Novel Drug Delivery System: Resealed Erythrocytes

Vishal Vats[1], Manju Bala[1], Anju Dhiman [*1], Rohit Kumar [2], Sandeep Dhiman [3], Chhavi Singla [4]**

[1] Department of Pharmaceutical Sciences, Maharshi Dayanand University, Rohtak-124001, Haryana, India
[2] Department of Pharmaceutical Engineering and Technology, IIT BHU, Varanasi, Uttar Pradesh-221005
[3]Kinapse India Scientific Services Pvt. Ltd., Gurugram -122001, Haryana, India
[4] Department of Pharmacy, School of Health Sciences, Sushant University Erstwhile Ansal University, Gurugram, Haryana- 122003, India
[*]*Corresponding author: Dr. Anju Dhiman*
E-mail: anju.dhiman@mdurohtak.ac.in
Phone: +91-8295951007
[**]*Co-corresponding author: Dr.Chhavi Singla*
E- mail: chhavisingla@sushantuniversity.edu.in
Phone: +91-9268659221

Article History: Received: 13 March 2020; Accepted: 05 August 2020; Published online: 28 August 2020

Abstract

To overcome drawbacks of conventional drug delivery system, modern drug delivery approach such as resealed erythrocytes can be a potential delivery system. Resealed RBC's are biodegradable, biocompatible and have prolonged life cycle. A number of mammalian RBC's are being utilized in drug encapsulation and resealing for their utilization in enzyme and drug delivery. Resealed erythrocytes can be used for drug targeting, carrier for drug, enzyme, macromolecules and proteins. For getting drug loaded erythrocytes collect the sample of blood, then separate erythrocyte from plasma; then loading of drug into erythrocyte and the resulted drug loaded erythrocytes resealed.There are different methods of drug loading such as dialysis method, dilution method, endocytosis method etc.The survival time of RBC is dependent on the shape, size, senescent cells antigen, surface charge and haemoglobin content lost at the time of loading. The survival time decide their entrapment efficiency. Resealed RBC's have potential treatment in hepatic tumour, parasitic disease, removal of toxic agents, replacement therapy, oxygen deficiency therapy. Therefore, resealed can be better delivery method for target drug delivery.

Keywords

Biocompatible, Dialysis method, Drug targeting, Hepatic tumor, Oxygen deficiency therapy, Replacement therapy, Resealed erythrocytes.

Introduction

Conventional dosage form faces several drawbacks like first pass effect, instability, rapid release of drug, plasma drug fluctuations and require high dose. As a result, the fascinating type of drug targeted novel drug carrier system has attracted a lot. Among the various targeted oriented delivery system,

the cellular carrier, microparticulate and vesicular can avoid immune response intravenously by mimic body's intrinsic component. Erythrocytes, granulocytes, leucocytes and lymphocytes are different cellular carriers. Amongst them erythrocytes can administer DNA enzymes (Ihler et al. 1973), therapeutically active drug, pesticides etc. The term RBC i.e Red blood cell comes into existence in 1979. For getting drug loaded erythrocytes collect the sample of blood, then separate erythrocyte from plasma; then loading of drug into erythrocyte and the resulted drug loaded erythrocytes resealed. The alternative carriers for drug entrapment are Leucocytes and platelets. The resealed RBC carriers are biodegradable, biocompatible and have prolonged life cycle. It can be used for diagnosis and treatment of several disorders. Clinical research has become sophisticated due to the problems arising during its storage production and regulations. Besides this, a large number of pharmaceutical industries are performing preclinical study and clinical trial for resealed RBC (Chessa et al. 2014) (Domenech et al. 2011) (Leuzzi 2015) (Hunault-Berger et al. 2015).

Erythrocytes/ Red Blood Cells (RBC)

A healthy female and male has approximately 4.8 millions and 5.4 millions male RBC/ microlitre of blood. RBC are biconcave in shape with thickness of 2.2 micrometer and diameter of 7-8 micro meter. They are necessary for delivery of oxygen in blood organs. Mature RBC have no mitochondria which provide more area for transportation of oxygen. They live only for 120 days. In some species, old RBC's are recognized depending upon senescent cell antigen and then destroyed by phagocytic cells (in spleen). While in others, RBC are removed from circulation in a random manner.

Characteristics of modified RBC

The pharmacological role of RBC carriage is to boost bioavailability and increase the circulation of drug. But RBC carriage inhibit the circulation of already present longer lasting agents like IgG. e.g. RBC inhibit the endothelial FcRn mediated immunoglobin recycling mechanism by interacting with them (Sockolosky et al. 2015). Moreover, RBC coated with immunoglobin endured phagocytosis through different mechanism such as opsonization, multivatent involvement of FcRn-gamma and the mechanism is based on degree and nature of modification of RBC. RBC carriage also inhibit the functions of some cargoes. RBC carriage also alter the distribution of drugs in blood circulation in different ways like

- Increase the glomerular filtration and eructation through endothelial intracellular and intercellular pathways.
- Redistribution in blood from marginal plasma layer to main blood stream.
- Increased the intake via spleen (Villa et al. 2016) .

RBC also changed the excretion of drugs are excreted through urine, bile by shifting to hepatobiliary and reticuloendothelial intake (Anselmo et al. 2013).

Isolation and source

A number of mammalian RBC's are being utilized in drug encapsulation and resealing for their utilization in enzyme and drug delivery. Many of them are formed by using RBC's of dogs, cattles, goats, monkeys, mice, pigs and rabbits. The blood sample is collected by using heparinised tubes and ethylene diamine tetra acetate and heparin is added to prevent their coagulation. After that RBC's

harvested and centrifuged. Then obtained cells are suspended by buffering at distinct hematocrit value and sorted in buffer (acid citrate-dextrose buffer) at 4^{o} C for 48 hrs before use.

Requirements of encapsulation

Biological therapeutic substances should be hydrophilic for their encapsulation. The salt form of non polar substances is main requirement for their encapsulation. e.g.andromycin dipropionate entrapped in bovin RBC (DeLoach et al. 1980). Encapsulation is not possible for those substances which cause detrimental effect on membranous structure. e.g. Daunomycin (Kitao et al. 1973). Membrane permeating peptides (MPP's) is a new method for encapsulation of drug in erythrocyte. Few laboratories are using this method without making any demage to RBC (He et al. 2014). MPP's can be used for the intracellular delivery of small peptides in nanoparticles (Komin et al.2017). As RBC's are susceptible to lysis and can be destroyed by a single pore. The MPP's obtained from haemolytic venoms agglomerates and lead to formation of pores which looks like a pore as formed by complement. The main feature of MPP's activity can be determined by the minimum concentration require for the haemolysis. The physical and pathological stresses encountered by RBC in blood stream can be resisted by encapsulation. Cationic MPP's when interact with RBC glycocalyx produce undesirable consequences.

Different methods of drug loading

These methods have been employed for drug loading in erythrocytes:

1.Elecron-insertion/Electro encapsulation method

2. Hypo-osmotic lysis method

a) Dialysis method

b) Dilution method

c) Isotonic osmotic lysis method

d) Preswell method

3. Membrane perturbation Method

4. Endocytosis method

5. Lipid fusion method

Electroinsertion/ electro encapsulation method

In 1973, Zimmermann develop an electric pulse technique (electroporation) for encapsulation of bioactive substances (Kinosita et al. 1977). The technique utilize electric shock by bringing irreversible alternation in RBC's membrane. Kinosita and Tsog utilize transient electrolysis. The RBC membrane opened by dielectric breakdown. Then resealed pore through incubation at 37^{O}C in buffer. This technique depends on the difference in potential across the membrane which is created directly by using intra and intercellular electrode and indirectly by application of electric field to cells. The formation of pores takes place after breaking of RBC through electro chemical compression. This depends on ionic strength of medium pulse duration and strength of uniform loading of cells as

compared to osmotic technique (Kinosita et al. 1978). But there is need of speed of instrument and also sophisticated process. The entrapment efficiency is about 35%. The examples of compounds entrapped by this method are urease (Zimmermann et al. 1976) and sucrose.

Hypo-osmotic lysis method

This method depends on the ability of RBC's toplacedin isotonic saline solution. The exchange of intracellular and extracellular RBC's takes place through resealing and lead to osmotic lysis. Finally drug encapsulated in RBC.

a) Dialysis Method

Klibansky utilize this technique for the entrapment of proteins then modified by Jarde (Muldoon et al 1987). First a suitable hematocrit is made by making a mixer of drug and RBC suspension which is then collected in dialysis tubes. The inner side volume is inflated by using airbubble and then sealed so that the suspension of RBC cover only 75% of inner side volume. The role of air bubble is very critical. The dialysis tubes are placed in bottle containing swelling solution (100ml) for desired lysis and shaked for their proper mixing at 4^{O}C. After that dialysis tube placed in resealing solution (100ml) at 20-30^{o} C, then give washing to loaded RBC by cold phosphate buffer solution at 4^{O}C. then again resuspended in phosphate buffer solution. This method has entrapment efficiency 30-50% and recovery percentage is 70-80%.

b) Dilution Method

In it RBC placed in 0.4% Nacl (hypotonic solution) which lead to their rupturing and allow escaping of cellular contents then swelling of cells takes place. (i.e. 6 times of their original weight). This swelling allows the formation of pores having diameter 200-500Å (Ihler et al. 1987). For equilibrium of intracellular and extracellular concentration treat 1 volume of RBC with 2-20 volume of solution for allowing their loading in hypotonic solution at 0^{O} C for 5 minutes. Tonicity of solution can be resorted by mixing hypertonic solution [35, 36]. This method is used for low molecular weightt drugs rapidly. It has very low entrapment efficiency i.e. 1-8%. Dilution method is applicable for enzymes like arginase (Adriaenssens et al. 1976), asparaginase (Updike,et al. 1983), β-galactosidase (IhlerGMet al 1973), β-glucosidase (IhlerGMet al 1973) and bronchodialotors like sulbutanol.

c) Isotonic osmotic lysis method

This method depends on the transient RBC membrane permeability and utilize polyethylene glycol (Billah et al. 1977). Diffusion of biologically active drug maintain equilibrium inside the environment . RBC are incubated under isotonic solution then resealed the equilibrated RBC's. This method is applicable for very small molecules and utilize urea solution, polyethylene glycol and ammonium chloride for isotonic osmotic lysis.

d) Preswell method

Rechesteiner (Rechsteiner M. C. 1975) used this method firstly and then modifications performed by pitt et al (Pitt et al. 1983). This include the principle swelling of RBC in slightly hypotonic solution. Swollen cells recoverd by using centrifution then aqueous solution of drug is added in small amount until lysis takes places. The swelling of cells takes places slowly which result in best retention of cellular content. This method is simple and form RBC carrier

with best life life span *invivo*. This method is applicable to insulin (Bird,et al.1983), levothyroxine, asparaginase (Alpar et al. 1985), cyclophosphate (Pitt et al. 1983), propanolol , metronidazole, isoniazide , methotrexate.

3) Membrane perturbation method

This method is used by Hattori and ketao for entrapment of daunomycin in human RBC and mouse RBC. The permeability of RBC membrane is initiated on exposure to amphotericin B.

4**) Endocytosis method**

Schrier et al explain the endocytosis is method for entrapment of drug in RBC carrier 1 volume of RBC is placed in 9 volume of buffer (2.5m MATP,1mm $CaCl_2$, 2.5mm $MgCl_2$) then incubated at 25^OC for 2 minute. Creation of pores they are resealed by use of 154mm Nacl then incubated at 37^O C for 2 minutes. The endocytosed material separated from cytoplasm by vesicle membrane and prevent it from RBC and vice versa. This method is applicable for entrapment of drugs like vitamin A, chloropromazine, primaquine, phenothiazine, tetracaine, hydrocortisone and vinblastine .

5) Lipid fusion method

Drug entrapped in vesicular lipid carrier mixed with human RBC and human result in exchange of encapsulated drug. Gresoneleand Nicholau applied this method for entrapment of inositol monophosphate for improvement of O2 transport capacity. This method has very low entrapment efficiency be 1%.

***In vivo* characterization of resealed RBC**

The characterstics of resealed RBC depends on following parameters:-

1. **Physical characterisation**
   a)Surface and Shape Morphology: The shape is examined by comparing the ghost RBC untreated RBC for this Scanning electron microscope, TEM is used for this purpose.
   b) Drug Release: Drug release is calculated by diffusion and dialysis method.
   c) Percentage encapsulation of drug content : First, by using acetonitrile/ methanol, membrane of loaded RBC are deproteinized. Then centrifused at 3000 rpm. The clear supernatant is used for calculation of drug content.
   d) Electrical surface potential and surface pH : Surface pH and electrical surface potential is calculated by use of pH sensitive probes and zeta potential measurements.

2. **Cellular characterisation**
   a) *In vitro* haemoglobin content and release of drug
   From drug loaded cells, haemoglobin and *in vitro* release of drug can be estimated. Cell suspension (5% hematocrit in phosphate buffer solution) are stored in ambered colour glass container at 4^O C and observed for haemoglobin drug content.
   a) Percent cell recovery

It can be estimated by by "counting the number of intact cells per cubic mm of packed erythrocytes". The equipment used for this technique is Neubeur's chamber and Hematological analyser.

b) Osmotic Fragility

It is estimated by calculating the resistance of erythrocytes uptohemolysis after exposing a step wise dilute saline solution.

c) Osmotic shock

After dilution of RBC suspension with distilled water, centrifuged for 15 min at 3000 rpm. The resulted clear supernatant is measured by drug and HB content in using spectrophoto meter.

d) Turbulence shock

It is estimated when cell suspension is passed through hypodermic needle (10ml/min) for calculation of drug and Hb content.

e) ESR (Erythrocyte sedimentation rate)

It is estimated by using ESR apparatus by determining the stability of suspension in plasma. It depends on relatives concentration of plasma proteins number and size of RBC.

3.Biological Characterisation

It involve sterility testing in which testing of pyrogens is carried out by checking response for rabbit fever or LAL i.e limulus amoebocytes lysate and animal toxicity test.

Devising RBC derivative for drug delivery

Size plays an important role for determine the property of erythrocytes maximum in microns in blood circulations. Large size retain the drug but difficulty in accessing the extravascular target like tumour. In past years, scientist tried to invent nano and microparticle from RBC e.g, Submicron vesicles of RBC loaded with cargoes. In recent years ,*in vitro* study techniques have been explored for coating of synthetic nano particle with particles of RBC .

This sophisticated techniques is used for covering of nano particle with RBC membrane. RBC cloaked nano carriers act as a valuable tool for eliminatings the adverse effect and safe delivery of drug. Membrane vesicular carrier or exosomes serves as a promising tool for novel specific drug delivery system.

Routes of administration

The RBC carriers can be delivered by various routes like intraarterial, intravenous, intraperitoneal, subcutaneous.

Release mechanisms

Drug can be released through different mechanism like

- Special membrane release cell. Drug accumulated in macrophages following drug release.
- Passive diffusion.

- Through subcutaneous administration, in which RBC deposit in lymph node for drug following hemolysis.

Immunological consideration

Immunological consideration can occur in 2 ways:

Immunogenicity of RBC carriers : The capacity of RBC for preventing the encapsulated drug from detection of immunological detection. So the method of lysis applying for encapsulation can bring out few cryptic antigens (Atukorale et al. 2015)

***In vitro* storage and in vivo survival**

- ***In vitro* storage**
- The challenge facing by resealed RBC is their storage. The media for storage involve acid citrate dextrose and Hank's balanced salt solutions at 4^{O} C. At this temperature, cells remain in their countable form for 2 weeks. For improving their life span, some purine nucleosides and calcium chelating agents can be added. For increasing their stability, membrane stabilizing agets like DMSO (dimethyl sulfoxide), glutaraldehyde, dimethyl-3,3-dithiobiopropionate and toluene-2,4-disocynate are added. But the appreciable amount of stabilizing agents interfere with life span of RBC.

***In vivo* survival**

The survival time of RBC is dependent on the shape, size, senescent cells antigen, surface charge and haemoglobin content lost at the time of loading. The survival time decide their entrapment efficiency. Longer life span is required in case of rapid phagocytosis. But for a particular target in RES organs, shorter is needed. The life span can be estimated by using fluorescent markers like fluorescein isothiocyanate. Normally the survival time for RBC is 60-140 days in humans. In which 15% are destroyed during handling in in vitro method. The 15% of cells are lost those which are damaged during in vitro handling. The second phase has half-life in order of weeks. The RBC carriers are made up of RBC of dogs, cattle, pigs, mice, sheep, and monkeys. While resealed RBC are made by RBC of rabbits and rats.

Application of resealed erythrocytes in biomedical field

There are various applications of resealed RBC in designing medicine both in human and animals.

1. **As Drug\Enzyme carrier**
   RBC as enzymes/drug carrier can be used for novel drug delivery system.
   a) **As carrier for enzyme**
      As enzyme carrier resealed RBC can be used to prevent inherited metabolic diseases. The different enzymes for carrier are asparaginase, acetaldehyde dehydrogenase, alcohol dehydrogenase, beta glucoronidase, glutamate dehydrogenase.
   b) **As carriers for drugs**
      RBC can entrap various medicinal drugs like antibiotics, antineoplastic, antiparasitic, antiameoebic and steroids etc.

c) **As carrier for macromolecules and proteins**.
RBC can entrap various proteins and peptides like recombinant human erythropoietin, mycotoxin, insulin and recombinant interleukin -2 etc.

2. **For drug targeting**

Resealed RBC carriers can be designed into target specific drug delivery system. Surface modified RBC are used to target organs of mononuclear phagocytic system.

a) To the RES organs

The destroyed RBC are engulfed by the phagocytosis process in liver and spleen. They can be made target specific or selective by modifying their surface characterstics The methods are for modifications are as following (Ganguly et al.2007) :

- By modifying surface characterstics with antibodies.
- By modifying surface characterstics with glutaradehyde.
- By modifying surface characterstics with carbohydrates like sialic acid.
- By modifying surface characterstics with sulphydryl.

b) **To the liver**

I. Enzyme deficiency (Replacement therapy)

Enzyme replacement therapy is used to replace the absence of enzymes in disease. But this therapy is very sophisticated because of side effects to normal cells allergy short half life. So RBC carriers loaded by enzymes minimize all these drawbacks. B-Glucosidase, β-glucoronidase and β-galactosides are enzymes used for this method.

II Treatment of Hepatic tumour

RBC encapsulated by anti tumour drugs are designed to target liver cancer (Gupta et al. 2019) (Jijja et al. 2017). The antineoplastic drugs like asparaginase, andromycin, methotrexate and bleomycin etc. used by RBC carriers.

III Treatment of parasitic disease

Parasitic disorder involve the residence of parasite in RES organ. RBC carriers encapsulated by antiparasitic drugs like metrodazole, pantamidine, antiamoebic antileishmanial and antimalarial(Pitt et al 1983). can be used to prevent parasitic disorders.

IV Removal of Toxic agents

Cannon et al specify that the murine carrier RBC including bovine rhodanes and sodium thiosulphate show antagonistic activity cynide.

V Removal of RES Iron overload

On repeated transfusion of blood, iron accumulated in RES cells which is main site for it. Green et al designed desferoxamine, a iron chelating agent encapsulated in RBC for removing iron overloading in RES organs. This technique for management of hemolysis was approved by united states in 1984.

c)To organs other than those of RES

This utilize different apparatus like

- Antibody anchoald RBC
- Magnet responsive RBC ghost
- Photosensitized RBC
- Ultrasoung mediated delivery of RBC loaded drugs

3. As circulation bioreactors

RBC carriers can be used for the immobilization of enzymes and also act as circulation bioreactors.

a) Delivery of Antiviral agents

Resealed RBC entrap antiviral agents and act as target specific delivery system. Because a large number of antiviral drugs contain nucleoside which need to be converted into purine and pyrimidine analogues. Resealed RBC are applicable for the delivery of azidothymidine derivatives, acyclovir (Rossi et al. 1988),deoxycyclidine derivatives (Chiarantini et al 1975), azathioprine, recombinant herpes simplex type 1 and fludarabine phosphate (Fraternale et al. 1996).

b)Thrombotic Therapy

RBC can entrap thrombolytic agents like heparin (Eichler et al. 1986), brinase (Flynn et al. 1994) and aspirin (Orekhova et al. 1990) for a thrombolytic therapy.

c)Delivery of Interleukins

Interlukin-3, a cytokine control the functions and production of macrophage and granulocyte and also control the production of blood cells. The entrapment of RBC carrier with interlukin-3 is more in hypotonic method than in isotonic incubation method (Olmos et al. 2000).

4. In oxygen deficiency therapy

Inositol hexaphosphate (IHP) less ability to entrap in RBC. Its encapsulation in RBC lead to increase in oxygen release and decrease in cardiac output. It also alter the oxygen and haemoglobin affinity but in case of RBC encapsulation, it initiate the transportation of $CO_2$and O_2 between tissues and lungs.

The use of IHP encapsulated RBC is necessary in following conditions like

- Low partial pressure of O_2 in high altitude regions.
- The resistance to O_2 diffusion is more in lungs.
- O_2 transport capacity is reduced.
- Mutations or chemical modification including low O_2 affinity for haemoglobin.
- The sensitivity of radiation sensitive tumours increased.
- O_2 carrying capacity of blood is restored
- Myocardial ischemia

5.Microinjection of macromolecules

RBC lack complex structure like nucleus which is necessary property for encapsulation protein, RNA and DNA. The macromolecules loaded in RBC ghost by using hypotonic hemolysis technique

following fusion with HVJ (hemagglutionating virus of Japan) positive cell lines. Lymphocytes act as target. The fusion efficiency of HVJ is greater than that of polyethylene glycol as fusing agent.

6.Biotinylated erythrocytes

Biotin is a vitamin with low molecular weight. In biotinylation process, amino group of RBC membrane joined covalently with N-hydroxy succinimide ester of biotin so that biotin has higher stability on RBC surface. This technique was firstly explored by Mishra and Jain (Mishra et al.2002) and used for selective drug delivery. Surface modification makes it more biocompatible. Angiotensin converting enzymes (Danilov et al. 1991), intracellular adhesive molecules, thrombomodulin (McEvoy et al. 1987) (Burrows et al. 1994), E-selection (Pouliot et al.2002) and tumour endothelial antigen (Spragg et al. 1997) are designed by biotinylation process.

Novel apparatus

Erythrosomes

Erythrosomes are made by modifying reverse phase evaporation process. In this method, the human RBC's are used having cross linkage on which lipid bilayer is coded. This encapsulation system is useful for large molecular drugs (Pouliot et al.2002).

Nanoerythrosomes

Nanoerythrosomes have average diameter of 100nm. These nano-vesicle are made from membrane of erythrocytes by breakdown method such as sonification, extrusion, electrical breakdown to form uniform size. Mishra and Jain reported that reverse bio-membrane vesicle having doxorubicin drug (Mishra et al. 2003). Nanoerythrosomes have gave potential significance in clinical treatment.

Major risk and limitations

1)Problem in drug loading

a)For this formulation there in a requirement of blood and during manufacturing process of isolation, encapsulation. There is a risk of damage to erythrocytes due to which the resulted formulation will have fraction of initial amount of blood used. This problem limits the manufacturing and availability of the formulation.

b) Another problem is of quality control and storage parameters and regulation of drug loaded in erythrocytes. Drug loaded RBC's have phospholipid bilayer with glycocalyx (Svetina S., 2012). This complex allows RBC to circulate up to 3 months.

c) Drug loading on RBC's causes reduction of compatibility with biological system up to some extent.

d) Some of the drugs may diffuse out from RBC's and can interact with blood, cells etc (Bossa et al. 2013).

2)This delivery system results in loss of plasticity of RBC up to some extent.

3)Due to RBC based drug delivery system there is a inhibition of functions of CD47.

4)In, Modified RBC's elimination gets reduced because of adhesion to endothelium and entrapment in microvascular. Due to which inflammation may arise (Burnouf et al. 2015) (Antonelou et al.2016)

5)*In vitro* testing of this formulation such as ionic strength, osmotic resistance shows alteration of RBC's membrane physiology (Mock et al. 2014) (Pan et al. 2016).

Disadvantages of Resealed erythrocytes

Disadvantages of Resealed erythrocytes formulation are summarized as followed(Hamidi et al. 2007) (Millán et al. 2004)

- Biodegradibility of natural cells and materials and this causes toxicological effects in biological system.
- Dose dumping and clumping of cells may take place.
- Alteration of physiological of erythrocytes.
- Possibility of leakage of encapsulation drugs in from loaded RBC's.

Advantages

Resealed erythrocytes have following advantages:

- Biocompatibility.
- Large number of bioactive agents can be incorporated.
- Large amount of drug can be load in small volume of cell.
- Excretion, degeneration, inactivation of premature protein can be prevented.
- Resealed erythrocytes have long systemic activity.
- Target specific.

Conclusion

Resealed erythrocytes have potential for treatment in hepatic tumour, parasitic disease, removal of toxic agents, replacement therapy, oxygen deficiency therapy. Therefore, resealed RBC's can be a potential method for drug delivery system and can help to overcome over barrier's and drawbacks of conventional and current drug delivery system (Hamidi et al. 2007).

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