidation of the induced aldehyde groups of the erythrocyte membrane by biotin hydrazide. Comparison of these different procedures by Magnani *et al.* showed that biotinylation by NHS–biotin provides the highest cell recovery (N90%); the binding of approximately 1000 biotin molecules per cell (mouse RBC) and the 24 h survival in circulation was unaffected. Avidin–biotin bridges have been used for reversible membrane binding of uricase [62], HIV-1 tat protein, bovine serum albumin and several other biopharmaceuticals.

Therapeutic applications:

The potential therapeutic applications of carrier erythrocytes as a drug delivery system cover a wide spectrum of pharmacologic as well as therapeutic targets mainly based on the intravenous slow drug release as well as the targeted drug delivery [11–14]. Most of the drug delivery studies based on drug-loaded erythrocytes are in the preclinical phase. However, in some cases the successful clinical trials on this delivery system have been reported [11, 14]. Considering the scope of this article, in the following we will review the therapeutic possibilities of carrier erythrocytes in the delivery of biopharmaceuticals.

Amino acid-based biopharmaceuticals

Peptides and peptidomimetics

Enalaprilat, a peptide-like drug, is an angiotensin-converting enzyme (ACE) inhibitor widely used as its esterified orally absorbable prodrug, enalapril, in the management of hypertension and congestive heart failure. Using a hypotonic preswelling method, it has been shown that human erythrocytes loaded by enalaprilat release the drug *in vitro* according to zero-order kinetics. The *in vivo* results in rabbit model have indicated that the area under the ACE inhibition curve versus time over the entire course of study was significantly greater following the administration of the erythrocyte-encapsulated drug compared to the free drug. In addition, encapsulated drug inhibited the serum ACE with a slow trend, more efficiently, over a considerably longer time and in a more reproducible manner, than the free drug, thereby emphasizing the role of carrier erythrocytes as slow-release systemic drug delivery system for enalaprilat. Glutathione. Glutathione is an important naturally occurring tripeptide which has a critical role in oxidoreduction reactions within the body. Recent evidence has

Proteins.

Insulin. Diabetes mellitus was identified more than 2000 years ago as a fetal disease. However, Banting and Best have changed this scenario in 1921 with the discovery of insulin [56]. Subsequently, and at the beginning of the 1980s, large scale production of this therapeutic hormone in *E. coli* became feasible using recombinant DNA technology [64, 65]. Erythrocytes are among the numerous delivery systems exploited so far for the delivery of

insulin to improve pharmacokinetic parameters of this important biopharmaceutical as well as to facilitate the drug application. As a result of the presence of some potent intracellular proteases within the erythrocytes, encapsulation of insulin in these cells has not been of therapeutic significance. However, in one study, the co encapsulation of a protease inhibitor, tolbutamide, with insulin in erythrocytes has resulted in an entrapment efficacy of 5%. Erythrocyte ghosts have also been used for buccal and oral administration of human insulin to Wistar rats with some promising results.

Erythropoietin Recombinant human erythropoietin is an important haematopoietic factor widely used in the treatment of certain serious forms of anaemia. In vitro and in vivo studies have been performed using recombinant human erythropoietin encapsulated in human and mice erythrocytes, to achieve a higher stability with slow release of erythropoietin. Garin *et al.* have reported the prolongation of plasma half-life of erythropoietin in mice from 30 min to 35 h using carrier erythrocytes. These results suggest that the erythrocyte-encapsulated erythropoietin may serve as a promising alternative to the administration of free recombinant human erythropoietin in clinical settings.

Nucleic Acid-Based Biopharmaceuticals

Nucleosides, Nucleotides and Their Analogues:

The use of carrier erythrocytes containing nucleosides, nucleotides and their analogues, especially anti-retroviral agents, constitutes one of the most promising therapeutic applications of these cellular carriers. Because most antiviral drugs are nucleotide or nucleoside analogues, their entrapment and release through the cell membrane needs careful consideration. Nucleosides are rapidly transported across the membrane whereas nucleotides are not and, thus, exhibit prolonged release profile. The release of nucleotides requires conversion of these moieties to purine or pyrimidine bases. It is a well-known fact 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 key role in infection by HIV-1. These cells, which become infected immediately after exposure to HIV, are relatively resistant to virus attack and constitute an important reservoir for the virus [41]. Both AZT, an analogue of thymidine, and DDI, a nucleoside analogue, are reverse transcriptase inhibitors and both are prescribed as antihuman immunodeficiency virus (HIV) drugs. One therapeutic strategy has been based on protecting the macrophages against infection by HIV-1 using AZT and DDI co-encapsulated in erythrocytes using a murine AIDS model.

Mice treated with AZT–DDI–GSH-loaded erythrocytes, presented a proviral DNA content in the brain and in macrophages that was significantly lower than in mice treated with a combination of AZT and DDI [41]. Disseminated infection by *M. avium* complex (MAC) is one of the most common serious opportunistic infections in patients with AIDS. MAC is not killed by any standard antituberculosis drug except ethambutol at concentrations achievable in plasma. For in vivo killing of MAC, the drug must penetrate macrophages as well as the MAC cell wall. In practice, there is a need for therapeutic strategies to be able to inhibit HIV and *Mycobacterium* replication that permit reducing toxicity and prolonging administration. Carrier erythrocytes containing prodrugs for slow delivery of AZT and ethambutol have been successfully tested in in vitro studies [46]. Immunogenic biopharmaceuticals

Model Antigens.

The use of adjuvants is usually required to induce strong immunological responses to biotechnology- derived antigens like proteins and, especially, subunit peptide vaccines [67, 68]. However, in many cases, this adjuvant cannot be extensively used in human and veterinary vaccination because of associated inflammatory reactions or granuloma formation. Magnani *et al.* have reported that model protein antigens (bovine serum albumin, hog liver uricase, and yeast hexokinase); coupled to autologous erythrocytes by means of a biotin–avidin–biotin bridge, elicit an immunological response in mice similar to or even higher than that obtained by the use of Freund's adjuvant. Quantities as low as 0.5 µg/mouse are high enough to generate these immunological responses. They concluded that the delivery of antigens by autologous erythrocytes is an effective way to avoid the use of adjuvant for producing anti-peptide antibodies and possibly to generate peptide vaccines.

Bacterial Toxoids.

Erythrocytes may be of interest in the field of vaccines as natural carriers and/or adjuvant for antigens. Several attempts have been made accordingly. Polvani *et al.* have encapsulated three bacterial toxoids, a mutant of the diphtheria toxin, the tetanus toxoid and a double mutant of the pertussis toxin in murine erythrocytes by a hypotonic dialysis method.

Viral Subunit Vaccines.

As mentioned previously, the use of adjuvant is usually mandatory for the delivery of subunit peptide vaccines [67, 68] such as viral subunit vaccines. To minimize the use of adjuvant, erythrocyte carriers are good candidates for the delivery of retroviral subunit vaccines.

Cancer Vaccines.

It has been shown previously in several tumor systems that C-reactive protein (CRP) is an effective agent for generation of macrophage-mediated tumoricidal activity by means of suitable delivery systems like multilamellar vesicles (MLV). Gautam *et al.* have shown that resealed erythrocyte ghosts can function in the same manner. In their study, CRP associated with erythrocyte ghosts inhibited established lung metastases of T241 fibrosarcoma in C57B1/6J mice. This finding, along with their *in vitro* findings, indicated that erythrocytes could be considered as another delivery system for biological response modifiers for cancer vaccination to prevent metastases.

Conclusion

The controlled and/or targeted release of biopharmaceuticals is among the mostly attractive applications of erythrocyte carriers in drug delivery in the recent decade. In this context, a series of successful studies have been carried out to extend the benefits of these cellular carriers to peptide/protein, gene/oligonucleotide and vaccine delivery. Most of the studies in this area are in the *in vitro* phase and the ongoing projects worldwide remain to step into preclinical and, then, clinical studies to prove the capabilities of this promising delivery system.

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