REVIEW ARTICLE

Nanoerythrosomes: Engineered Erythrocytes as a Novel Carrier for the Targeted Drug Delivery

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Abstract

The emerging advances in the development of novel drug delivery technologies are likely to have a significant impact on the drug industry. Among the various carriers used for targeting of 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. Leukocytes, platelets, erythrocyte, and nanoerythrocytes have been proposed as cellular carrier systems. Among these, the erythrocytes have been the most investigated and have found to possess great potential in novel drug delivery. Most of the nano erythrocytes used as drug carriers are rapidly taken up from blood by macrophages of the reticuloendothelial system (RES), which is present in liver, lung, and spleen of the body. Once in the RES, the erythrocyte is attacked by lysosomal enzymes that cause the breakage of the cellular membrane and the degradation of the hemoglobin by the heme-oxygenase enzyme. The use of red cells as carriers of drugs constitutes a field of work that has been barely explored, especially when compared to other carrier systems. From a therapeutic perspective, erythrocytes may be employed for two main purposes. To act as a reservoir for the drug, providing the sustained release of the same into the body, this enables the posology of the drugs to be modified by altering the dose and increasing the dosage intervals. To selectively direct the drugs to the RES (monocyte-macrophage system) of the liver, spleen, and bone marrow, which constitute the usual sites form the destruction of nanoerythrocytes. Nanoerythrosomes (NERs) are prepared by different erythrocyte ghosts to produce small vesicles with an average diameter of 100 nm. Since the NERs are particles, phagocytosis may be involved in their mechanisms of potentiating drugs such as antineoplastic and angiotensin converting enzyme inhibitors and systemic corticosteroids and prodrug. Such cells could be used as circulating carriers to disseminate a drug within a prolonged period in circulation or in target-specific organs, including the liver, spleen, and lymph nodes. A majority of the drug delivery studies using drug-loaded erythrocytes are in the preclinical phase.

Key words: Nanoerythrosomes, erythrocytes, carrier, targeted drug delivery

INTRODUCTION

Realizing the potential of nanoparticles in medicine, researchers worldwide have put tremendous efforts into the development of nanoparticles based drug carriers resulting in an exponential accumulation of novel nanoparticle systems and related research data. Importantly, many nanotechnology-based systems are rapidly advancing toward preclinical and clinical trials for cancer diagnosis and therapy. In the last decade, nanotechnology has tremendously impacted the field of medicine through advances in drug delivery. Nanotechnology-based drug delivery aims to target the drug payload to the right place, at the right time, at the right (optimal) dose. In general, nanodrug delivery systems are submicron-sized particles with one or more therapeutic agents that are dispersed, adsorbed, or covalently bound in encapsulated vesicles, capsules, or polymer matrices.[1] Nanodrug delivery systems enhance the bioavailability of each drug reducing deleterious side effects caused by related toxicities. In medicine, many of the nanotechnology breakthroughs have occurred in cancer therapy, including

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drug delivery systems based on polymer nanoparticles or liposomes and image contrast agents in nanoscale dimensions to aid diagnostic imaging or image-guided therapy. These nanoscaled formulations are non-targeted drug delivery systems designed to improve the pharmacokinetics and bioavailability of encapsulated therapeutics. The current pharmaceutical scenario is aimed at the development of drug delivery systems with maximum therapeutic benefits for safe and effective management of diseases.[2] The concepts are based on controlled drug delivery, biotechnology, and polymer sciences, which surpass all the barriers of diseases. The emerging advances in the development of novel drug delivery technologies are likely to have a significant impact on the drug industry. Among the various carriers used for targeting of 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.[3] Leukocytes, platelets, erythrocyte, and nanoerythrocytes have been proposed as cellular carrier systems. Among these, the erythrocytes have been the most investigated and have found to possess great potential in novel drug delivery.

Most of the nanoerythrocytes used as drug carriers are rapidly taken up from blood by macrophages of the reticuloendothelial system (RES), which is present in liver, lung, and spleen of the body. Once in the RES, the erythrocyte is attacked by lysosomal enzymes that cause the breakage of the cellular membrane and the degradation of the hemoglobin by the heme-oxygenase enzyme.[4] The use of red cells as carriers of drugs constitutes a field of work that has been barely explored, especially when compared to other carrier systems. From a therapeutic perspective, erythrocytes may be employed for two main purposes.[5]

To act as a reservoir for the drug, providing the sustained release of the same into the body, this enables the posology of the drugs to be modified by altering the dose and increasing the dosage intervals. To selectively direct the drugs to the RES (monocyte-macrophage system) of the liver, spleen, and bone marrow, which constitute the usual sites form the destruction of nanoerythrocytes.[5]

Nanoerythrosomes (NERs) are prepared by different erythrocyte ghosts to produce small vesicles with an average diameter of 100 nm. Since the NERs are particles, phagocytosis may be involved in their mechanisms of potentiating drugs such as antineoplastic and angiotensin converting enzyme inhibitors and systemic corticosteroids and prodrug.[6]

NERs have applications in fields of human and veterinary medicine. Such cells could be used as circulating carriers to disseminate a drug within a prolonged period in circulation or target-specific organs, including the liver, spleen, and lymph nodes. A majority of the drug delivery studies using drug-loaded erythrocytes are in the preclinical phase. Antineoplastic drugs such as methotrexate, bleomycin, asparaginase, and adriamycin have been successfully delivered by erythrocytes. Removal of RES iron overload, removal of toxic agents, and delivery of antiviral agents are some other applications of resealed erythrocytes.[7] Biopharmaceuticals, therapeutically significant peptides and proteins, nucleic acid-based biological, antigens, and vaccines, are among the recently focused pharmaceuticals for being delivered using carrier erythrocytes.[8]

IDEAL PROPERTIES REQUIRED IN A DRUG CARRIER

The drug carrier must fulfill the following requirements:

1. It should be of appropriate size (s) and shape to permit the passage through the capillaries;
2. It should possess specific physicochemical properties by which the desired target site could be recognized;
3. It should be biocompatible and should have minimum toxic side effect;
4. The degradation product of the carrier system, after release of the drug at the selected site, should be biocompatible;
5. Minimum leaching or leakage of drug should take place before target site is reached;
6. The drug should be released at the target site in a controlled manner;
7. It should possess the ability to carry a broad spectrum of drugs with different properties;
8. It should be physicochemically compatible with the drugs;
9. It should have sufficient space to carry and eventually to permit the delivery of clinically adequate amount of drugs, and;
10. The carrier system should have an appreciable stability during storage.[9]

PHYSIOLOGYICAL FUNCTION OF ERYTHROCYTE

When erythrocytes undergo shear stress in constricted vessels, they release adenosine triphosphate (ATP), which causes the vessel walls to relax and dilate so as to promote normal blood flow. When these hemoglobin molecules are deoxygenated, erythrocytes release S-nitrosothiols, which also act to dilate blood vessels, thus directing more blood to areas of the body depleted of oxygen. Erythrocytes can also synthesize nitric oxide enzymatically, use L-arginine as substrate, as do endothelial cells. Exposure of erythrocytes to physiological levels of shear stress activates nitric oxide synthase and export of nitric oxide, which may contribute to the regulation of vascular tonus.[10] Erythrocytes can also produce hydrogen sulfide, a signaling gas that acts to relax vessel walls. It is believed that the cardioprotective effects of garlic are due to

erythrocytes converting its sulfur compounds into hydrogen sulfide. Erythrocytes also play a part in the body's immune response: When lysed by pathogens such as bacteria, their hemoglobin releases free radicals, which break down the pathogen's cell wall and membrane, killing it.[7]

BIOCHEMICAL FUNCTIONS OF NERS

Enzyme therapy

Many metabolic disorders related to deficient or missing enzymes can be treated by administering these enzymes as NERs, e.g., β-glycoside, β-glucuronidase, β-galactosidase.[11]

Respiratory function of erythrocytes

Oxyhemoglobin is formed during respiration when oxygen binds to the heme component of the protein hemoglobin in red blood cells (RBCs). This process occurs in the pulmonary capillaries adjacent to the alveoli of the lungs, and the oxygen then travels through the bloodstream to be dropped off at cells where it is utilized in aerobic glycolysis and in the production of ATP by the process of oxidative phosphorylation.[12] It does not, however, help to counteract a decrease in blood pH. Ventilation, or breathing, may reverse this condition by removal of carbon dioxide, thus causing a shift up in pH.

Glucose transport (GLUT-1) through RBC membrane

Glucose is transported through RBC membrane glucose by a facilitated diffusion by GLUT-1. GLUT-1 is independent on insulin, i.e., insulin does not promote GLUT-1 to RBCS.[13]

Genetic defects in enzymes of glycolysis

In genetic defects, one of the enzymes of glycolysis in RBCs results in a reduced rate of glycolysis in RBCs and by this way will deprive RBCs of the only means for producing energy. As a result, hemolytic anemia will be a consequence as RBCs will not be able to keep the biconcave flexible shape which allows it to squeeze through narrow capillaries with an end result of hemolysis (destruction of RBCs).[14]

Pentose phosphate pathway

RBCs contain an active pentose phosphate pathway (PPP) for glucose that supplies NADPH (PPP is the only source for NADPH in RBCs). NADPH is important in keeping glutathione in the reduced glutathione. Reduced glutathione plays a very important role in the survival of the RBCs (prevents oxidation of membrane).[15]

PROPERTIES OF NANO ERYTHROSOMES

1. Biodegradability
2. Circulate throughout the circulatory system
3. Has sufficient to carry large quantities of material can be encapsulated within them
4. Can be utilized for organ targeting within RES
5. A wide variety of bioactive agents can be encapsulated within them
6. Erythrocytes are biocompatible cells are used in patients; there are no possibility of triggered immunological response
7. Appropriate size, shape
8. Biocompatible and minimum toxic side effects
9. Minimum leakage before target site is achieved
10. Specific physicochemical properties
11. Appreciable stability during storage period
12. Be released at target site
13. The degradation product of the carriers system, after release of the drug at the selected site, should be biocompatible
14. It should be physicochemically compatible with drug.[16]

MECHANISM OF DRUG RELEASE FROM NERS

It is an eminent fact that, in physiologic conditions, as a consequence of the gradual inactivation of the metabolic pathways of the erythrocyte by aging, the cell membrane loses its natural integrity, flexibility, and chemical composition. These changes, in turn, finally result in the destruction of these cells upon passage through the spleen trabeculae. The other effective site for the destruction of the aged or abnormal erythrocytes is the macrophages of the RES, including peritoneal macrophages, hepatic Kupffer cells, alveolar macrophages of the lung, peripheral blood monocytes, and vascular endothelial cells. It has been known that aging and a series of other factors (e.g. stress during non-gentle loading methods) make the erythrocytes recognizable by the phagocyting macrophages via changing the chemical composition of the erythrocyte membrane, i.e., the phospholipids component.[17]

NERs can preferentially accumulate in the tumor via the enhanced permeability and retention (EPR) effect. Due to the leaky vasculature and dysfunctional lymphatic drainage in the tumor tissues, nano-sized drug carriers preferentially extravasate across the tumor endothelium and elevate the drug exposure in the tumor, while having very limited access to normal organs because of their tightly-knitted endothelial lining. (Figure 1) Finally, NERs are amenable to the surface modification with the targeting ligands, which specifically recognize the receptors overexpressed on the surface of tumor cells and tumor endothelium, resulting in highly efficient intracellular delivery of NERs drug.[18]

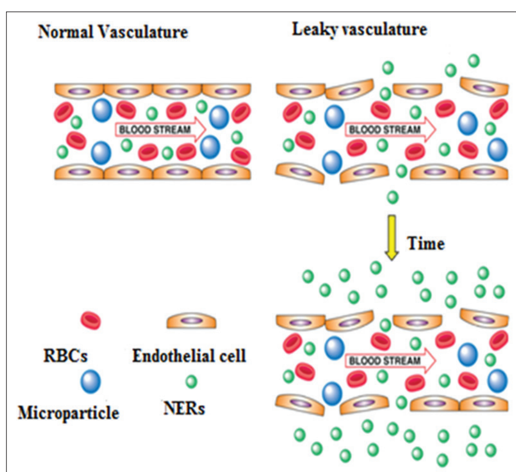

Figure 1: Drug release mechanism from nanoerythrosomes

RBC GHOST PREPARATION

To prepare erythrocyte ghost, blood mix with anticoagulant and gently transfer the blood to a 1.5 ml snap top conical tube, rinse the syringe with osmotically balanced 0.15 M NaCl solution and made for centrifuge your conical tube for 1 min on the highest setting to pellet the RBCs and leave the major portion of the yellow plasma on top. Using a fresh pipette, remove the yellow plasma top layer from the conical tube and discard into bleach bucket. This top plasma layer may also be referred to as the supernatant (a liquid overlaying a solid). This will leave you with a small red cell pellet at the bottom of your conical tube. Use a fresh plastic pipette and add 0.15 M NaCl (sodium chloride) up to the 1.5 ml line on the conical tube. Vortex the conical tube for 10 s to resuspend the RBCs. Centrifuge your conical tube for 1 min on the highest setting to repellet the red cells and wash off plasma that was between the red cells in the initial red cell pellet using a fresh pipette, remove the top layer (plasma/supernatant wash) from conical tube and discard pipette into bleach bucket. Again, this will leave with a small red cell pellet at the bottom of your conical tube. Add deionized (DI) water using a fresh pipette to your conical tube containing the RBC pellet up to the 1.5 ml mark. This step and the next will lyse (burst) the RBCs. Set vortex to max and vortex conical tube for 10 s to resuspend the red cells and encourage their bursting. Set centrifuge to max and centrifuge conical tube for 5 min (This step will create a pellet of RBC ghost membranes). The RBC ghost membrane pellet is very small and may be hard to observe. Obtain a fresh plastic pipette and remove the red hemoglobin supernatant by slowly pipetting down to the 0.3 ml mark on your conical tube. Discard the red hemoglobin supernatant into the bleach bucket. Be careful not to remove all of the red hemoglobin supernatant as you may lose your RBC membranes. Now RBC ghost is prepared for test.[19-21]

STEPS FOR FORMATION OF NERS

Hypotonic hemolysis and isotonic resealing methods

This method is based upon hypotonic lysis of cells in solution containing the drug/enzyme to be entrapped followed by restoration of tonicity to reseal them. Three types of ghosts can be distinguished: Type 1 ghosts who reseal immediately after hemolysis; Type 2 ghosts which reseal after, reversal of hemolysis by addition of alkali ions; and Type 3 ghosts which remain leaky under different experimental conditions. Erythrocytes have an exceptional capability for reversible shape changes.[5,22-25]

Dilutional hemolysis

Population of erythrocytes when exposed to hypotonic saline solution (0.4% NaCl) swells until it reaches a critical value of volume or pressure where membrane ruptures and becomes permeable to macromolecules and ions, therefore, permitting the escape of cellular components. When these RBC are placed in the hypotonic (0.4% NaOH), they get ruptures permitting escape of cellular content and equilibrium is achieved within 1min, which results in swelling up to 1.6 time its initial volume. At 0°C, opening permits to attain equilibrium for inter- and extracellular fluids through the pore size of 200-500Å. Increasing the ionic strength at 37°C results in resealing of cell membrane and restoring the osmotic property. This method is simple and faster but has very low encapsulation efficiency (1-8%) and loses of cytoplasmic constituents during osmotic lysis. Low molecular weight drugs such as ß-glucosidase and ß-galactosidase can be encapsulated.[26]

Preswell dilutional hemolysis

The technique is based upon initial controlled swelling of erythrocytes without lysis by placing them in slightly hypotonic solution followed by centrifugation at low “g” to take them up to point of lysis. Finally, the addition of small volume of drug solution to attain drug-loaded resealed erythrocytes.

Hemolysis and resealed is implemented in 3 steps:

- First, one volume of washed cells is suspended in 5 volumes of slightly hypotonic buffer.
- Second, after incubation at 37°C for 5 min, the cells were recovered by gentle centrifugation.
- Third, the swelling procedure is further extended by the addition of a volume of hypotonic buffer equal to one-half the volumes of the swollen cells to effect lysis of the cells.

This hypotonic buffer medium also contains the material to be loaded, and the cells are allowed to remain lysed for 10 min at 37°C. This is followed by restoration of tonicity and resealing of the membrane. The efficiency by this technique has been reported as 72%, e.g., thyroxin, ibuprofen, etc.

Dialysis method

The major limitation of dilution procedure is low entrapment efficiency. It can be overcome by carrying out lysis and resealing within a dialysis tube.[27] All the dialysis based methods work on common principle that the semipermeable dialysis membrane maximizes the intracellular: Extracellular volume ratio for macromolecules during lysis and resealing, but also allows for free flow of small ions, responsible for lysis and resealing of the erythrocytes.

Hemolysis and resealing is implemented in 3 steps:

- First, washed erythrocytes (hematocrit 85-95%) are mixed with phosphate buffer saline (0.15 M NaCl, 5 mM sodium phosphate, pH 7.4) containing the drug to be incorporated. This mixture is then placed in the dialysis bag.
- The dialysis bag is inflated with an air bubble and sealed in such a way that the erythrocyte suspension occupies no more than 75% of internal volume.
- The sealed dialysis bag is then placed in a bottle containing at least 200 ml of lysis buffer (0.1%w/v NaCl) and placed on a mechanical rotor for 2 h at 40°C. After the initial lysis, the dialysis bag is transferred to a bottle containing at least 200 ml of resealing buffer at room temperature for 30 min.

Use of red cell loader

Entrapment of non-diffusible drugs into erythrocytes may done by developing a piece of equipment called a "red cell loader." With as little as 50 ml of a blood sample, different biologically active compounds were entrapped into erythrocytes within a period of 2 hrs at room temperature under blood banking conditions. The process is based on two sequential hypotonic dilutions of washed erythrocytes followed by concentration with a hemofilter and an isotonic resealing of the cells. There was 30% drug loading with 35-50% cell recovery. The processed erythrocytes had normal survival *in vivo*. The same cells could be used for targeting by improving their recognition by tissue macrophages.[28]

Isotonic osmotic lysis

This method, also known as the osmotic pulse method, involves isotonic hemolysis that is achieved by physical or chemical means. The isotonic solutions may or may not be isotonic. If erythrocytes are incubated in solutions of a substance with high membrane permeability, the solute will diffuse into the cells because of the concentration gradient. This process is followed by an influx of water to maintain osmotic equilibrium. Chemicals such as urea solution, polyethylene glycol, and ammonium chloride have been used for isotonic hemolysis. However, this method also is not immune to changes in membrane structure composition. A method that involved suspending erythrocytes in an isotonic solution of dimethyl sulfoxide (DMSO). The suspension was diluted with an isotonic buffered drug solution. After the cells were separated, they were resealed at 37°C. (Figure 2)

Membrane perturbation

This method is based on the observation that the permeability of the erythrocytic membrane is increased when it is exposed to some chemical agents. This allows small molecular weight substances to get entrapped. Antibiotics such as amphotericin B damage microorganisms by increasing the permeability of their membrane to metabolites and ions. This property could be exploited for loading of drug into erythrocytes. Amphotericin B interacts with the cholesterol of plasma membrane of eukaryotic cells causing a change in permeability of the membrane.[29]

Loading by lipid fusion

Lipid vesicles containing drug can be directly fused with human erythrocytes leading to an exchange of lipid entrapped drug. This technique is used for loading of inositol hexaphosphate into erythrocytes for the increased oxygen carrying capacity.[30]

Formation of NERs

NERs are small vesicular structures formed by the consecutive extrusion of erythrocyte ghost suspension through a polycarbonate filter membrane under nitrogen pressure, and they have an average diameter of 100 nm.[31]

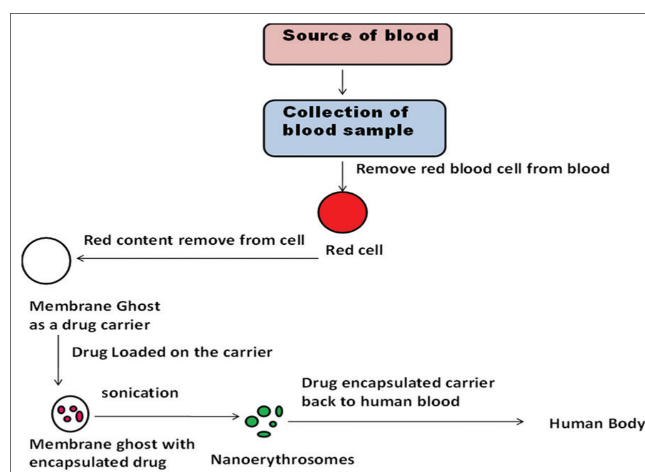

Figure 2: Steps involved in formation of nanoerythrosomes

PREPARATION OF NER AND DRUG LOADING

There are three methods for loading of drugs in NERs.

Extrusion

The erythrocyte ghost suspension (50% hematocrit) is extruded through the 25 mm polycarbonate membrane filter (0.4) pore obtained by 8-10 consecutive extrusions under nitrogen pressure. The ghosts obtained are stained with uranyl acetate, and they are observed under a microscope. The extrusions are performed in a thermostatically controlled extrusion device at 37°C, and the final preparation is stored in a refrigerator at 4°C. The extrusions are performed at 37.8°C in a thermostatically controlled extrusion device. In general, the size of the vesicles seems to be independent of the temperature of the solution but decreases significantly as the extrusion pressure increases. It is not possible to extrude significant numbers of erythrocyte vesicles at or below 30 psi. At these pressures, some fluid does pass through the polycarbonate filter membranes, but only at an exceedingly low flow rate, and the fluid that is extruded does not contain sufficient vesicles upon which to perform light scattering experiments. Thus, there is a minimum pressure P_{min} required to extrude vesicles through narrow pores. The minimum pressure does not vary significantly with temperature.[32]

Sonication

Erythrocyte ghosts are converted into small vesicles using a dismembrator. There are two types of sonicators that can be used namely Bath Sonicator and Probe Sonicator.

Sonication process of converting an electrical signal into a physical vibration that can be directed toward a substance. This device creates a signal that powers a transducer. This transducer converts the electric signal using piezoelectric crystals or crystals that respond directly to the electricity by creating a mechanical vibration the vibration molecules in origin, is carefully preserved and amplified by the sonicator, until it is passed through the probe. The sonication probe transmitted vibration to the solution being sonicated and size reduction process is done by the vibration.[33]

Electrical breakdown method

It is used to convert ghosts into small vesicles under the influence of electric potential. The NERs can also be considered as lipoproteosomes (vesicles constituted of both lipids and proteins) by analogy with liposomes. Because of their high surface to volume ratio (approximately 80-fold higher than the parent RBC) and they remain in suspension for prolonged periods of time are called buoyant vesicles. This was a long reduction in the resistance of an electrical insulator when the voltage applied across it exceeds the breakdown voltage. This result in the insulator becoming electrically conductive. The electrical breakdown may be momentary event. There are several possible mechanisms for breakdown in liquid: Bubbles, small impurities, and electrical super heating have all been proposed. The process of breakdown in liquids seems to be more complicated because of hydrodynamic effects since the additional pressure is exerted on the liquid by the non-linear electrical field's strength in the gap between the electrodes.[34]

MACROPHAGIC UPTAKE OF LOADED NERS

As expected, modifications that altered RBC surface antigens and membrane plasticity (e.g., treatment with crosslinking agents) resulted in their rapid phagocytosis by macrophages in the RES, providing a mechanism to deliver encapsulated drugs into lysosomes in these and other cell types with active internalization processes, including tumor cells. Pilot studies showed that microparticles made from RBC ghosts facilitate delivery of cytotoxic agents to malignant cells. (Figure 3)

CHARACTERIZATION

Physiological characterization

Drug content

Packed-loaded erythrosomes are first deproteinized with acetonitrile and subjected to centrifugation at 2500 rpm for 10 min. The clear supernatant is analyzed for the drug content.[10]

In vitro drug and hemoglobin release

Normal and loaded erythrosomes are incubated at 37±2°C in phosphate buffer saline (pH 7.4) at 50% hematocrit in

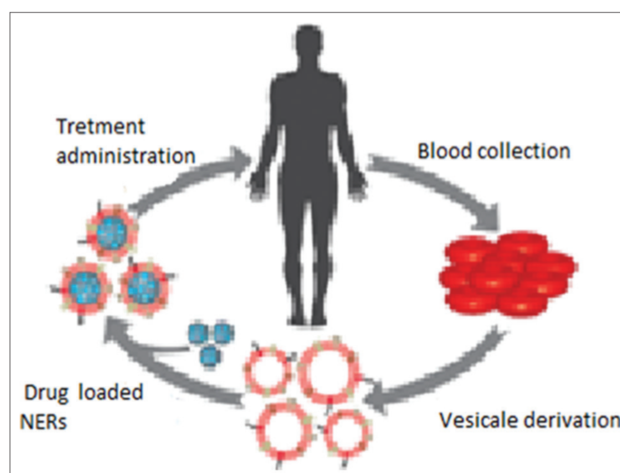

Figure 3: Illustration of personalized therapy using the red blood cell membrane-coated nanoerythrosomes platform[32]

a metabolic rotating wheel incubator bath. Periodically, the samples are withdrawn with the help of a hypodermic syringe fitted with a 0.8 µ spectropore membrane filter.[15] Percent hemoglobin can similarly be calculated at various time intervals at 540 run spectrophotometrically.

Osmotic fragility

It is reliable parameter for *in vitro* evaluation of carrier erythrocytes with respect to shelf life, *in vivo* survival and effect of encapsulated substances. When RBC are exposed to solution of varying toxicities, this shape changes due to osmotic balance. To evaluate the effects of varying toxicities, drug-loaded erythrocytes are incubated with saline solutions of different toxicities' at 37°C ± 2°C for 10 min.[16] The suspension after centrifugation for 15min, 2000 rpm is assayed for drug or hemoglobin release.[17]

Osmotic shock

Osmotic shock describes a sudden exposure of drug-loaded erythrocytes to an environment, which is far from isotonic to evaluate the ability of resealed erythrocytes to withstand the stress and maintain their integrity as well as appearance. Incubating the resealed erythrocytes with distilled water for 15 min followed by centrifugation at 3000 rpm for 15 min may cause the release of hemoglobin to varying degrees, which could be estimated spectrophotometrically.[17]

Turbulence shock

This parameter indicates the effects of shear force and pressure by which resealed erythrocyte formulations are injected, on the integrity of the loaded cell.[18] Loaded erythrocytes are passed through a 23-guag hypodermic needle at a flow rate of 10 ml/min. After every pass, aliquot of the suspension is withdrawn and centrifuged at 300 g for 15 min, and hemoglobin content, leached out is estimated spectrophotometrically.[19]

Morphology and percent cellular recovery

Phase contrast optical microscopy, transmission electron microscopy, and scanning electron microscopy are the microscopic methods used to evaluate the shape, size, and surface features of loaded erythrocytes. Percent cell recovery can be determined by assessing the number of intact erythrocytes remaining per cubic mm with the help of hemocytometer.[20]

Percentage cell recovery

May be determined by counting the number of intact cells per cubic mm of packed erythrocytes before and after loading the drug.

Determination of entrapped magnetite

Atomic absorption spectroscopic method is reported for determination of the concentration of a particular metal element in a sample.[21] The HCl is added to a fixed amount of magnetite-bearing erythrocytes and content are heated at 60°C for 2 h. Then, 20% w/v trichloroacetic acid is added and supernatant obtained after centrifugation is used to determine magnetite concentration using atomic absorption spectroscopy.[13]

Deformability

It evaluates the ease of passage of erythrocytes through narrow capillaries and the RES there by evaluates the lifespan of the cells. The deformability is measured by passage time of the definite volume of cells through capillary of 4 mm diameter or polycarbonate filter with average pore size of 45 mm. This is done to determine the rheological behavior of the cells and is dependent on the viscoelasticity of the cell membrane, viscosity of the cell contents, and the cellular surface-to-volume ratio.

Viscosity and sedimentation volume

Viscosity is determined using a rotator Brookfield viscometer. Sedimentation volume of the formulation was obtained by measuring the height of sediment in a graduated measuring cylinder.[22]

$$F=vu/v0$$

F=sedimentation volume; Vu=ultimate volume of sediment; V0=original volume of formulation.

Centrifugal stress

For this, ne drug conjugates are centrifuged at variable rpm in a refrigerated centrifuge at 4°C for 15 min. Drug leakage in supernatant solution was estimated.

HEMATOLOGICAL TESTS

Mean corpuscular volume

Mean corpuscular volume is a measure of the average volume of a red blood corpuscle (or RBC). The measure is attained by multiplying a volume of blood by the proportion of blood that is cellular (the hematocrit) and dividing that product by the number of erythrocytes (RBCs) in that volume. The mean corpuscular volume is a part of a standard complete blood count. In a laboratory test that computes MCV, erythrocytes are compacted during centrifugation.[35]

Mean corpuscular hemoglobin (MCH)

The MCH or mean cell hemoglobin is the average mass of hemoglobin per RBC in a sample of blood. It is reported as part of a standard complete blood count. MCH value is diminished in hypochromic anemia. It is calculated by

dividing the total mass of hemoglobin by the number of RBCs in a volume of blood.[36]

Erythrocyte sedimentation rate (ESR)

The ESR, also called a sedimentation rate or Westergren ESR, is the rate at which RBCs sediment in a period of 1 h.[37] It is a common hematology test and is a non-specific measure of inflammation.[23] To perform the test, anticoagulated blood was traditionally placed in an upright tube, known as a Westergren tube, and the rate at which the RBCs fall was measured and reported in mm/h.[38]

BIOCHEMICAL CHARACTERIZATION

Extraction of lipids

The red cell membrane collected was again centrifuged for 10 min at 15,000 g. The supernatant was decanted and the pellet obtained was suspended in 1 ml of methanol and homogenized. The solution was made up to 5 ml with the same methanol and centrifuged for 15 min at the same speed. The supernatant was decanted, added 14 ml of chloroform to the tube and transferred into a flat bottom flask. The content of this flask was evaporated in a fume hood. To the evaporated content, added 5 ml of chloroform-methanol mixture to dissolve the solid and the resulting solution is poured into a labeled centrifuge tube and covered with paraffin.[39] These tubes are centrifuged at 6000 rpm, and two differed layers of fluid were visible. The upper aqueous layer was aspirated and discarded while the lower layer was retained for lipid testing. Aliquots of this layer were used for the estimation of cholesterol, phospholipids, and TG.[40,41]

Membrane cholesterol

Lipid extract of 0.6 ml was evaporated. Different standard concentrations ranging from 20, 40, 60, 80, 100, and 120 µg were used. Each standard of 0.05 mL was transferred from respective tubes to the tubes labeled S1-S6 and evaporated. To these tubes (including the test) was added 3 ml of ferric acetate uranyl acetate. The blank was run simultaneously. Sulfuric acid ferrous sulfate 2 ml was added, and the contents were mixed properly. The tubes were left standing for 20 minutes, and their color intensity was read at 560 nm. The cholesterol concentration of the test sample was determined using a standard graph plotted.

Membrane phospholipids

Lipid extract of 0.6 ml was evaporated in a fume hood. Standard phosphate solutions of five different concentrations were used. A blank was run simultaneously. To all the tubes added 1 ml of perchloric acid, 0.5 ml of 3% ammonium molybdate (freshly prepared), and 0.5 ml ascorbic acid (freshly prepared). The total volume of all the tubes was made up to 6 ml with deionized water. All tubes were kept in boiling water for 6 minutes and the blue developed was red at 710 nm. The phospholipid concentration of the test sample was determined using a standard graph plotted.[42]

Membrane Na^+K^+ATPase

The membrane collected was used for the Na^+K^+ATPase. The membrane was made up to 1 ml with isotonic buffer and added 0.2 ml of 50 mM $MgSO_4$, 50 mM KCl, 1 mM EDTA, and 40 mM ATP solution. Blank was run along with it which contained the entire reagent except the red cell membrane. The tubes were equilibrated at 37°C for 10 min. To all the tubes added 0.1 ml of enzyme solution at intervals of 3 minutes, and the tubes were incubated at 37°C for 1 h in a water bath. After 1 h, 1 ml of ice-cold 10% TCA and 0.1 ml of ANSA were added. The blue developed was read at 620 nm. The Na^+K^+ATPase concentration of the test sample was determined using a standard graph.

Membrane protein

Membrane suspension of 0.1 ml was made up to 1.0 ml with deionized water. 5.0 ml of alkaline copper reagent was added, mixed well, and allowed to stand for 10 min at room temperature. Standards solutions of bovine serum albumin range from 10 to 100 µg and blank containing 1 ml water were treated similarly. Folin-Ciocalteu reagent of 0.5 ml was added very rapidly and mixed immediately. After 2 min, the blue developed was read at 640 nm. Protein concentrate ions were expressed in mg/ml of the membrane preparation.

PHARMACOKINETIC CHARACTERIZATION

The albino mice of either sex (average weight 20-25 g) were divided into five groups each comprising six mice. The first group was administered with drug solution.[25] The second to fourth group was administered "NERs-drug conjugated" formulation. The fifth group was kept as control. The blood samples were collected from retro-orbital plexus using a heparinized syringe at different time intervals, and the samples were analyzed for drug content by polarography.[35,42]

STABILITY

Stability studies of the prepared NERs were carried out by storing all the formulations at 4°C and at room temperature for 2 weeks and 1-month period.[23,27]

IN VITRO STORAGE

Storage is one of the limiting factors in case of drug-loaded erythrocytes. The common storage media is Hank's balanced salt solution and acid-citrate dextrose at 40°C.[28] The addition of calcium chelating agents or purine nucleosides improve circulation survival time of cells upon reinjection. Exposure of resealed erythrocytes to membrane stabilizing agents such as DMSO, dimethyl 3,3-di-thio bispropionamide, gluteraldehyde,toluene-2-4 diisocyanate followed by lyophilization was reported to enhance stability.[22,23,27]

TOXICITY OF NERS

No toxic effects have been reported in animal studies directly due to the use of erythrocytes as circulating blood carriers. In human studies, no untoward effects were found in his patients receiving desferrioxamine in erythrocyte ghosts.[30] Patients gave normal values when tested for liver function and coagulation values. In another clinical trial, where patients were infused with glucocerebrosidase encapsulated in erythrocytes as a replacement therapy in Gaucher disease, no side-effects were observed and the author commented that the experiments showed that resealed erythrocytes could be safely infused into patients and if "loaded" at a high hematocrit value (e.g. as packed cells) circulated *in vivo* for a prolonged time.[32] Side effects directly related to the drug are reduced since the encapsulation process effectively reduces the levels of free or protein bound drug in the circulation.[33]

APPLICATION

Slow drug release

For sustained delivery of antineoplastics, antiparasitics, veterinary antiamoebics, vitamins, steroids, antibiotics, and cardiovascular drugs.[34]

Drug targeting

Surface-modified erythrocytes are used to target organs of mononuclear phagocytic system/RES.[43]

Targeting RES organs

Resealed erythrocytes, by modifying their surface characteristics with antibodies, glutaraldehyde, sialic acid, sulfydryl, and surface chemical cross-linking, e.g. delivery of 125 I-labeled carbonic anhydrase loaded in erythrocytes cross-linked with sulfosuccinimidyl propionate.[35]

Targeting the liver-enzyme deficiency/replacement therapy

The enzymes used include ß-glucosidase, ß-glucuronidase, and ß-galactosidase. If glucocerebrosidases are accumulated in the liver and spleen can be treated by glucocerebrosidase-loaded erythrocytes.[5]

Treatment of hepatic tumours

Daunorubicin diffuses rapidly from the cells upon loading, hence covalently linking with erythrocytic membrane using glutaraldehyde or cisaconitic acid as a spacer is useful.[35]

Treatment of parasitic diseases

Because of selective accumulation of resealed erythrocytes within RES organs such as liver and spleen make them useful tool for delivery of antimalarial, antileishmanial, and antiamoebic drugs. Removal of RES iron overload desferrioxamine-loaded erythrocytes have been used to treat excess iron accumulated because of multiple transfusions to thalassemic patients.[35]

Targeting organs other than those of RES

The various approaches include entrapment of paramagnetic particles, photosensitive material along with the drug and antibody attachment to erythrocyte membrane to get specificity of action.[40]

CONCLUSION

This review addresses the use of NERs as biological carriers of therapeutic agents, such as drugs, enzymes, and peptides, as well as mainly used drug loading methods for erythrocytes encapsulation, and further potential applications of the erythrocyte carrier system. The various methods currently existing for encapsulating substances in erythrocytes mainly based on the osmosis-based methods and especially on hypo-osmotic dialysis, which is at present the method of encapsulation most widely used. The use of resealed erythrocytes helps in a safe and effective delivery of various drugs for passive and active targeting. The same concept also can be extended to the delivery of biopharmaceuticals. In near future, erythrocytes based delivery system with their ability to provide controlled and site specific drug delivery will revolutionize disease management. For the present, it is concluded that NERs carriers are "golden eggs in novel drug delivery systems" considering their tremendous potential.

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