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Review Article

Resealed erythrocytes a specified tool in novel drug delivery system: A Review

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ABSTRACT

Among the various carriers used for targeting drugs to various body tissues, the cellular carriers meet several criteria desirable in clinical applications, among the most important being biocompatibility of carrier and its degradation products. Leucocytes, platelets, erythrocytes, Nano erythrocytes, hepatocytes, and fibroblasts etc have been proposed as cellular carrier systems. Among these, the Resealed Erythrocytes have been the most investigated and have found to possess greater potential in drug delivery. The main objective of this review is to explore the various features, drug loading technology, and biomedical applications of resealed erythrocytes. It deals with the various research work done on Resealed Erythrocytes providing an overall review about the different advantages and applications of Resealed Erythrocytes in different fields. Wide varieties of drugs like Anti-inflammatory, Steroidal and Chemotherapeutic agents are seen to have reduced side effects upon incorporation into these carriers. The morphology, isolation techniques, properties and methods of drug loading are highlighted in this paper along with the characterization and applications of resealed erythrocytes.

1. Introduction

Erythrocytes, the most abundant cells in the human body,have potential carrier capabilities for the delivery of drugs. Erythrocytes are biocompatible, biodegradable, possess very long circulation half-lives and can be loaded with a variety of chemically and biologically active compounds using various chemical and physical methods**[1, 2]**.

Erythrocytes or Red blood corpuscles (*erythro-* red; *cyte-*cell) are biconcave discs with diameter of 7–8 μm. Erythrocytes contain the oxygen-carrying protein haemoglobin, a pigment that gives whole blood its red colour**[3]**. Erythrocytes are highly specialized for theiroxygen transport function. These are produced in the bone marrow by regulatory effect oferythropoietin**[4]**. Around 760g of haemoglobin in total which is about 10% of total protein content of the body is contained in the erythrocytes**[5-7]**. The enclosed haemoglobin transport oxygen for prolonged period**[8]**. The sources of energy to the erythrocytes are glycolysis and hexose monophosphate shunt. Erythrocytes travel 250 km throughout the cardiovascular system in their life span of 100 – 120 days**[9]**. The processes of erythrocyte production within the body are called as erythropoiesis; the erythrocytes are produced in red bone marrow under the regulation of a hemopoeitic hormone known as erythroprotein**[10]**.

1.1 Isolation of erythroytes**[12]**

Various types of mammalian erythrocytes have been used for drug delivery, including erythrocytes of mice, cattle, pigs, dogs, sheep, goats, monkeys, chicken, rats, and rabbits. To isolate erythrocytes, blood is collected in heparinized tubes by vernipuncture. Fresh whole blood is the blood that is collected and immediately chilled to 400°C and stored for less than two days. The erythrocytes are then harvested and washed by centrifugation. The washed cells are suspended in buffer solutions at various haematocrit values as desired and are often stored in acid–citrate– dextrose buffer at 400°C as long as 48 hours before use.

1.2 Reasealed erythrocytes**[14, 15]**

Resealed erythrocytes are the part of parental control release formulation, RBCs have been used extensively studies for their potential carrier & capability for delivery of drug loaded microsphere. In this carrier erythrocytes are prepared and the blood sample is collected from the organism of interest, the erythrocytes are separated from the plasma, then the entrapping drug in the erythrocytes & resealing the cellular carriers. Hence the overall process is based on the response of these cells under osmotic condition. Through the process of reinjection, the drug loaded erythrocytes provide slow circulating depots & target the drug to a disease tissue organ.

1.3 Advantages of resealed erythrocytes**[16- 20]**

Resealed Erythrocytes possesses number of advantages which involves the modification of the pharmacokinetic and pharmacodynamics parameters of the drug according to the need of disease. Considerable increase in drug dosing intervals with drug concentration in the safe and effective

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level for a relatively long time had also established with the use of resealed erythrocytes. Completely controllable loading procedure is involved. Desirable size range and considerably uniform size and shape can be achieved. Ideal kinetic release i.e. zero order release is followed with the use of resealed erythrocytes.

1.4 Disadvantages of resealed erythroytes[21-23]

The main problem encountered with this drug carrier is that they remove in vivo by RES, which limits their usefulness as drug carriers and in some cases it may cause toxicological problems. 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. There may be chances of clumping of cells and dose dumping. It may also involve the rapid leakage of certain encapsulated substances from the loaded erythrocytes.

Table No 1: Buffers Used for Isolation[13]

S. No.	Species	Washing Buffer	Centrifugal Force
1.	Rabbit	10mmol $KH_2PO_4/NaHPO_4$	500-1000
2.	Dog	15mmol $KH_2PO_4/NaHPO_4$	500-1000
3.	Human	154mmol NaCl	<500
4.	Mouse	10mmol $KH_2PO_4/NaHPO_4$	100-500
5.	Cow	10-15mmol $KH_2PO_4/NaHPO_4$	1000
6.	Horse	2mmol $MgCl_2$, 10mmol glucose	1000
7.	Sheep	10mmol $KH_2PO_4/NaHPO_4$	500-1000
8.	Pig	10mmol $KH_2PO_4/NaHPO_4$	500-1000

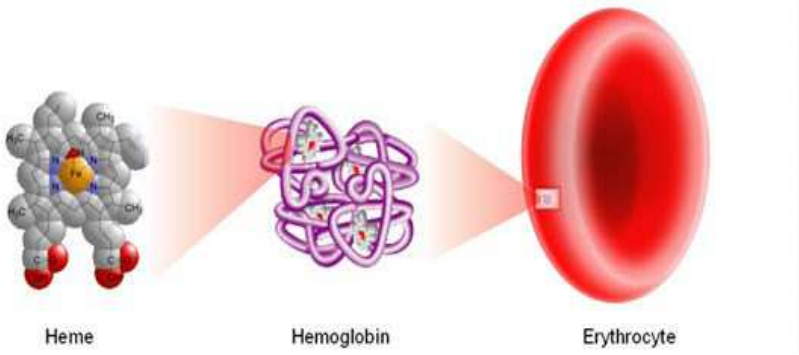

Figure No 1: Introduction of Erythrocytes[11]

2. Methods for drug loading of resealed erythrocytes

Various types of methods are employed for the drug loading of resealed erythrocytes which involves different technique for the lysis of erythrocytes, followed by drug loading and finally the resealing of drug loaded erythrocytes. Most commonly used method is Hypo- osmotic Lysis method which is further divided into four different types. Following chart enlist different methods for drug loading of resealed erythrocytes:

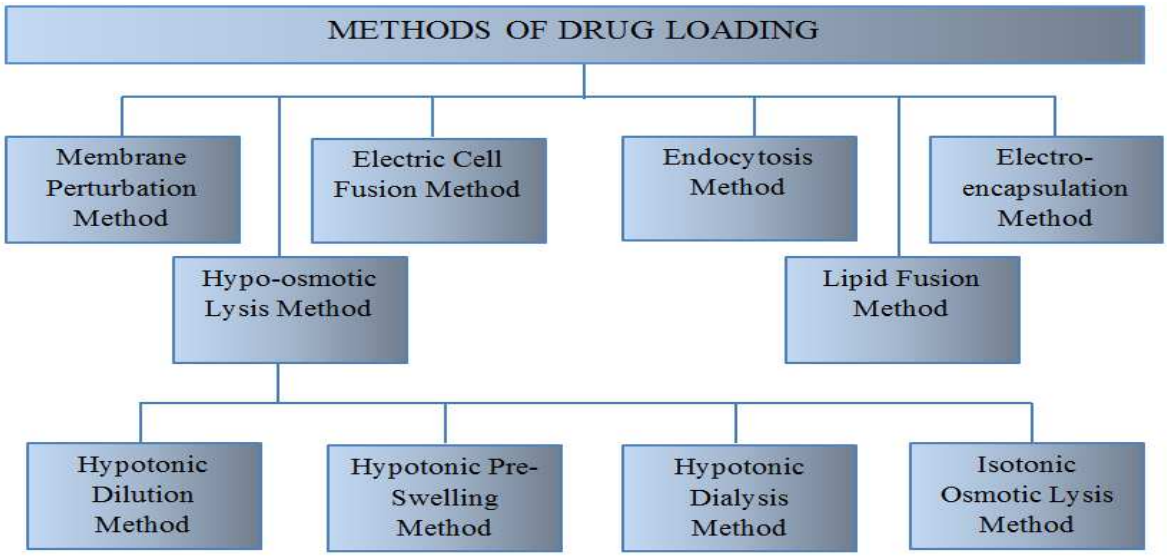

Figure No 2: Methods of Drug Loading

3. Overview of different methods of resealed erythrocytes

1. Membrane Perturbation Method[24, 25]

It is based upon the increase in membranepermeability of erythrocytes when the cells are exposed to certain chemicals. In 1973, Deuticke showed that the permeability of erythrocytic membrane increases upon exposure to polyene antibiotic such as Amphotericin B. In 1980, this method was used successfully by Kitao and Hattori to entrap the antineoplastic drug Daunomycin in human and mouse erythrocytes.

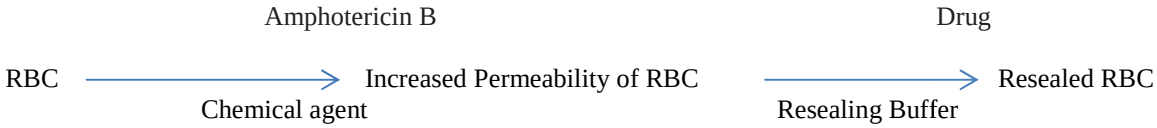

Figure No 3: Loading of drug by Chemical Membrane Perturbation Method[26]

2. Electric Cell Fusion Method[27, 28]

This method involves the initial loading of drug molecules into erythrocyte ghosts followed by adhesion of these cells to target cells. The fusion is accentuated by the application of an electric pulse, which causes the release of an entrapped molecule. An antibody against a specific surface protein of target cells can be chemically cross-linked to drug-loaded cells that would direct these cells to desired cells.

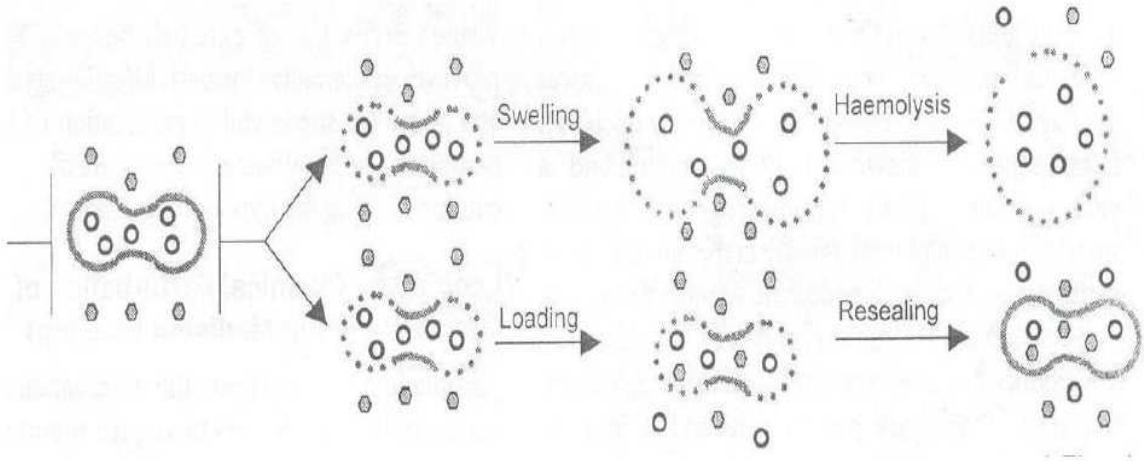

Figure No 4: Loading of drug by Electric Cell Fusion Method[29]

3. Endocytosis Method[30]

It was reported by Schrier in 1975. Endocytosis involves addition of one volume of washed erythrocytes was added to nine volumes of buffer containing 2.5 mM ATP, 2.5 mM $MgCl_2$, and 1 mM $CaCl_2$followed by incubation for 2 minutes at room temperature. The pores were created and resealing of pores by using 154 mM of NaCl and incubation at 37°C for 2 minutes. By endocytosis the entrapment of material occurs. The vesicle membrane separates endocytosis material from cytoplasm thus protecting it from the erythrocytes and vice versa. The various chemicals entrapped by this method include primaquine and related 8-amino-quinolines, vinblastine, chlorpromazine and related phenothiazine, hydrocortisone, propanolol, tetracaine, and vitamin A.

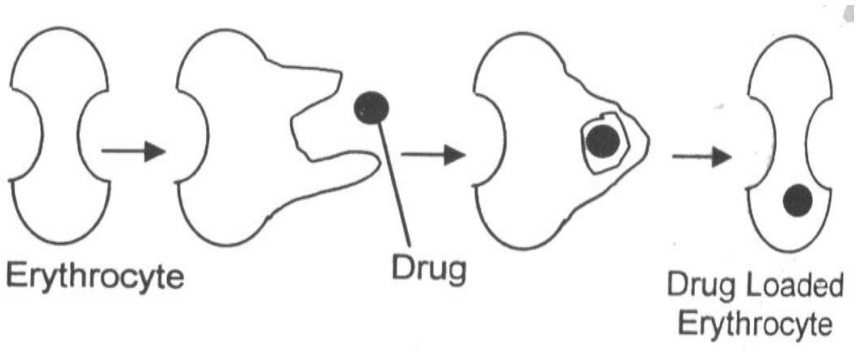

Figure No 5: Loading of Drug by Endocytosis Method[31]

4. Electro-encapsulation Method[32, 33]

Electro encapsulation method is also known as electroporation, the method consist of creating electrically induced permeability changes at high membrane potential differences. In 1977, Tsong and Kinosita suggested the use of transient electrolysis to generate desirable membrane

permeability for drug loading. Electrical breakdown is achieved by membrane polarization for microseconds using varied voltage of 2kv/cm is applied for 20 μ sec.

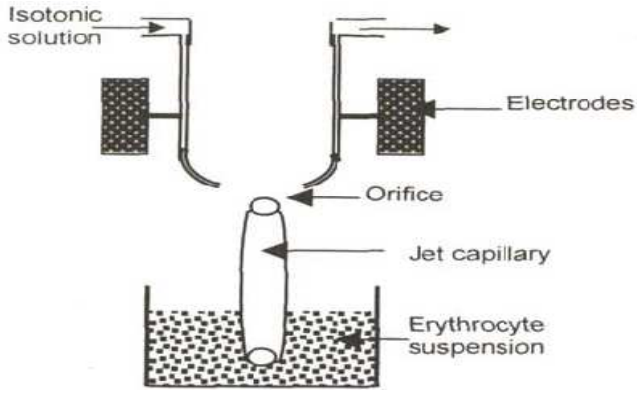

Figure No 6: Loading of Drug by Electro- encapsulation Method[34]

5. Hypo-osmotic Lysis Method[35]

Hypo-osmotic lysis of cells in a solution containing the drug/enzyme to be entrapped followed by restoration of tonicity to reseal them. Increase in volume initially leads to conversion in swollen erythrocytes. These have little capacity to resist volume greater than50-75% of the initial volume and when placed in solution less than about 150mOsm/Kg, the membrane ruptures, permitting escape of the cellular constituents. Erythrocyte are resealed on addition of sufficient 1.54 M KCl, which restores isotonicity. Where preservation of energy metabolism within the cells is enviable, 4mM magnesium salts (e.g. $MgCl_2$, $MgSO_4$), 10 mM glucose and 2mM adenosine are included during resealing to attain above final concentrations.

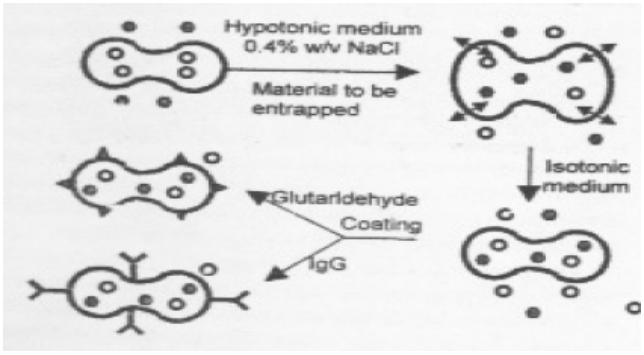

Figure No 7: Loading of Drug by Hypo- osmotic Lysis Method[36]

4. Comparison between various hypotonic osmotic lysis baed methods[37]

The comparison between different hypotonic osmotic lysis based methods gives the idea about the difference in various aspects of these methods such as percentage of drug loading, advantages and disadvantages and provides the brief overview regarding these methods. Following table gives description about the various based on hypotonic osmotic lysis based methods:

Table No 2: Advantages and Disadvantages of various Hypotonic Osmotic Lysis Method[37]

S.No	Method	% Drug Loading	Advantages	Disadvantages
1.	Dilution Method	20- 40	Fastest and simplest especially for low molecular weight drugs	Entrapment efficiency is less
2.	Dialysis Method	30-45	Better in vivo survival of erythrocytes better structural integrity and membrane	Time consuming, heterogeneous size distribution of resealed erythrocytes
3.	Pre- swell Method	30- 90	Good retention of cytoplasm and good survival in vivo	
4.	Isotonic Osmotic Lysis	-	Better in vivo survival	Impermeable only large molecules, process is time consuming

5. Storage[38]

The encapsulated preparation was stored by suspending in Hank's balanced salt solution [HBSS] at 4°C for two weeks without the loss of integrity. The Blood group 'O' [universal donor] cells were used and by using the pre-swell or dialysis technique, batches of blood for transfusion. Standard blood bag may be used for both encapsulation and after loading of therapeutic agent on erythrocytes, the carrier cells are exposed to physical, cellular as well as biological evaluations.

6. Characterisation of resealed erythrocytes

Resealed Erythrocytes can be characterised by both in vivo and *in-vitro*. The evaluation of resealed erythrocytes involve the physical evaluation which include characterisation of size and surface area, biological evaluation which include sterility testing and cellular evaluation which include cell recovery, percent cell volume, ESR etc. The in vivo evaluation of resealed erythrocytes involves stability studies and tissue distribution studies. Following chart enlist the different evaluation parameters of resealed erythrocytes:

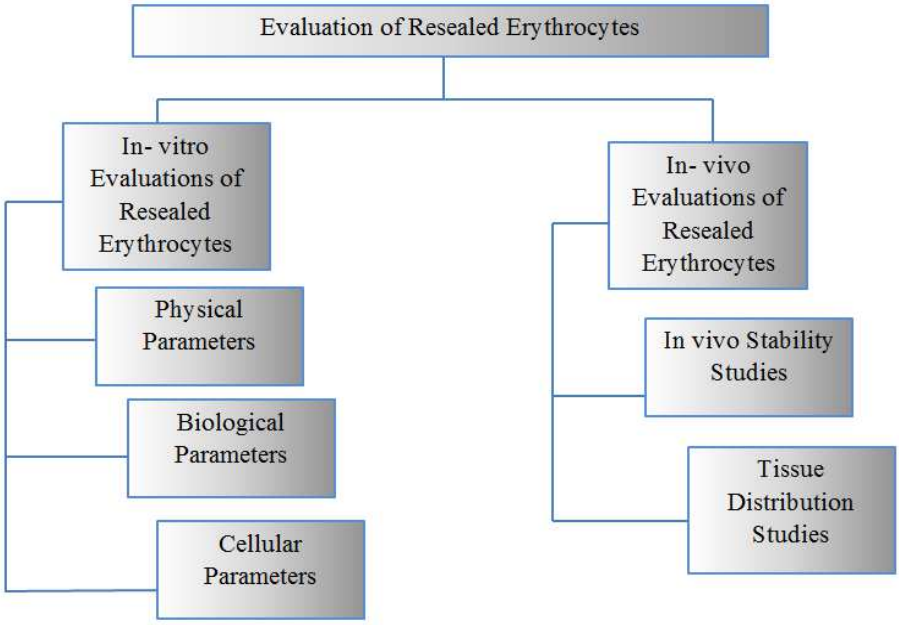

Figure No 8: Parameters for Evaluation of Resealed Erythrocytes

6.1. *In- vitro* evaluations of resealed erythrocytes

The resealed erythrocytes can be characterised and evaluated in vitro by different parameters such as Physical parameters, Biological parameters and Cellular parameters. Different techniques had been employed in these parameters for the evaluation of the resealed erythrocytes. In vivo evaluation studies are also carried out which involves in vivo stability studies and Tissue Distribution studies. Following table enlists the various parameters involved in the in- vitro evaluation of Resealed Erythrocytes:

Table No 3: *In- vitro* Evaluation Parameters of Resealed Erythrocytes[39-41]

S.No.	PARAMETERS	Techniques used
1.	**PHYSICAL PARAMETERS**	
a)	Size, Shape, Surface Morphology	Transition Electron Microscopy, Scanning Electron Microscopy, Optical Microscopy, Phase Contrast Microscopy
b)	Vesicle size and shape	Transition Electron Microscopy, Optical Microscopy
c)	Drug Release	Diffusion Cell Dialysis
d)	Drug Content	Deproteinization of cell membrane followed by the assay of released drug
e)	Surface Electrical Potential	Zeta Potential measurement by Photon Correlation (PCS)
f)	Surface pH	pH sensitive probes
g)	Deformity	Capillary Method
2.	**BIOLOGICAL PARAMETERS**	
a)	Pyrogenicity	LAL Test , Rabbit Method
b)	Sterility	Aerobic and Anaerobic cultures used and involve Sterility testing methods

c)	Toxicity	Toxicity Testing Methods
3.	**CELLULAR PARAMETERS**	
a)	% Haemoglobin Content	Deproteinization of cell membrane followed by haemoglobin assay
b)	Cell Volume	Laser Light Scattering
c)	% Cell Recovery	Haematological Analyser, Neubaeur's Chamber
d)	Osmotic Fragility	Stepwise incubation with isotonic to hypotonic saline solution and determination of drug and haemoglobin
e)	Osmotic Shock	Dilution with distilled water and determination of drug and haemoglobin
f)	Turbulent Shock	Passing Cell Suspension through a 23 gauge needle, hypodermic needle (10ml/min), and estimation of residual drug and haemoglobin
g)	Erythrocytes Sedimentation Rate (ESR)	ESR Apparatus

7. Current research work done on the preparation of resealed erythrocytes using different techniques

Anna Chorzalska *et al.* prepared the resealed erythrocytes by isotonic lysis method and studied the effect of the full-length Ankyrin- binding domain of β-spectrin on natural erythrocyte and HeLa cell membranes was tested. It appeared that these barrier properties varied between ghosts resealed in the presence or absence of polypeptides corresponding to the full-length and truncated Ankyrin-binding domain. The erythrocyte ghosts were resealed in the presence of a protein corresponding to either the full-length or truncated Ankyrin-binding domain to test the possibility of endogenous spectrin release through a competitive mechanism. It results in the induction of partial release of endogenous spectrin from the erythrocytes ghosts which were quantified by Densitometry analysis. The presence of full-length (DWA and N1C) Ankyrin-binding domain polypeptides during the resealing of the erythrocyte membrane induced a decrease in the barrier properties and result in the change of permeability properties**[42]**.

In a research conducted by Mohammed F Ibrahim *et al.* had introduced a new carrier system for Salbutamol, maintaining suitable blood levels for a long time, as atrial to resolve the problems of nocturnal asthma medication. The human erythrocytes have been successfully loaded with salbutamol using endocytosis method either at 25°C or at 37°C. Salbutamol loaded erythrocytes have morphology and fragility like natives, proving that both Salbutamol and endocytosis are less destructive to erythrocytes and preserve the cells fragility and morphology. Salbutamol release from loaded erythrocytes obeying zero order kinetics and may persist in the body for more than 4 days. It concluded that salbutamol is successfully entrapped into erythrocytes with acceptable loading parameters and moderate morphological changes, this suggesting that erythrocytes can be used as prolonged release carrier for salbutamol**[43]**.

In an attempt made by Ranuka Sarma Mallela *et al.* had prepared paclitaxel resealed erythrocytes. Thestudy involved the usage of cross linking agents like DMF, DMSO and Glutaraldehyde for the formulation of Paclitaxel resealed erythrocytes. The procedure considered for the formulation is Preswell dilution technique as this method led to high entrapment efficiency. The formulated resealed erythrocytes were evaluated for parameters like scanning electron microscopy, zeta Potential, particle size analysis, drug assay, osmotic fragility studies, turbulence fragility studies, osmotic shock studies, percentage haemoglobin release and in-vitro drug release studies and all the values were found to be within the prescribed limits Finally it was concluded that prepared resealed erythrocytes of Paclitaxel using Glutaraldehyde as cross linking agent had proved to be potential candidate for safe and effective sustained drug release over an extended period of time which can reduce dose frequency**[44]**.

One of the investigations also involves the erythrocytes which were loaded with prednisolone using preswell dilution technique with two different cross-linking agents, glutaraldehyde and dimethylsulphoxide. Carrier erythrocytes, having acceptable loading parameters showed increased percentage drug content with theaddition of cross-linking agents. Formulation containing glutaraldehyde as cross-linking agent showed maximum drug entrapment efficiency. The results of present study showed that the carrier erythrocytes having considerable loading parameters, release their drug content with zero order kinetics. Osmotic fragility using 0.3% w/v concentration of sodium chloride (Hypotonic solution) showed maximum drug entrapment and haemoglobin content. Targeting efficiency of drug loaded erythrocytes over free drug is higher, which may provide increased therapeutic index and drug targeting to various organs. It may help in the reduction of dose required for the therapy and there by dose related systemic side effects could also be minimized. Present work was a preliminary satisfactory in designing prednisolone resealed erythrocytes for site specificity and prolonged release of therapy**[45]**.

The erythrocytes loaded Ribavirin was also prepared with the aim to benefit the reticulo endothelial system targeting potential of the carrier cells. Endocytosis technique was used for Ribavirin loading in erythrocytes and the entire loading procedure was evaluated and validated. The highly changed erythrocyte shape and morphology evidenced in this study, being one of the main determinants in erythrocytes disappearance kinetics in circulation, can be potentially beneficial in terms of successful cell targeting to RES, which, in turn, leads to the improved Ribavirin effects on RES-mediated immune responses**[46]**.

Mahshid Foroozesh and Abdolhossein Zarrin *et al.* had worked on a novel combinatory paradigm for chronic hepatitis C treatment using liver-targeted carrier erythrocytes

co-encapsulated with inter-feron alpha-2b, ribavirin and boceprevir. The erythrocytes were co-encapsulated with interferon alpha-2b, ribavirin and boceprevir by means of hypotonic preswelling encapsulation method with post-loading modifications of obtained drug-carriers, which results in the preparation of liver targeted-triple combination therapy for HCV particularly in difficult patients (non-responders, co-infected patients with HIV and the ones with decompensated liver disease)**[47]**.

Successful preparation of resealed murine erythrocytes which encapsulate phosphodiesterase by hypnotic dilution method is another exapmle of fine resealed erthrocyte preparation. These resealed erythrocytes are capable of hydrolysing paraoxon. This results in the rapid hydrolysis of the organophophorus substrate, as the high lipid solubility of paraoxon contributed to its rapid diffusion of the through the membrane. The encapsulation of phosphotriesterase into resealed erythrocytes and rapid hydrolysis of paraoxon by these resealed erythrocytes appeared to show as a new approach to antagonise the toxic effect of organophosphorus compounds**[48]**.

One of the group of researchers had prepared amikacin loaded resealed erythrocytes which were prepared by hypnotic dialysis method. The administration of the antibiotic using carrier erythrocytes elicited asustained release effect, with an increase in the plasma half-life and in the area under the curve of the antibiotic and leads to the significant changes in the pharmacokinetic behaviour of the antibiotic a greater accumulation being observed in RES organs such as liver and spleen. This shows that loaded erythrocytes are potentially useful for the delivery of antibiotics in phagocytic cells located in the RES. The selective uptake of carrier erythrocytes by phagocytic cellsand thehigher accumulation of the drug in RES organs such as the liver and spleen when amikacin is incorporated into erythrocytes show that carrier erythrocytes can be used as alternatives to other delivery systems, such as liposomes, and that they are potentially useful for intracellular infections caused by aminoglycoside-sensitive germs**[49]**.

James M.May *et al.* had prepared resealed erythrocytes which were resealed in the presence of ascorbic acid. The aim was to determine the mechanism of ascorbate-dependent trans or intramembrane electron transfer in human erythrocytes and to determine whether such transfer protects against membrane lipid peroxidation. The results showed that the ferricyanide does oxidize a small amount of arachidonic acid that is presumably located in the outer membrane bilayer and that intravesicular ascorbate protects against this oxidation**[50]**.

7.1 Summarization of different work done by researchers

Many research works had been carried out on resealed erythrocytes which are summarised in following table. Different techniques had been employed for the preparation of the resealed erythrocytes such as isotonic lysis method, endocytosis method, preswell dilution method which one or another way had enhanced the better and targeted delivery of drugs.

Table No 4: Research Work done on Resealed Erythrocytes

S.No.	Formulation	Active ingredient	Other ingredients	Preparatory method	Results	References
1.	Preparation of full length Ankyrin resealed erythrocytes	Full length Ankyrin binding domain of ß-Spectrin	Isopropyl ß-D-1-thiogalacto pyronoside, Urea, Sodium Chloride	Isotonic Lysis Method	Decrease in Barrier Properties and Change in Permeability properties	**[42]**
2.	Preparation of Salbutamol loaded resealed erythrocytes	Salbutamol	Adenosine 5-Triphosphate, Acetonitrile, Sodium Chloride	Endocytosis Method	Prolonged Release carrier for Salbutamol for the treatment of nocturnal asthma	**[43]**
3.	Preparation of Paclitaxel loaded resealed erythrocytes	Paclitaxel	Glutaraldehyde, Dimethyl Formamide, Methanol, Sodium Hydroxide	Preswell Dilution Method	Potential candidate for safe effective sustained drug release for the treatment of cancer	**[44]**
4.	Preparation of Prednisolone loaded resealed erythrocytes	Prednisolone	Glutaraldehyde, Dimethylsulphoxide	Preswell Dilution Method	Increased Therapeutic index and drug targeting to organs for prolonged release to treat arthritis and other skin conditions	**[45]**
5.	Preparation of Ribavirin loaded Resealed Erythrocytes	Ribavirin	Adenosine 5-Triphosphate, Disodium Hydrogen Phosphate, Calcium Chloride	Endocytosis Method	Drug Targeting to RES and thus improving Ribavirin effect for the treatment of Hepatitis C	**[46]**

6.	Preparation of combinatory paradigm using Resealed erythrocytes with Interferon alpha 2b, Ribavirin and Boceprevir	Interferon –α, Ribavirin, Boceprevir	Polyethylene Glycol	Hypnotic Preswell Dilution Method	Liver Targeted Triple combination therapy for Hepatitis C	[47]
7.	Preparation of Resealed Murine Erythrocytes encapsulating Phosphodiesterase	Paraoxon	Dichloromethane, Sodium Chloride, Dextrose, Magnesium Chloride	Hypnotic Dilution Method	Beneficial & New Approach to antagonise toxic effects of Organo phosphorus compounds	[48]
8.	Preparation of Amikacin loaded Resealed Erythrocytes	Amikacin	Glutathione, Sodium Pyruvate, Adenine	Hypnotic Dilution Method	Elicits Sustained release effects of antibiotic and higher accumulation in RES organ like spleen and liver	[49]
9.	Preparation of Resealed Erythrocytes with Ascorbic acid	Ascorbic Acid	Dehydroascorbate, Tridecylamine	Hypnotic Preswell Dilution Method	Prevention of oxidation of cell membrane and limits the membrane lipid peroxidation	[50]

8. Applications of resealed erythrocytes[51, 52]

1. Resealed Erythrocytes are used for the slow drug release anti neoplastic, anti-parasitic, vitamins, steroids and cardiovascular drugs.
2. Surface modified Erythrocytes are used to target the organs of mononuclear phagocytic system/ RES because the change in the membrane is recognised by the macrophages.
3. Reserve of cyanide intoxication with murine carrier erythrocyte containing bovine rhodanase and sodium thiosulfate can be used.
4. Usually loading anticancer drugs into carrier reins improves their delivery to tumours.
5. Various types of enzymes can be delivered by the use of carrier erythrocytes for eg Amyloid β-degrading peptidases, adenosine deaminase and pegademase, alcohol dehydrogenase and aldehyde dehydrogenase, alcohol oxidase.

9. Future perspectives[53, 54]

Following are some future perspectives of Resealed Erythrocytes:

- A large amount of valuable work is needed so as to utilize the potentials of erythrocytes in passive as well as active targeting of drugs.
- Diseases like cancer could surely find its cure.
- Genetic engineering aspects can be coupled to give a newer dimension to the existing cellular drug carrier concept.

10. Conclusion

In this paper, various numerous applications have been proposed for the use of resealed erythrocytes as carrier for drugs, enzyme replacement therapy etc. The use of resealed erythrocytes looks promising for a safe and sure delivery of various drugs for passive and active targeting. The Resealed erythrocytes had also been employed for effective delivery of numerous drugs as depicted by many researchers for the treatment of cancer, tumour, and arthritis and also for effective treatment of the poisoning. However In near future, erythrocytes based delivery system with their ability to provide controlled and site specific drug delivery will revolutionize in effective treatment of various disease. For the present, it is concluded that erythrocyte carriers are "Nano Device in field of Nanotechnology" considering their tremendous potential and prospective.

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