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DRUG LOADED ERYTHROCYTES:OPPORTUNITIES AND APPLICATIONS

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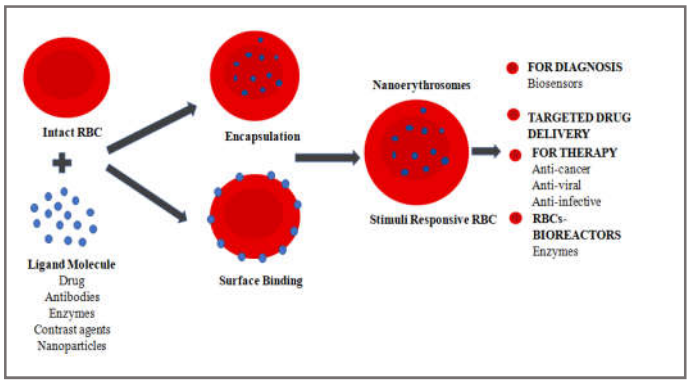
ABSTRACT

Erythrocytes as an endogenous, biocompatible, multi-purpose carrier molecule have been explored for many years. Therapeutic molecules loaded erythrocyte delivery system has enhanced delivery of many synthetic and biological agents. Loading of these agents include internal loading as well as surface attachments. Various therapeutic agents have been used to deliver through Red Blood Cells which include anti-malarial drugs, anti-diabetic drugs, enzymes, proteins and peptides, antibodies etc. Advances in RBC-based delivery systems include RBC's as nano carriers with the concept of nanoerythrosomes, design of stimuli responsive carriers etc. Hence, erythrocytes loaded drug delivery systems can be used as a multi-purpose cargo for delivery of various therapeutics agents, diagnostic agents etc. thereby enhancing clinical aspects of this delivery system.

Keywords: carrier, hitchhiking, nanoerythrosomes, stimuli sensitive, Theranostics.

INTRODUCTION



The major drawbacks with the use of injectable delivery systems are related to its circulation time and tissue distribution issues leading to its lower therapeutic potential. Development of novel carrier systems to deliver drugs is the current major challenge in the treatment of a variety of diseases. The ideal drug carrier must be safe, economical, scalable, non-immunogenic, target specific, having good drug loading capacity and good bio distribution throughout the body tissues. Drug carrier systems can be categorised as natural biological systems and synthetic biological mimetic systems. Various synthetic and polymeric systems are in current use such as liposome's, polymeric micelles, microspheres, etc. However, it is difficult for any synthetic system to completely mimic the body cells or membranes e.g. liposome's. Hence, biological systems have gained major attention to act as carriers for a variety of molecules. Natural Bio macromolecules have greater compatibility with the body cells and thus can be easily accepted by the body without any immunogenic responses.[1-3] Amongst the natural bio molecules, RBCs are considered to be the potential candidate for delivery of several types of drugs and cargoes including anti-infective agents, anticancer drugs, proteins and peptides, enzymes, vaccines, anti-diabetic drugs, drugs for wound healing, etc. The aspect of targeted delivery of drugs to the liver and reticuloendothelial system can also be achieved with the help of loaded erythrocytes. This potential to entrap or load a variety of cargoes makes RBC a versatile carrier. This cellular carrier also meets the most desirable qualities that are, biocompatibility and its safe degradation products.[4] Since there is rising demand for accuracy and diversity in drug delivery systems, it becomes difficult to fulfill all the drug delivery demands by mere use of naturally available RBCs as carriers. Hence, the natural erythrocytes are further modified depending on their intended function as a carrier. The RBCs can be modified as Pristine RBCs, RBC membrane cloaked nanoparticles and RBC mimetic nanoparticles.[2]

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Isolation of RBCs

RBCs for drug delivery are isolated from mice, rats, dogs, sheep, monkey, cattle, pigs, goats and rabbits. In order to isolate these mammalian erythrocytes, the blood is collected by vain puncture and put into heparinised tubes. It is found that the encapsulation capacity of freshly collected erythrocytes is more than that of old erythrocyte samples. Hence, fresh blood is generally used for loading purpose. Thus, fresh blood is collected and immediately stored at 4˚C and used within two days. The next step is harvesting and washing the erythrocytes by centrifugation. The washed erythrocyte samples are then suspended in buffer solution at different haematocrit values which generally includes acid citrate dextrose buffer at 4˚C for two days before use.[5]

Advantages and Disadvantages:

[1],[3],[6] Erythrocytes as drug carrier has various advantages and limitations as listed below.

Advantages:

1. High degree of biocompatibility.
2. Absolute biodegradability and no issue of toxicity that results due to carrier degeneration.
3. High life span of the resealed drug loaded erythrocytes compared to any other synthetic carriers.
4. Circulation of drug throughout the circulatory system.
5. Protection of the loaded drug against premature degradation and inactivation and also protect the host from any toxic immunological reaction due to drug.

6. They are non-immunogenic.
7. Large volume of drug can be incorporated in small volume of erythrocytes.
8. A wide range of chemicals such as enzymes, peptides, proteins and broad categories of drugs can be incorporated.

Disadvantages:

1. The major disadvantage associated with biodegradable carriers is their easy removal by RES in vivo.
2. Possibility of drug leakage from the cells.
3. Possibility of dose dumping from the cells.
4. Liable to bio contamination.
5. Physiology of RBCs may be altered sometimes.
6. The storage of loaded and resealed erythrocytes becomes a problem.

Ideal properties of drugs for RBC loading

The unique features of red blood cells make it appropriate for loading various therapeutic agents. Due to its elastic, biconcave shape it can easily sweep through narrow capillaries thus forming a specialised carrier system in the body. For loading molecules into or on the exterior of Red blood cells some special characteristics are essential. The agents should be hydrophilic, polar in nature. Hydrophobic molecules when present in salt form can also be incorporated. The agents should possess least physical and chemical interaction with erythrocytes membranes. Erythrocytes can bag vast heterogenous agents including drugs, enzymes, antibodies, etc. The size of molecules can vary (5000-600,000 Daltons in size).

MECHANISM OF DRUG LOADING: [6-8]

Several methods are investigated to ensure proper loading of cargoes within the inner RBC cavity or by binding them with RBC surface as depicted in Fig.1a, 1b and 1c. The drug loading is explored in order to ensure maximum loading of the drugs into the erythrocytes.

Encapsulation Methods:

Hypo osmotic lysis:

This method is based on the concept of reversible swelling of erythrocytes when placed in hypotonic solution. The cells absorb water and swell, but are able to maintain their integrity up to a certain extent after which they tend to burst and release their content out. Hence just before the point of cell lysis, the pores are generated in the membrane and drug can be loaded in erythrocytes. It includes various methods like dilution method, dialysis method, Isotonic Osmotic Lysis Method, Preswell Method.

- **Dilution Method:** Here, the erythrocytes are placed in hypotonic solution leading to swelling of the cells. This swelling results in pore formation on the cell membrane. The drugs can be thus loaded into the cells. The tonicity of the cells can be further adjusted by the addition of hypertonic buffer solution. This is a very simple and cost-effective method for drug loading. However, the entrapment efficiency using this method is very less to about 1-8%.
- **Dialysis Method:** In this method, dialysis bag is used which is filled with a mixture of RBC suspension and the drug to be loaded and the bag is being sealed off from both the ends. While tying the bag, 75% portion must be occupied by the the suspension mixture while in the rest part the air should be entrapped in the form of a bubble. This bubble formation is the critical step during drug loading procedure as it helps in creating proper blend of the contents. The bag is placed in swelling solution and after a certain period of lysis, it is transferred to a resealing buffer solution wherein the loaded erythrocytes are resealed. This method is used for loading of enzymes such as fl-glucosidase and 8-galactosidase c.
- **Pre swell Method:** This method involves swelling of RBCs first by placing them into hypotonic solution and then adding drug solution into it at the point of lysis thereby entrapping the drug into the erythrocytes.
- **Isotonic Osmotic Lysis Method:** This method is employed for incorporation of small molecules. Agents such as urea and polyethylene glycol are used for isotonic hemolysis. The RBCs are placed in a solution with high erythrocytic membrane permeability. The solute then enters into the cells due to chemical gradient developed. This is followed by water uptake until the osmotic gradient is restored and then the cells are resealed.

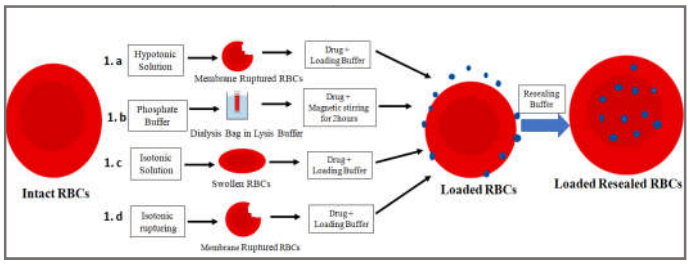

Figure 1 Hypo osmotic method of drug loading

Electro encapsulation method:

This method is based on the concept that application of electrical shock brings about irreversible changes in the RBC membrane. Hence transient electrolysis can be conducted to make desired membrane permeability and load drugs into erythrocytes. The pores of RBC membrane can be sealed off using resealing isotonic media. The cells are suspended in a medium and an external electrical field with pre-determined voltage is applied as explained in Fig.2(2). The optimum intensity of electric field is decided to bring necessary changes in the erythrocyte membrane. The drug to be entrapped is also added into the cell suspension. This method provides uniform distribution of the loaded cells and hence is advantageous over osmotic methods. However, it requires sophisticated equipment and process and hence its usage is limited. WenruiJin et. al. employed this method in order to incorporate Diclofenac sodium into human erythrocytes and its entrapment was determined further qualitatively and quantitatively.[9]

Membrane perturbation method:

The permeability of the RBC membrane is found to increase when exposed to certain chemical compounds. This logic was taken forward to entrap drugs into the erythrocytes. Fig 2 (3) depicts the effect of chemical agents on the permeability of RBC's. Compounds such as Amphotericin B and polyene antibiotics can be used to increase the membrane permeability.

Lipid fusion method:

Lipid vesicles loaded with drug are directly fused with RBCs. This leads to the exchange of drug from the vesicles into the erythrocytes as shown in Fig 2 (4). Inositol hex phosphate is incorporated into erythrocytes by this method to increase the oxygen carrying capacity of the cells.[10] The major disadvantage associated with this method is its low drug entrapment capability.

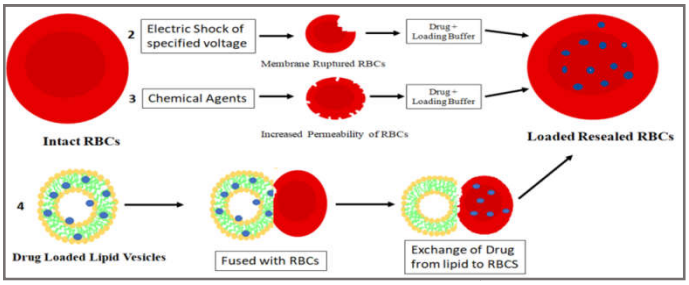

Figure 2 Electro encapsulation, membrane perturbation and lipid fusion method of drug loading

Surface Binding Method and RBC Hitchhiking:

Cargoes can be loaded onto the surface of erythrocytes either by covalent or non-covalent bonding. The binding of drugs to the RBC surface can help to increase its circulation in the body and alter their tissue distribution as well. This method is advantageous in case of drugs with higher molecular size wherein their encapsulation into RBCs via small membrane pores becomes difficult. Hence, binding of drugs onto RBC surface is preferred in such cases. Covalent binding of cargoes to the RBC surface helps to prolong the circulation life of the attached cargoes. Avidin–biotin interaction strategy is used for covalent binding of several bioactive agents to the RBC membrane. Biotinylation of RBCs is done using N-hydroxysuccinimidobiotin (NHS-biotin) by Magnani et.al. They reported that about 1000 biotin molecules per mouse RBC was obtained as highest recovery (about 90%) and their circulation was unaffected.[11] Another strategy used in covalent binding of cargoes is similar to the targeting approach. Here, initially affinity ligands are bound to the membrane such as antibodies or peptide chains. Further, the cargoes are loaded by covalent bond formation so that they can be delivered to the target site.[12] Non covalent bonding of nanoparticles to the erythrocyte surface is also referred as RBC hitchhiking.[13] Studies have reported that the adsorbed particles due to RBC hitchhiking remain in circulation until they remain adsorbed on RBC surface and are released due to shear forces and cell-cell interactions and cleared from the liver.[14] Fig.3 depicts the adsorption and release of particles. RBC hitchhiking helps to boost the delivery of nanoparticles to the target organs. Hitchhiking strategy improves the delivery of wide range of nanocarriers including viral vectors. Studies in mice and ex vivo human lung also shown higher targeting and less offside toxicity and hence the technology can be said to be clinically translatable.[15] By combining the advantage of circulating cells such as RBCs and synthetic nanoparticles, advanced drug delivery systems have been developed by Samir Mitragotri et. al. by adopting the concept of cellular hitchhiking.[16] Adsorbing nanoparticles on RBC surface has increased the circulation behavior of RBCs. Samir Mitragotri et. al. studied the delivery of nanoparticles to the lungs by adsorbing them on RBC surface in order to prevent their liver and spleen accumulation. This approach helped in increasing the blood persistence of nanoparticles to about 3 folds and about 7-fold higher lung accumulation.[17] Further, M. P. Nikitin et. al. carried out nanoparticle-based delivery using RBCs as carrier system by hitchhiking method. This approach was employed for the delivery of nanoparticles to the lungs for inhibition of lung metastases growth of aggressive melanoma B16-F1.Their findings concluded that the efficiency of nanoparticles is not governed by their circulation time, instead it is dependent on the complexation of nanoparticles to the RBCs via hitchhiking. Thus, this method for loading of nanoparticles can be further extended for the treatment of small cell lung cancer and other lung disorders as well.[18] Intravenously injected nano pharmaceuticals sometimes show adverse cardiopulmonary reactions. Hence, the work by Moghimi et. al. focused on reducing these side effects by surface modifications and complexing them with erythrocytes via hitchhiking strategy.[19] Samir Mitragotri et. al. in another work conducted Erythrocyte leveraged chemotherapy (ELeCt) which demonstrated the adsorption and assembly of nanoparticles on erythrocyte membrane. This strategy was found to be useful in combating lung metastasis which otherwise with conventional chemotherapy was found to be ineffective due to its poor tumour accumulation and inefficient targeting.[20] Thus, RBC hitchhiking can be considered as an excellent upcoming strategy to improve the effectiveness of nanoparticulate drug delivery systems.

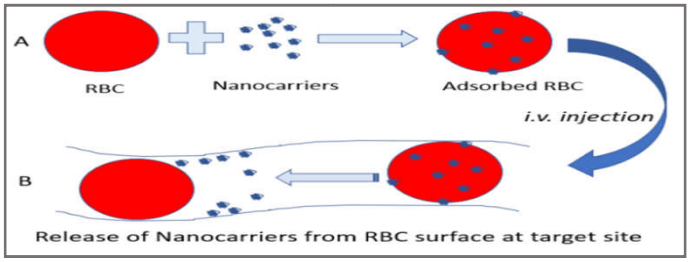

Figure 3 A. Ex-vivo adsorption of Nanocarriers onto RBC surface
Figure 4 B. Transfer of loaded Nanocarriers from RBCs to first downstream organ's capillaries

DRUG RELEASE MECHANISMS

Drug release mechanism from resealed erythrocytes involves:

1. Passive diffusion
2. Specific membrane linked carrier transporters
3. Slow drug release from internal cavity of macrophages upon phagocytosis of drug loaded erythrocytes. Subcutaneous administration of drug loaded erythrocytes gets accumulated in lymph nodes there by release drug after hemolysis.

In-vitro release studies vary depending on the properties of drug. Different drugs show different release patterns.

1. Lipophilic drugs like primaquine, methotrexate, dexamethasone shows increased drug release from RBC's when compared with rate of hemoglobin release from intact RBC's3.
2. Polar drugs like enalprilat, gentamicin, enzymes like as paraginase shows comparable release with hemoglobin release from RBC's. Also release of such drugs is not by diffusion process but dependent on complete hemolysis of RBC's there by initiating drug release. Drugs showing such release patterns can be used as controlled release systems and for targeting RES organs.[21], [22]
3. Drugs like propranolol, isoniazid shows intermittent drug release between diffusion and drug release initiated after hemolysis.[23]

CHARACTERIZATION OF DRUG LOADED ERYTHROCYTES : [6], [24], [25]

Loading erythrocytes with drugs involves various parameters to be studied and characterized simultaneously. Cellular, biological and physical aspects are the important parameters which get altered during and after the drug loading into or on the surface of the erythrocytes.

The characterization of resealed erythrocytes is summarized in table no. 1.

Table 1: Characterization parameters for drug loaded erythrocytes

	Parameters	Methods
A.	**Cellular Parameters**	
1.	%Haemoglobin Content	Deproteinization of cell membrane followed by haemoglobin assay
2.	Osmotic Fragility	Stepwise incubation with isotonic to hypotonic saline solution and determination of drug and haemoglobin
3.	Turbulent Shock	Passing Cell Suspension through a 23-gauge needle, hypodermic needle(10ml/min), and estimation of residual drug and haemoglobin
4.	Erythrocytes Sedimentation Rate (ESR)	ESR Apparatus
5.	% Cell Recovery	HaematologicalAnalyser, Neubaeur's Chamber
6.	Cell Volume	Laser Light Scattering
B.	**Biological Characterization**	
1.	Pyrogenicity	LAL Test, rabbit fever response test
2.	Sterility	Aerobic and Anaerobic cultures used and involve Sterility testing methods
3.	Toxicity	Animal toxicity testing
C.	**Physical Parameters**	
1.	Size, Shape, Surface Morphology	Transition Electron Microscopy, Scanning Electron Microscopy, Optical Microscopy, Phase Contrast Microscopy
2.	Vesicle size and shape	Transmission (TEM) electron microscopy, optical microscopy
3.	Drug Release	Dialysis bag method or diffusion cell assembly
4.	Drug Content	Deproteinization of cell membrane followed by the assay of released drug
5.	Surface Electrical Potential	Zeta Potential measurement by Photon Correlation (PCS)
6.	Surface pH	pH sensitive probes
7.	Deformity	Capillary Method

IN VITRO STORAGE OF CARRIER ERYTHROCYTES

Proper storage conditions are vital for maintaining the survival, integrity and drug content of the loaded RBC. The storage media frequently used involves Hank's balanced salt solution and acid-citrate-dextrose at 4°C.At lower temperatures the integrity of cells and the carrier attributes remain viable at least for a fortnight. To improve the survival time after reinjection into the system agents like calcium-chelating agents or the purine nucleosides can be added. Cryopreservation of RBC loaded agents at liquid temperature can also be considered.[24,25]

LIFE-SPAN OF ERYTHROCYTE LOADED DRUGS

The durability of the drug loaded erythrocytes after re-injection into the human body is one of the vital parameters for effective drug delivery into the circulation. Prolonged life-span of loaded erythrocytes is required for sustained drug delivery in the circulation. The life span of drug loaded erythrocytes inside the systemic circulation depends on surface charge, size, shape, leakage of hemoglobin and other constitutional changes during the process of drug loading. Several methods have been reported for determining the survival time of loaded erythrocytes. Methods include labeling the carrier cells by Cr51 or the fluorescence markers such as fluoresce in isothiocyanate (FITC). Compounds like gentamicin, C-14 containing sucrose when encapsulated in drug loaded erythrocytes, remain in the cell till the survival time of the erythrocytes. Lysis of carrier erythrocytes is indicated by leakage of such compounds in plasma. Initially, rapid disappearance of drug loaded erythrocytes in circulation is reported up to 24 hours after re-injection due to abnormal morphology of the cells followed by slow decline phase of half-life varying from days to weeks depending upon the individual test organism.[3,23]

APPLICATIONS OF DRUG LOADED ERYTHROCYTES:

RBCs have potential role as a carrier system in the delivery of variety of drugs, proteins and peptides, enzymes and several other types of cargoes as well.[26,27] Fig. 4 summarizes applications of RBCs. Various small as well as large molecules can be either encapsulated or adsorbed on the RBC surface to ease their delivery into the body. Therapeutic application of erythrocytic drug carriers focus on potential drug delivery as slow drug releasing intravenous systems and drug targeting to reticuloendothelial System.[28,29]

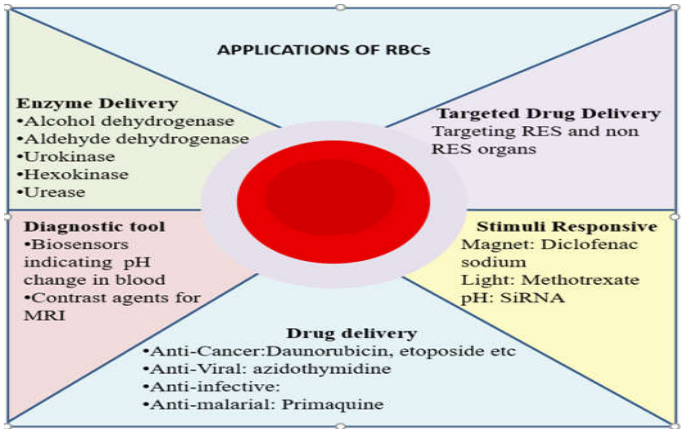

Figure 4. Applications of RBC as a carrier

This passive drug targeting can be achieved by surface modification in order to develop selectivity and specificity towards the target cells.[29] This surface modification can be brought about by making the use of several agents such as antibodies, glutaraldehyde, carbohydrates, sulfhydryl and chemical cross linking by adsorbing on the RBC membrane. Convenient modifications by inserting bifunctional antibodies and cross linking have been exploited for delivery of antibodies to target organs for inducing immunogenicity and immune tolerance.[30] Since enzyme administration is associated with several disadvantages such as their shorter half-lives, frequent administration and degradation and instability, loading them into RBC carriers can be proved effective. Variety of enzymes such as alcohol dehydrogenase, aldehyde dehydrogenase, urokinase, hexokinase, urease, uricase, rhodanese, etc. have been tried as prototype enzymes by loading them into RBCs to see the effectiveness of erythrocytes as enzyme carriers.[31,32] Erythrocytes can be used as natural carriers and adjuvants of antigens. Xiao Han et. al. developed nanoerythrosomes in which tumor antigens were loaded by fusing cell membranes with antigens. These fused antigen-nanoerythrosomes shown tumor inhibition in vivo with B16F10 and 4T1 tumor models. Thus, the antigens can be delivered this way for enhanced chemotherapy.[33] Purulent diseases are the complications of wound infections which can be effectively treated by encapsulating cytokines and IL into erythrocytes by hypotonic preswelling method and bring about their targeted delivery. According to Kulzhan Berikkhanova et al. RBCs can carry cytokines and other drugs to the infected site wherein the erythrocytes undergo phagocytosis thereby releasing the free drug. The wound healing dynamics assessment was done and significant wound healing was observed based on visual and microbial results by improving pharmacokinetics of RBC

ghost.[34] Delivery of Metformin oral tablets is associated with some gastrointestinal effects along with problems of poor bioavailability. Development of controlled release parenteral formulation by loading metformin in erythrocytes by endocytosis method was suggested by Aneesh TP et al. as a way to overcome this issue. The near zero order release kinetics, comparable haematological indices with normal erythrocytes and maintenance of lipid asymmetry of erythrocytes makes them a perfect carrier for parenteral slow release depot formulation of metformin.[35] Since chemotherapy is the most commonly used treatment strategy for cancer, attempts are made to entrap several anticancer agents such as Etoposide, [36] daunomycin, [37] methotrexate, [38] doxorubicin, carboplatin via RBCs. Researchers have tried to increase the circulation time and enhance tumor targeting. Several anti-infective categories of drugs can also be entrapped into RBCs. N. K. Jain et. al. encapsulated antimalarial drug Primaquine into erythrocytes for prophylaxis and radical cure of malaria. [39] Other agents such as Gentamycin,[40] metronidazole [41] and systemic corticosteroids such as dexamethasone [42] can also be incorporated into erythrocytes for their effective delivery. Resealed erythrocytes also have biomedical applications such as nanosponges for detoxification and nanotoxoids for safe and effective toxin vaccination by adsorbing toxic molecules in the body.[43] Erythrocytes are also potential vectors for loading and carrying proteins and peptides, modified oligonucleotides and genes, and help in their targeted delivery. Several peptides including Anti-HIV peptides, antineoplastic peptides, etc. can be loaded into RBCs.[44] Anti-viral agents such as azidothymidine and antimycobacterial drug ethambutol can also be encapsulated into erythrocytes.[45] RBC encapsulation strategy is also used for the delivery of agents such as aspirin and heparin for the thrombotic treatment. Also, it has increased application in case of oxygen deficiency therapy by the incorporation of Inositol hexaphosphate into the RBCs. Agents for the treatment of other parasitic diseases such as antileishmanial drugs, antimalarial drugs and anti-amoebic drugs can also be successfully encapsulated into erythrocytes.[46], [47] Since a wide range and variety of agents and cargoes can be encapsulated into RBCs, it serves as a potential carrier in modern medicine.

NOVEL SYSTEMS DEVELOPED IN ERYTHROCYTES DRUG DELIVERY SYSTEMS:

Apart from encapsulating or surface attaching of therapeutic agents, studies have reported some novel approaches for delivery drugs through erythrocytes. Encapsulation of nanoparticles containing drugs in erythrocytes has been tried to improve the circulation time of drugs and further improve its targeting properties. Apart from its use as nanocarriers, stimuli responsive delivery of therapeutic agents using erythrocytes as carriers has been reported.

RBC's as nanocarrier:

Nanosystems like liposomes, nanoparticles, polymeric nanoparticles, dendrimers have varied application. Their properties and specificity can be enhanced by addition of hydrophilic polymers, targeting moieties etc which might include use of stimuli responsive methods like temperature, pH, enzyme responsiveness. The properties can also be enhanced by changing morphological and physiological aspects like size shape, elasticity etc. Still there exits certain challenges in delivery of drugs through nanocarries which includes unpredictable response in blood stream, stability in blood stream etc. Hence combining nanocarriers with RBC's to generate a drug delivery system will serve as a platform technology for various developed nanocarriers facing similar problems.

Nanoerythrosomes are rapidly taken up by RES system where lysosomal enzymes destroy the erythrocytes thereby causing rupture of the cells and releasing the active molecule. Encapsulating drugs in nanoerythrocytes will act as a depot for drug storage causing slow release of drug along with modification in drug dose and dosing intervals. Nanoerythrosomes find importance in various biochemical processes of our body. With application such as modulating functions of enzymes, carrying out respiration process.

Formation of Nanoerythrosomes:

Most nanoerythrosomes are produced using extrusion technique where in smaller vesicles of ghost erythrocytes are obtained under extrusion cycles carried out under inert conditions through polycarbonate filters. The brief procedure for formation of nanoerythrosomes with drug loading is listed in Fig5.

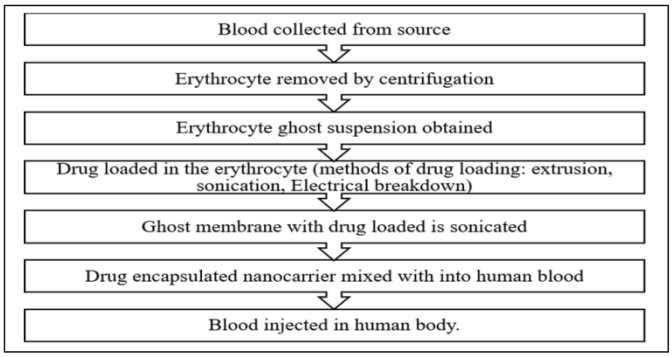

Figure 5. Formation of Nanoerythrosomes

Application of Nanoerythrosomes:

Nanoerythrosomes have demonstrated various applications which include modifying drug release, drug targeting, targeting the liver-enzyme deficiency/replacement therapy, treatment of hepatic tumours, treatment of parasitic diseases, enzyme therapy etc. [48] Nanoerythrosomes can be a part of various biochemical functions of our body. Enzymes can be administered through these nanocarriers for enzyme therapies required for enzyme deficiency, metabolic disorders etc. Examples of enzymes include β-glycoside, β-glucuronidase.[49] Nanoerythrosomes can be used as an excellent carrier system to encapsulate drugs and deliver them via inhalation route in order to bring about targeted delivery of drugs. Several drugs such as bronchodilators, rho kinase inhibitors, vasodilators, etc. can act as good substrate to be encapsulated into such nano engineered erythrocyte ghosts to deliver drugs by inhalation. Nilesh Gupta et al. used Fasudil as an investigational drug to see the effect of nano erythrocytes as a delivery carrier by comparing it with the effect of free drug. Fasudil is a rho kinase (ROCK) inhibitor which helps in reducing the symptoms of pulmonary arterial hypertension due to its signalling pathway. This drug was encapsulated into erythrocytes by hypotonic lysis–extrusion method and further the size was reduced to nano range. This was further incubated with rat pulmonary arterial membrane to see the uptake by the cells. The nanoerythrosomes were observed in the cytoplasm of these cells suggesting the uptake of encapsulated drug into the cells. The ROCK inhibition study was also carried out by Nilesh Gupta et al. to understand the inhibition and therapeutic potential of the encapsulated drug over free drug. It was found that the ROCK inhibition potential of plain Fasudil dropped the rho kinase levels to 65.57±7.89%. Whereas, the ROCK inhibition by nano erythrosomes containing Fasudil was found to lower the rho kinase levels to as low as 53.39±8.99%. These values suggest that there is increase in ROCK inhibition with nanoerythrosomes loaded

with drug. Thus, entrapment of Fasudil and similar drugs into nano erythrosomes can be a good approach to target inhalation route by development of formulation with favorable stability, entrapment and in vitro drug release profiles.[10] Jaya Agnihotri1 et al., focused on combining the concept of nanoerythrosomes with anti-malarial drug, artesunate.[50] Artesunate, when given intravenously, has a short half-life of 2.7 min. with renal toxicity. To overcome this toxicity and increase its half-life, artesunate was incorporated into nanoerythrosomes through glutaraldehyde cross links. The complexation was confirmed using FT-IR spectrum. The particles obtained were intact and spherical in shape. When the particle size of plain nanoerythrosome and artesunate loaded nanosrythrosomes was compared, it was found that artesunate loaded nanoerythrosomes showed increase of 5nm in particle size with respect to plain nanoerythrosomes with 0.5 poly dispersibility index. In-vitro drug release studies were carried our using dialysis bag method. Drug release kinetic studies showed zero order drug release from nanoerythrosomes up to 8 hours in an extended manner with increase in drug release after 8 hours indicating loss of integrity of the nanocarrier. In-vivo plasma concentration of artesunate drug solution and aretsunate loaded nanoerythosome was administered intravenously in albino rats. After 4 hours plain drug solution showed plasma concentration of 1.50µg/ml whereas artesunate loaded nanoerythrosomes showed plasma concentration of 5.52µg/ml. One of the desired properties for intravenously administered formulation is its re-dispersibility. Nanoerythrosome loaded artesunate formulation exhibited good dispersibility with saline.[48]

RBCs as stimuli sensitive Drug Delivery System and Theranostics:

Some drugs face difficulty to pass across intact RBC membrane and hence show a slow release pattern due to gradual degradation of RBC membrane. Similar to the other synthetic drug delivery systems, an attempt is made to modify RBCs as long circulating, modified release and stimuli responsive systems to deliver drugs at target site.[2] This modification is made based on the target cells especially tumor sites. Surface modifications help the RBCs to achieve selectivity and specificity towards the target cells. Engineered RBCs by coating with tumor targeting molecules such as folic acid and magnetic nanoparticles can be used even for capturing circulating tumor cells.[51] These modified RBCs further not only act as a mere drug delivery system, but also provide additional diagnostic values such as image-guided diagnosis and therapy and hence can be classified as Theranostics.[52] Table No. 2 summarizes examples of stimuli sensitive drug delivery systems using erythrocytes.

Table No.2: Stimuli sensitive drug delivery systems loaded in erythrocytes

SR.NO.	STIMULI	LOADED CARGOES	REFERENCES
1	Magnet	Diclofenac sodium, ibuprofen, anticancer drugs	55, 56,54
2	Light	Methotrexate	60, 61
3	pH	SiRNA	66
4	Ultrasound	Perfluoropentane (PFP) and Perfluorobutane (PFB) nanodroplets	58, 59
5	Glucose	Insulin	64
6	Enzyme	Doxorubicin	65

Magnetically stimulated RBCs:

Development of RBCs that is responsive to applied external magnetic field help in targeting of the entrapped drug at a pre decided location or target. These drug delivery systems are designed by coating the RBC membrane with magnetic nanoparticles and ferrofluids by adsorbing them on RBC membrane. It is possible to entrap magnetite particles in erythrocyte ghosts. U. Sprandelet. al. identified the influence and effect of ferrofluids on RBC membranes.[53] The magnetically active RBCs can be used for successfully targeting tumor sites to deliver anticancer agents thereby preventing non target toxicity. Caterina Cinti et. al. developed a drug delivery system with viral spike fusion protein and studied this site-specific localization of drugs. The higher entrapment potential of RBCs can reduce the dose to about 10 times.[54] S. P. Vyas and S. K. Jain studied the efficacy of ibuprofen loaded erythrocytes and diclofenac sodium loaded RBCs. The effect of external magnetic field, safety and tolerance towards various conditions was also studied.[55],[56] Apart from acting as mere drug delivery systems, magnetically activated RBCs also act as an effective agent for imaging guided therapy due to their intrinsic magnetic sensitive properties and hence can serve as an effective theranostic tool. Zhuang Liu et.al. developed a multifunctional drug delivery system which shows enhanced delivery of drugs in combination with image-guided therapy of cancer. They successfully loaded Doxorubicin into the erythrocytes in order to for a stable and long circulating drug delivery system. Here, the RBC membrane was precoated with iron oxide nanoparticles provide magnetically responsive RBCs.[57]

Ultrasound Stimulated RBCs:

Ultrasound responsive RBCs are developed with respect to their higher penetration potential and non-invasiveness. To study the sensitivity of RBCs towards ultrasound, a model drug was entrapped into erythrocytes and was conjugated with perfluoropentane (PFP) and perfluorobutane (PFB) nanodroplets. The ultrasound triggered release of model drug was evaluated. These acoustic stimulations can be further extended for its photoacoustic (PA) imaging application as well.[58] The method involves fabrication of perfluoropentane nanodroplets and conjugating them with loaded RBCs. The biodistribution and organ specific accumulation of RBCs in murine tumor cell was also studied. The photo acoustic imaging is done based on incorporation of Indocyanine green into the RBCs and the measurement of photoacoustic signal amplitudes was done to know the signal fluctuations as a tool for tumor imaging.[59]

Light Stimulated RBCs:

Near infrared light is majorly used as a trigger stimulus for targeting sensitive RBCs. A.P. McHale et. al. developed photosensitive erythrocytes loaded with methotrexate for targeted delivery to cancerous cells. The photosensitization was induced in erythrocytes with the help of hematoporphyrin derivative. Light dependent drug release was also studied using Hela cells as a target.[60] Ultrasensitive light-responsive erythrocytes were prepared by Zhuang Liu et.al. for light-controlled chemotherapy using Doxorubicin loaded RBCs.[61] Photo activated RBCs were prepared for synergistic chemo and photodynamic therapy. 5,10,15, 20-tetraphenylchlorin (TPC) was used as a photosensitizer which helps in promoting the photo trigger and tumor accumulation.[62] Zhuang Liu et. al conjugated Doxorubicin with an albumin bound near infrared dye and targeting was achieved by surface modification of RBC membrane with targeting proteins. Under the influence of externally applied near infrared laser, the RBC membrane gets ruptured thereby releasing

the incorporate drug at the target site. Hence, synergistic photo thermal and chemotherapeutic activity can be achieved.[63]

Other Stimuli responsive RBCs:

Apart from the major stimuli imparting elements, some other elements are also being tried to create response dependent targeting and drug release from RBCs. For example, Zhen Gu et. al. studied about insulin delivery via RBCs in response to the glucose levels in the body. Thus, glucose responsive RBCs prepared by membrane modification can be a potential delivery system to deliver 'smart insulin' at desired site.[64] Other triggers such as enzyme stimulus can also be used for stimuli dependent drug targeting and release as experimented by Kui Luo et. al. for doxorubicin delivery in conjugation with peptide dendrimer using enzyme as a trigger element.[65]

CONCLUSION

Drug loaded erythrocytes have proven to be a promising multi-purpose drug delivery system which is natural, biocompatible and safe. With continuous ongoing research in this field certain unmet technologies like blood cell-mimicking synthetic biomaterial particles and nano crystallization of blood cells needs to be focused on. RBC's as drug carriers can be a potential delivery system where in the transfer of molecules in the form of nanocarriers can reach the target sites of cells/tissues using affinity ligands. Although most of the studies are undertaken at lab level, efforts will be required for transferring the technology at commercial stage. With technology advancement, blood cell loaded therapeutic molecules can be a satisfying delivery system for the medical and diagnostic applications.
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