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*Review*

# Erythrocytes and Nanoparticles: New Therapeutic Systems

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**Abstract:** Nano-delivery systems represent one of the most studied fields, thanks to the associated improvement in the treatment of human diseases. The functionality of nanostructures is a crucial point, which the effectiveness of nanodrugs depends on. A hybrid approach strategy using synthetic nanoparticles (NPs) and erythrocytes offers an optimal blend of natural and synthetic materials. This, in turn, allows medical practitioners to exploit the combined advantages of erythrocytes and NPs. Erythrocyte-based drug delivery systems have been investigated for their biocompatibility, as well as the long circulation time allowed by specific surface receptors that inhibit immune clearance. In this review, we will discuss several methods—whole erythrocytes as drug carriers, red blood cell membrane-camouflaged nanoparticles and nano-erythrosomes (NERs)—while paying attention to their application and specific preparation methods. The ability to target cells makes erythrocytes excellent drug delivery systems. They can carry a wide range of therapeutic molecules while also acting as bioreactors; thus, they have many applications in therapy and in the diagnosis of many diseases.

**Keywords:** erythrocyte; nanoparticles; carrier into carrier



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## 1. Introduction

Chemotherapeutic drugs that are conventionally used in clinical trials show several drawbacks, e.g. side effects and lack of targeting. Nanotechnology can offer innovative methods to enhance the efficiency of such drugs. The loading of therapeutic agents into nanostructures can increase the cellular targeting, allowing lower therapeutic doses [1]. The most important challenges faced in nanomedicine are represented by drug delivery targeting to regions of interest while keeping the loaded NPs hidden under the radar of the immune system [1–5]. Several nano-delivery systems were developed for drug delivery purposes—and their biocompatibility, non-immunogenicity, non-toxicity and biodegradability have led to great therapeutic applications of these structures. Depending on their composition, all of these nano-systems can be classified into different categories: polymeric NPs, e.g., dendrimers, micelles, nanogels and protein NPs; nonpolymeric NPs, e.g., carbon nanotubes, nano-diamonds, metallic NPs and quantum dots.; lipidic NPs, e.g., liposomes; and solid lipid NPs. Red blood cell (RBC) exploitation could represent an optimal solution in the search for ameliorative solutions in the design of novel functional nanomaterials that achieve the desired therapeutic effects while avoiding reticuloendothelial system (RES) clearance.

Red blood cells have attracted great attention in the field of bio-delivery for their unique biological and biophysical properties. As tissue oxygen delivery cells, RBCs are the most abundant cells of the human body. After differentiating from erythroblasts in the bone marrow, they are released into the blood and survive in the circulation for a period that may vary between 70 and 140 days after which they are removed by the RES. In adults, RBCs correspond to approximately 70% of all cells by number, thus representing the most extended cellular surface accessible to blood [6]. The specialized structure of the membrane combines internal viscosity with cytoskeleton elasticity; this gives RBCs high deformability and allows their passage through the capillaries, as well as granting them resistance to shear stress. Additionally, an RBC membrane is characterized by several proteins that impart many vital functions, e.g., an active role in systemic metabolism, participation in redox homeostasis, important modulation of innate immunity, responsiveness to neurotransmitter stimulation or metabolism, release of macrovesicles and uptake and metabolism of circulating endogenous or exogenous bioactive molecules [7]. These features—along with their biocompatibility, low immunogenicity and high persistence—make RBCs an attractive platform for nanomedicine, in particular for drug encapsulation and delivery. In particular, the circulation time of loaded drugs is enhanced after this process thanks to the advantages of RBCs, which allow better pharmacokinetics. The most commonly used method to load drugs inside RBCs is the hypotonic method [8,9]. The creation of a hypotonic environment around the cells allows their swelling and the opening of temporary pores in the cellular membrane, thus allowing the drug/nanomaterials to penetrate and accumulate in the RBCs. Subsequently, a hypertonic solution is added into the cell suspension in order to release the previously created pores and to restore the original cellular size [10]. The swelling and resealing process used to encapsulate cargos into RBCs is the critical point—necessary for successful drug loading and the preservation of membrane biocompatibility. These factors are essential when the goal is to prevent a reduction in their circulation time [11]. In effect, although widely used, this method leads to a certain degree of change in cell morphology and membrane structure which results in hemolysis, thus impairing biocompatibility of prepared RBC carriers and, consequently, their use in vivo [10].

Another key problem could be the incompatibility of different blood groups. Human blood groups depend on the presence of specific surface antigens, such as sugars. This aspect must be taken into consideration during the administration of modified RBCs. If the receiving patient presents a different blood group from the donor, problems of agglutination or hemolysis may arise. For example, the 0-blood group (also called universal donor due to the absence of surface antigens) is characterized by the production of antibodies against the A and B blood group antigens. Additionally, an RhD-negative patient can suffer an immunogenic response if given RBCs from an RhD-positive subject. To avoid such problems, red blood cells should be sourced from healthy donors with the same blood group for which they are intended. In addition, where possible, autologous red blood cells should be removed, loaded and reinfused into the same subject, ensuring absolute biocompatibility.

Another strategy to overcome these drawbacks is based on the conversion of one blood group to another through the action of specific enzymes able to apply specific modification of the surface markers. In this regard, specific exoglycosidases have been used to convert blood group B to 0. In those cases, no clearance problems were detected for these modified RBCs once injected in the receiving patient. On the other hand, the same cannot be said for the conversion of blood group A to 0, because of very low conversion efficacy [12].

Alternatively, the hyperosmotic method [13] allows for the creation of temporary pores in RBCs. Via contemporary exposure to the cargo of interest, RBCs are loaded after the osmotic gradient. After that, by washing the cells, the external concentration of substance decreases, the pores close and the loaded molecules remain inside the RBCs [11].

Another method is electroporation, which is the encapsulation of therapeutic molecules in RBCs through a high-intensity electric field applied to the cell suspensions [12]. Despite the potential of employing RBCs as carriers for therapeutic agents, their use can

be hampered by different critical points during their manipulation. These should not be underestimated, and can include: damage to RBCs membranes during drug encapsulation, changes in osmotic pressure, contamination during manipulation, etc. [14]. A pivotal role in overcoming these drawbacks is played by the physicochemical properties of the drug. This is because loading molecules with high affinity toward RBCs leads to a reduction of the steps of cell manipulation that could compromise the biocompatibility of the RBCs [15]. The other principal approach to produce RBC vectors for therapeutic purposes is loading therapeutic molecules onto the cell surface. One possibility is the direct attachment of specific ligands onto the RBC surface by exploiting physical interactions such as van der Waals forces or hydrogen bonds [16]. Another option is coupling therapeutic molecules to ligands specific for the RBC membrane [9].

However, surface loading can compromise the biocompatibility of RBCs, leading to lysis and elimination through activation of complement pathways (and others). To overcome this drawback, one developed solution could utilize the employment of biotherapeutic agents (e.g., fibrinolytics) conjugated with antibodies for complement receptor type 1 (CR1), present on erythrocytes' surfaces [17]. Furthermore, there are other targets on RBCs which can be used for this purpose. One example is human glycophorin A (hGPA), which can interact with the single-chain variable fragment (scFv) fragment of the monoclonal antibody TER119, conjugated with different recombinant biotherapeutics [18]. For example, this antibody—when fused to complement-regulating proteins, including CR1—can enhance RBC resistance to lysis [19]. Similarly, by employing the endothelial anticoagulant and anti-inflammatory glycoprotein thrombomodulin, conjugated with TER119 scFv, a longer half-life in the bloodstream can be obtained compared with soluble thrombomodulin, improving the prevention of arterial and venous thrombosis [20]. Thus, by exploiting the advantageous characteristics of RBCs, it is possible to improve the treatment of various diseases, making RBCs suitable carriers of several therapeutic agents. As mentioned above, some concerns may arise from the difficulty in choosing the best method to load the therapeutic molecules. Encapsulating the molecules inside RBCs requires invasive procedures due to the need to generate transient pores in the membrane, which could cause an alteration in the cell shape, loss of glycocalyx, loss of ions or loss of water. On the other hand, surface conjugation of specific ligands could be less damaging—but could lead to a series of disadvantages including hemolysis, reduction of deformability of the membrane, alteration of biocompatibility, the blocking of protective components (CR1) or inhibition of the specific ligand CD47, which prevents phagocytosis [21,22].

## 2. Bio Hybrid Carrier Using RBC

Three types of nano/bio hybrid carriers arising from RBCs can be classified (Figure 1): whole red blood cells which have incorporated drug loaded NPs (carrier into carrier); NPs coated with RBC membrane; and NERs, which can be considered NPs derived from RBC.

The design and production of NERs could reduce the micrometer-sized RBC membrane to nanometer-sized while maintaining its interesting biological and biophysical properties. The biomedical application of NPs can be hindered if they are recognized by immune system cells—in particular, macrophages, which prevent NPs from reaching their final destination. Additionally, the activation of the complement system, which leads to inflammation and side effects, can be a hindrance. The encapsulation of NPs into RBCs is a camouflage tactic against the immune system [23,24]. Several clinical trials were performed by successfully loading doxorubicin [25] or valproate [26] into NPs and subsequently encapsulating them into RBCs. For example, using this method, Jiang and colleagues [27] reported an increase of the half-elimination time of doxorubicin from the bloodstream, compared to the administration of NPs alone. This external camouflage increases the efficiency and safety of NPs—even if these systems are limited by an increase in the stiffening and sensibilization of erythrocytes to damage by osmotic, mechanical and oxidative stress [28,29]. The employment of a carrier into carrier has a great advantage over classical RBC carriers with respect to preventing the rapid and uncontrolled

escape of the encapsulated therapeutic agent due to passive drug diffusion out of the RBCs. Recently, researchers have designed a cellular hitchhiking strategy. This new alternative method can improve blood pharmacokinetics and the vascular delivery of NPs while simultaneously avoiding uptake by tissues such as the liver or spleen. A recent work showed chitosan-based nanogels (loaded with the antiepileptic drug sodium valproate) to have high cell-escaping characteristics, especially during the encapsulation procedure [26]. These NPs were prepared through an ionotropic gelation, mediated by the interaction of the polycation chitosan with the polyanion sodium tripolyphosphate. Subsequently, using a hypotonic dialysis method, these nanocarriers were encapsulated into RBCs and showed a good profile of slow release of the agent after the administration of the carrier into carrier [26].

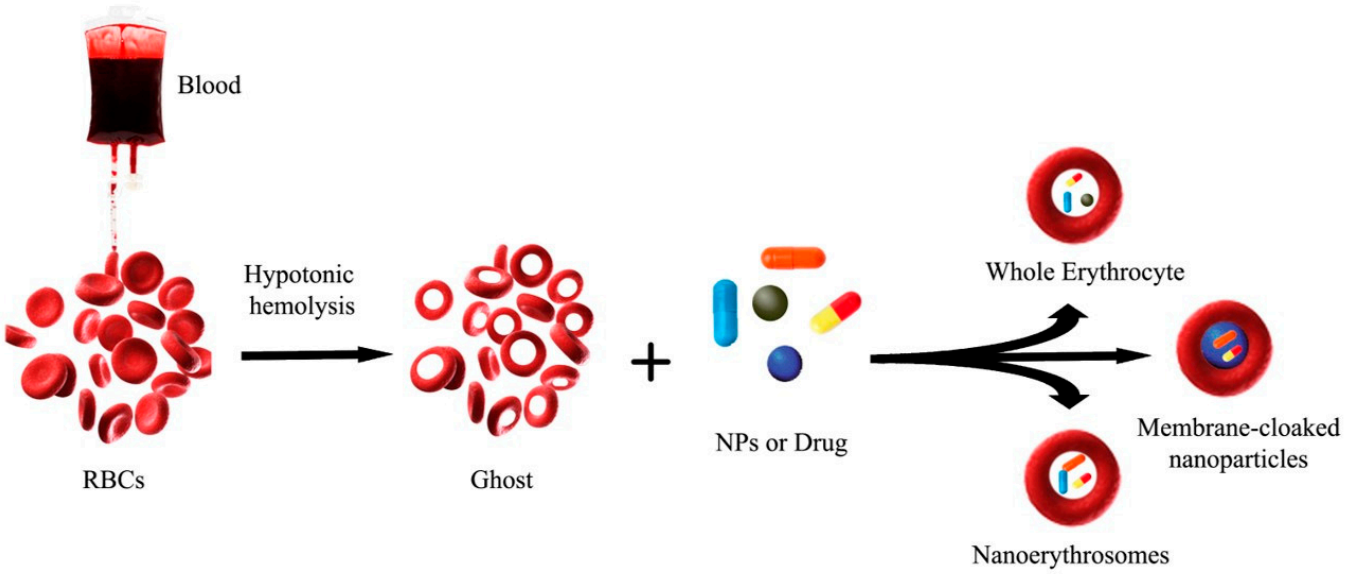


**Figure 1.** Scheme representing the preparation of whole red blood cell (RBC) loaded with nanoparticles (NPs) or therapeutic molecules (carrier into carrier), NPs coated with RBC membrane and nano-erythrosomes (NERs).

Another recent study [30] reported the encapsulation of two different molecules—5(6)-carboxyfluorescein and rhodamine (labeled dextran)—into RBCs using the hypotonic osmotic method, followed by incubation with gold NPs. The release of these molecules was triggered by exposure to a near-infrared laser which led to pore formation, allowing the cargo to be released from the RBC membrane. In a similar study, the stimulus was a magnetic field: doxorubicin was loaded into the RBC and chlorine e6 (Ce6) onto poly(ethylene glycol) (PEG)-coated iron oxide NPs; in turn, the NPs were conjugated onto the surface of Doxorubicin (Dox)-loaded erythrocytes. A site-specific and continuous Dox and Ce6 delivery toward tumor tissue occurred when an external magnetic field was applied [31]. Through this approach, this carrier into carrier (based on RBCs and magnetic NPs) blends the photodynamic therapy of chlorine with the chemotherapeutic effect of Dox, leading to the inhibition of tumor growth. Another application of carrier into carrier is diagnostic; recently, supermagnetic iron oxide NPs, used as contrast agents for magnetic resonance imaging, were encapsulated into RBCs under hypoosmotic conditions to overcome the fast phagocytosis to which superparamagnetic iron oxide nanoparticles (SPIONs) are subjected if administrated alone [32].

*Nanoerythrosomes (NERs)*

Another nano-system with applications in drug delivery is represented by NERs: small vesicles obtained from erythrocytes that offer additional benefits to the already well-known advantages of RBC-based systems. Because of their reduced size and higher surface-to-volume ratio, they can access locations unreachable for RBCs. They also have

prolonged circulation time [33], due to their avoidance of RES clearance [34]. Some authors have remarked that the characteristic shape of RBCs could also play an important role in the reduction of systemic clearance, as has been demonstrated for vesicles obtained from erythrocyte membranes containing anisotropic NPs [35]. Although some changes in their structure have been observed during the formation of NERs [36], they preserve enough similarity with the surface of native erythrocytes to maintain their inherent biocompatibility. Nevertheless, modifications of NERs have been proposed in order to increase vesicle suspension stability and circulation times [36,37].

NERs—classified by some authors as a particular type of RBC-mimicking particle [34,35]—are considered in this review as different nanoparticulated entities, due to their biological origin (in contrast to the synthetic NPs above mentioned).

Erythrocytes are the main extracellular vesicle producers in blood; in healthy individuals there is a physiological basal level of derived nanovesicles that can be increased under pathological conditions [38]. Nevertheless, these vesicles can also be obtained artificially, and different methods for NER production (e.g., sonication, electrical breakdown, extrusion and their combination) have been proposed.

As well as carrier erythrocytes, NERs have been studied as carriers of drugs, enzymes and other peptides, toxins, genetic material, contrast agents [39] and even other NPs [35]. They can be applied in drug targeting to the liver, the spleen, lymphatic ganglia and tumors [40].

NERs have also been proposed for cancer vaccination. NERs loaded with tumor-associated antigens (Nano-Ag@erythrosomes), obtained by fusion of NERs with the membrane of tumor cells, have confirmed their ability to reach splenic antigen-presenting cells and activate T cell immune responses. The administration of nano-Ag@erythrosomes is adjuvant in combination with immune checkpoint blockade (ICB) therapy-induced tumor regression in tumor models [41].

Combination of NERs with other systems have also been explored. Buss et al. demonstrated the feasibility of an active cargo delivery system designed by conjugation of NERs with genetically engineered peritrichously flagellated *Escherichia coli* by biotin–streptavidin interaction. These biohybrid micro-swimmers may be used in autonomous targeted cargo delivery applications, allowing (1) faster propulsion than those based in RBCs and (2) access to smaller locations, thanks to the nanosized dimensions of their components [42].

## 3. NPs and RBC

### *3.1. RBC and Solid Lipid NPs (SLN)/Nanostructured Lipid Carriers (NLC)*

The last decade has witnessed a rising interest in lipid-based nano-systems such as solid lipid NPs (SLNs) for drug delivery purposes due to their lipid nature and submicron size [43,44]. Because SLNs are made of natural lipids (triglycerides, fatty acids, sterols (usually cholesterol) and waxes), they are less toxic in comparison to polymeric NPs [45]. However, SLNs are characterized by a low drug loading efficiency with hydrophilic compounds such as peptides and proteins [46]. A new generation of lipid NPs, namely nanostructured lipid carriers (NLCs), has been developed to overcome the limitations of SLNs. Thus, an improvement in loading efficiency can be obtained using NLCs composed of a mixture of solid and liquid (e.g., short-chain triglycerides, vitamin E, lecithin) lipids that can ensure a less structured solid lipid matrix [45]. Another noteworthy aspect of these lipid NPs is that they can protect the drug from degradation and promote its transport across mucosal barriers [47].

To the best of our knowledge, no carrier in carrier system exploiting the potential of lipid carriers such as SLN and NLC in biological vectors (i.e., red blood cells) has been reported. However, some articles focusing on the interactions between lipid carriers and red cells are of interest, in view of the formulation of a vehicle where chemical advantages (i.e., from SLNs and NLC) and biological compatibility (as from red cells) can be combined.

In vitro experiments have been conducted on chloroquine-loaded and heparin surface-functionalized solid lipid NPs [48], brought to formulated SLNs with the goal of specifically

targeting parasitized red blood cells—as afforded by heparin. The investigated SLN NPs exhibited bigger in vitro activities than chloroquine (CQ) on CQ-sensitive parasites, showing antimalarial activity against chloroquine sensitive strains of *P. falciparum*. Moreover, lipid NPs of Precirol or Compritol were tested together with Precirol/squalene NLCs for application as delivery systems aiming at achieving parenteral camptothecin administration. Both carriers showed sustained drug release and strong cytotoxicity; also, limited hemolysis was provided (around 5%), suggesting a good tolerance to lipid NPs after injection [49].

Development and evaluation of lipid NPs for camptothecin delivery has also been carried out [49]. In view of targeting specific districts of the human body, SLNs and NLCs, once delivered from a biological carrier, should possess excipients capable of increasing contact with the same district. In this regard, in order to prolong the residence time of NLC in the nasal cavity following intranasal administration, Gartziandia et al. [50] modified the surface charge of lipid nanocarriers with polycation chitosan, giving rise to chitosan-coated lipid NPs (CS-NLC), in which the neurotrophic factor human IGF-I was efficiently encapsulated in view of administration in anti-Parkinson therapy.

### 3.2. RBC and Inorganic NPs

Various inorganic NPs have been used in anticancer therapy for drug delivery [2,51]. A coating of RBC membrane can improve the circulation time of these NPs, avoiding immune clearance by reducing macrophages' cellular uptake. $Fe_3O_4$ NPs have been proven to possess the good characteristics (e.g., controlled size, low toxicity and biocompatibility) necessary to achieve therapeutic applications [52]. In a recent study [53], researchers coated these NPs with RBC membranes, providing better blood retention, reduced macrophage uptake and less biodistribution into spleen and liver, compared with $Fe_3O_4$ NPs and PEG-$Fe_3O_4$ NPs. Another important aspect of these NPS regards the pharmacokinetic trend of the second injection of RBC-$Fe_3O_4$ NPs (despite PEG-$Fe_3O_4$ NPs), which showed a decrease in the Fe content and an accelerated immune clearance.

Mesoporous silica NPs are another type of inorganic NP which has found application in the anticancer therapy field [54]. To overcome the low efficacy of these NPs due to their aggregation and limited circulation time, researchers have exploited a camouflaged RBC, achieving high loading capacity of doxorubicin and of the near-infrared photosensitizer chlorin e6 loaded therein. [55].

In recent years ultrasmall nanomaterials (<100 nm)—with application as inorganic nano-antimicrobials—have been prepared utilizing the laser ablation approach [56]. RBC-membranes are used to improve antibiotic delivery systems consisting of gelatin NPs coated with vancomycin antibiotic [57]. The camouflaged RBCs seem to improve the immune evasion, reducing symptoms caused by bacterial infection due to the optimal release of drugs in the targeted site [58].

In this direction, gold (Au) NPs have recently been extensively studied as drug carriers [59,60]. In a recent study [61], Au NPs with the RBC membrane have shown a prolonged half-life in circulation, a better tumor deposition and, consequently, increased survival in treated mice. The process involved a hypotonic treatment of the RBC, followed by an extrusion through porous membranes, which led to the formation of RBC membrane-derived vesicles that were fused onto the Au NPs [62]. After the fusion process, the resultant RBC-Au NPs showed a core-shell structure with an increase in diameter and a less negative surface charge (Figure 2). Also, in this study, escape from macrophage uptake (after covering NP with RBC membrane) was confirmed, thanks to the presence of specific ligands, such as CD47 and sialic acid, on their surface.

All of these studies confirm the positive impact in functionalization when RBCs are uses to coat inorganic NPs.

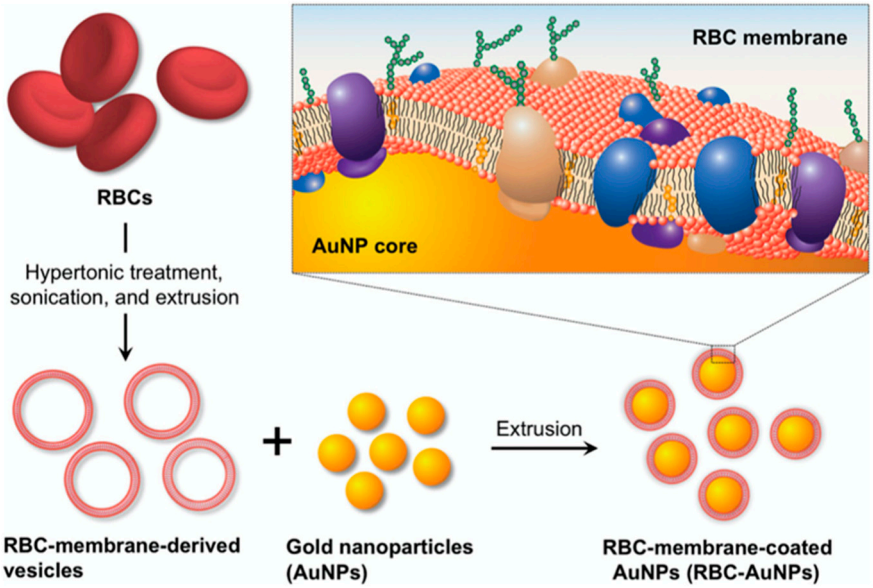


**Figure 2.** Preparation of gold NPs coated with RBC membrane. Reproduced with permission from [62] (Copyright John Wiley & Sons, 2013).

### *3.3. RBC and Polymeric NPs*

Similarly, polymeric NPs have been increasingly exploited for active delivery of therapeutic molecules [63–65].

Correspondingly, the use of polymeric NPs covered with RBCs has shown different advantages. In a recent study [66], RBC-camouflaged poly(caprolactone) NPs loaded with paclitaxel (PTX) and co-administered with tripeptide Arg-Gly-Asp (iRGD, a tumor penetrating peptide) have shown good results in metastatic breast cancer treatment (Figure 3). The antineoplastic agent PTX was able to guarantee a strong regression of metastases, acting for a longer time thanks to the increase in blood circulation time. In addition, to ensure an effective drug delivery [55], the possibility of modifying the polymeric core with thermosensitive lipid dipalmitoylphosphatidylcholine (DPPC) was also investigated.

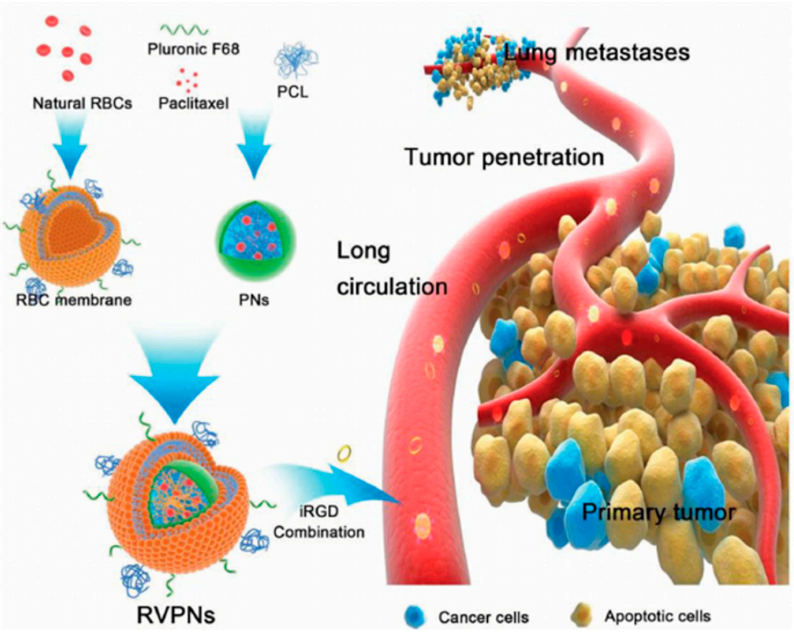


**Figure 3.** Scheme of preparation of polymeric NPs coated with RBC membrane. Reproduced with permission from [66] (Copyright John Wiley & Sons, 2016).

Poly (lactide acid) (PLA) represents another biocompatible and biodegradable polymer used in the field of drug delivery [26,67]. Recently, RBC membrane-coated PLA NPs loaded with doxorubicin for the treatment of acute myeloid leukemia have been explored [55]. These structures showed a sustained release of the drug compared with uncoated PEG-PLA NPs (with a release of 20% for RBC membrane-coated PLA NPs versus 40% for PEG-PLA NPs within 72 h).

Another polymer widely used to perform NPs is the poly(D,L-lactide-co-glycolide), PLGA, which fulfils several requirements for drug or gene delivery. In a recent study, RBC membrane-coated PLGA NPs loaded with perfluorocarbons (PFC) [68] showed a decrease in tumor hypoxia thanks to the high solubility of PFC. Similarly, good properties of PLGA have found application in colorectal cancer treatment.

In another work [69], cell proliferation of the gastrointestinal tract was inhibited after treatment with gambogic acid-loaded PLGA NPs, which were covered with RBC membrane. Mice treated with these NPs showed an increased survival time.

Although polymeric NPs covered with RBCs offer interesting opportunities in nanomedicine, a recurrent drawback is related to the lack of targeting of solid tumors, due to the outside shell represented by RBC membranes. Thus, surface modifications of RBC membranes—or thermosensitive lipid DPPC insertion in the inner core of polymeric NPs—could represent appropriate strategies for enhancing the efficacy of drug targeting and release. This could be thanks to a more rapid destruction of the core and a better release of the therapeutic agent [70].

### *3.4. RBC-Mimicking Particles*

Another strategy regarding NP-delivery systems involves the development of mimicking particles [71,72]. In particular, for erythrocyte-related delivery systems, these are synthetic particles that try to emulate the beneficial properties of erythrocytes (e.g., their morphological properties, membrane composition and surface) and hence their physiological behavior (e.g., resistance to phagocytosis and systemic clearance) [40].

Several materials have been studied in attempts to mimic the erythrocyte properties considered useful for drug delivery. Modifications from polystyrene or PLGA spheres led to RBC-shaped templates and cationic and anionic polymers deposited by means of the layer-by-layer technique [70]. Materials, such as silica, have been coated with lipids to emulate the erythrocyte outer leaflet, demonstrating an improvement of their hemocompatibility [73]. Other RBC-like particles based on cellulose derivatives have been synthetized by electro-spraying. The inclusion of magnetite NPs and fluorescent dye allowed the researchers to obtain a dual-imaging probe with a flexibility similar to RBCs [74].

Some of the most investigated materials in this field are hydrogel microparticles of different form and flexibility [75–77]. Particles—including PLGA based particles —mimicking flexibility and the ability to bind and carry oxygen, drugs and contrast agents have been obtained [70]. Discoidal polymeric nano-constructs (DNPs)—composed by a homogeneous mixture of poly(D,L-lactide-co-glycolide)-acid carboxylic terminated (PLGA-COOH) and poly(ethylene glycol) diacrylate (PEG-DA), conjugated to tissue plasminogen activator (tPA)—have demonstrated their lytic activity with an adequate deformability and blood circulating times that confirm their potential for thrombolytic therapies [78].

Particles based on 2-hydroxyethyl acrylate have been also designed to mimic murine erythrocytes, demonstrating that their biocompatibility, circulation times and biodistribution are conditioned by the particle elasticity [75].

Stimuli-responsive systems based on poly (methacrylic acid) (PMAA) particles with morphology and flexibility similar to erythrocytes have been developed in order to integrate advantages of these erythrocyte mimicking particles with the potential of drug delivery triggered by existing differences between pathological and healthy regions [77].

## 4. Innovative Application of RBC-Coated NPs versus Nano-RBCs

Due to widespread antibiotic resistance, the application of nano- red blood cells (nano-RBCs) as antimicrobial drug carriers can be exploited by using enzyme-responsive properties. Loading antibiotics such as vancomycin [58] into gelatin NPs and coating them with RBC membranes has been shown to produce a nano system that can be activated by the gelatinase present at the site of bacterial infection. This enzyme was able to degrade carriers, leading to the release of the therapeutic agent only at the site of infection -thus killing bacteria and achieving a reduction in immune clearance thanks to the external shell.

As explained above, introducing stimuli-responsive NPs onto RBC surfaces can induce specific cargo release. Additionally, by coating gold NPs (AuNPs) with RBCs-membrane, specific nano-RBCs for photothermal therapy can be produced. In this frame, RBC-coated AuNPs were injected into mice and irradiated with an infrared laser. A clear photothermal effect was observed together with the ablation of cancer cells within the irradiation zone [61]. In this way, creating a nano-RBC with the long blood circulation lifetime conferred by erythrocytes—together with the photothermal properties of AuNPs—allowed researchers to improve the treatment of tumors, thanks to the combined characteristics of the synthetic and natural carriers. Starting with one RBC, it is possible to obtain a great number of nano-RBCs through the classical hypotonic hemolysis, which leads to the formation of a ghost suspension. NERS are subsequently obtained by extrusion using a polycarbonate membrane filter pore and/or sonication (Figure 4).

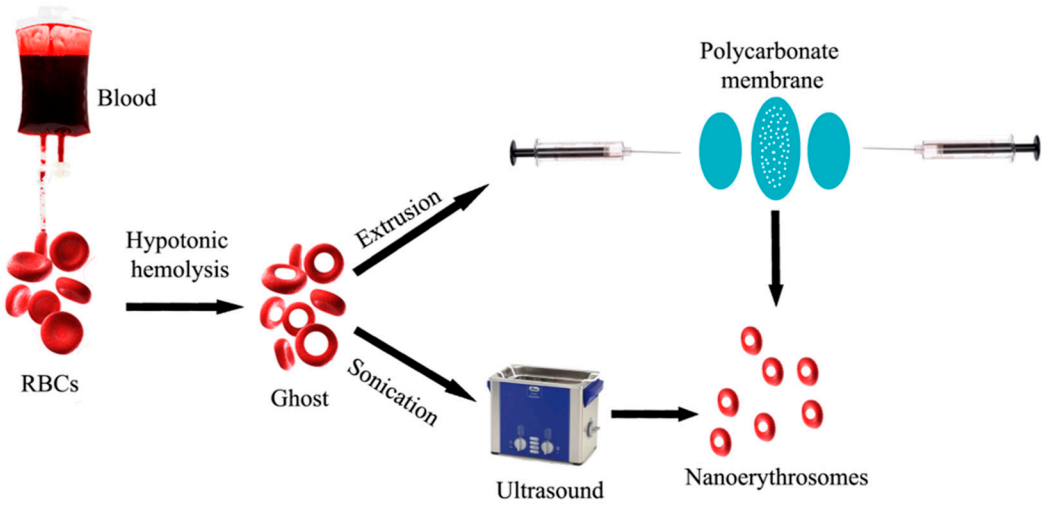


**Figure 4.** NERs synthesis using extrusion and sonication approaches.

The final diameter of the produced vesicles is about 100 nm. If compared with NPs coated with RBC membrane, they seem to show interesting advantages, due to their smaller size. Other advantages include their biocompatibility, biodegradability, easy preparation, and lack of immune response after administration. The therapeutic agent can be encapsulated inside, but it cannot be loaded on the surface.

Researchers employing nano-RBCs loaded with Fasudil (a therapeutic agent used for inhalation therapy in pulmonary arterial hypertension) have observed an increase in the half-life of drugs [33].

A recent study presented a design of nano-RBCs to deliver sodium tanshinone IIA sulfonate (STS) for the treatment of cardiovascular diseases [79], confirming the advantageous characteristics of this innovative carrier. The carrier preserved membrane proteins, thus avoiding the immune system, and showed an increased uptake from spleen and liver. It also improved the half-life time of the drug compared with the administration of the agent alone. Furthermore, the effectiveness of delivery was also influenced by the appropriate profile of drug release, showing a slower controlled release of the agent from nano-RBCs as opposed to the fast release of free STS.

Another recent study concerned the treatment of lysosomal storage disease [80] caused by a deficiency of β-glucocerebrosidase lysosomal enzyme, through its loading into erythrocytes. This strategy opens interesting perspectives for enzyme replacement therapy.

Alzheimer and Parkinson's are widespread neurodegenerative diseases which can probably be associated with hyperammonemia [81]. Researchers have shown that a promising solution could utilize the encapsulation of glutamate dehydrogenase or alanine aminotransferase into RBCs [82], which in turn could lead to a remarkable decrease in plasma ammonium concentration after administration.

A further meaningful biomedical application of nano-RBCs concerns the improved treatment of widespread human malaria disease [83]. As is well known, malaria is caused by parasites of genus *Plasmodium* belonging to species *P. vivax*, *P. ovale*, *P. malariae*, and *P. falciparum*. The female anopheles mosquito transmits the sporozoites of *Plasmodium* parasites into the blood of the host. Then, the sporozoites are transported to the liver where their conversion to merozoites occurs. Finally, they reach the RBCs, where they reproduce and infect other RBCs. The known therapeutic agents for treatment are quinine, chloroquine and artemether (ARM). The employment of the latter drug is limited by its very short half-life and poor aqueous solubility—thus, the need for new drug delivery systems for ARM. To address this, ARM-loaded nano-RBCs can be exploited for the ability of merozoites to attack RBCs, thus controlling the spread of the disease thanks to an intelligent release of the drug to the target. Testing three different methods to encapsulate ARM, (namely hypotonic dilution, hypotonic pre-swelling and modified hypotonic pre-swelling), researchers noted less damage to the cell membrane using the first method. The reason was adduced to a certain shrinkage of the cell due to the hypertonic treatment, followed by an incubation with hypotonic solution to restore isotonicity. This led to a better slow-release profile, allowing the drug to be retained for a longer period of time [83]. On the other hand, the hypotonic pre-swelling and modified hypotonic pre-swelling methods led to ARM-loaded nano-RBCs to be rapidly recognized by the RES system due to a decreased membrane integrity—which corresponded to a faster release of the drug.

## 5. RBCs Loaded with Biologically Active Compounds and NPs: New Perspective

Exploiting the advantages of biocompatibility, biodegradability, long-life in bloodstream, decreased side effects of therapeutic agents, immune evasion and the possibility of targeting specific organs such as liver, kidney and spleen, RBCs can be employed not only for targeted drug delivery, but also as bioreactors, as carriers with smart release, and also in diagnostics (Figure 5) [84,85].

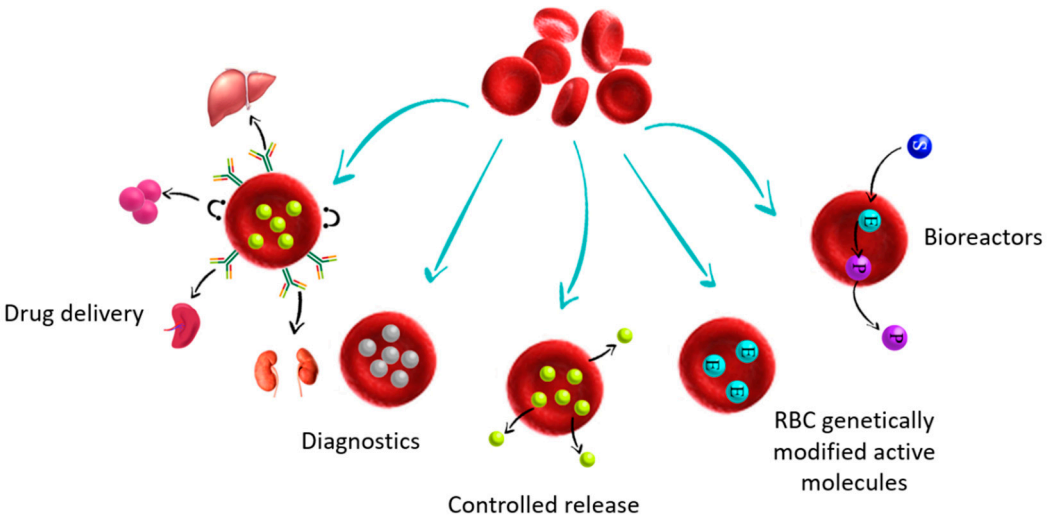


**Figure 5.** Different application of RBCs loaded with therapeutic agents and/or NPs.

The employment of RBCs as bioreactors has also found several applications in anticancer therapy. This employment has focused mainly on l-asparaginase, methioninase and arginine deiminase, which are able to decrease the blood level of amino acids (asparagine, methionine or arginine, respectively) necessary for biosynthesis during cell division. These are essential for cancer cells that are not able to synthetize asparagine or arginine on their own, since they do not contain asparagine synthetase [86]. Erythrocytes (as bioreactors) represent a new and interesting subfield for biomedical applications, giving researchers the opportunity to improve the treatment of specific diseases caused by the absence or decrease in the activity of certain enzymes that, if administrated alone, could cause an immune response or the rapid removal from the bloodstream. Despite these opportunities, the RBC membranes' impermeability represents a limit for their effectiveness in application as bioreactors. To overcome this barrier, specific membrane transporters can be exploited to allow the loading of the specific substrate of interest through an active transport. Otherwise, RBCs as bioreactors with a specific enzyme could allow entrance of cargo of interest. It is possible to utilize a non-diffusible form of the therapeutic agent, thanks to the creation of transient pores. Then the enzymatic conversion of the drug into the diffusible form is released in the blood, thanks the bioreactor's RBCs. An example of this strategy would be the prodrug dexamethasone 21-phosphate which, once inside erythrocytes, is subjected to conversion in the diffusible drug by the enzyme loaded inside [87]. Recently, the above-mentioned therapeutic strategies have found good results in the treatment of acute lymphoblastic leukemia, acute myeloid leukemia [88] and metastatic pancreatic cancer by loading RBCs with asparaginase. In the last case, a high effectiveness was successfully achieved for pancreatic cancer, with a reduction of the mortality rate of 40%, compared with chemotherapy treatment alone [89].

Nevertheless, the engineering of RBCs as carriers for the gradual release of anticancer drugs resulted in good performance in clinical trials. The administration of pharmacological agents such as anthracycline antibiotics (e.g., doxorubicin) can lead to cardiotoxicity and other undesired side effects on healthy tissues due to the production of reactive oxygen species [90]. These drawbacks have been overcome through the encapsulation of doxorubicin into erythrocytes, which were then employed for the treatment of acute lymphoblastic and myeloid leukemia. This engineered erythrocyte-based carrier led to a decrease in the peak plasma concentration of antibiotics compared with the administration of the free drug. Additionally, a targeted delivery to the liver, better effectiveness and a prolonged half-life in the bloodstream were reported, as were decreases in cardiotoxicity and the number of adverse reactions [91]. As is well known, senescent and damaged RBCs are removed upon passage through the spleen trabeculae and by the macrophages of the RES, including peritoneal macrophages, hepatic Kupffer cells and alveolar macrophages of the lung. These properties can be exploited for targeted drug delivery by a suitable modification of the RBC's surface in order to impart the damaged features. The exploited modification concerns the opsonization with antibodies of their membrane, the binding of complement component C3b or, alternatively, treatment with calcium ionophore [92]. In this regard, a targeted delivery of methotrexate to the liver was successfully carried out [93]. For the loading of the drug into RBCs, researchers have experimented with the electroporation, hypoosmotic and hyperosmotic methods. The prepared RBC carriers were administered, leading to decreased peak plasma concentration (which is important for a gradual release of the drug), along with increasing accumulation in the liver, higher methotrexate concentration in the bloodstream, and a longer half-life that treatment with the free drug.

In a similar way, RBC-based carriers envisaged a new contribution in the treatment of retroviral infections caused by the human immunodeficiency virus (HIV). The loading of antiretroviral drugs enabled inhibition of the activity of reverse transcriptase. Additionally, encapsulation of reduced glutathione (GSH) was shown to block viral replication and interfere with the protein envelope folding. These carriers were further modified in

order to target macrophages, preventing the transmission of HIV from macrophages to lymphocytes and reducing typical symptoms of the disease within 3 months [94].

Furthermore, the importance of targeted drug delivery is shown in other studies concerning immunization, cancer immunotherapy and immune tolerance [95]. The possibility of delivering antigens directly to macrophages or dendritic cells (DCs) can guarantee an improvement in the immunological response when compared to the response obtained using classical adjuvant. A classical study demonstrated these properties by the conjugation of glycoproteins B of Herpes Simplex virus type 1 onto the surface of red blood cells [96].

The ultimate goal of immunotherapy is to stimulate the immune system against cancer. With this in mind, loading specific tumor-associated antigens into RBCs could offer the opportunity to stimulate the activation of specific T-lymphocytes which act against tumoral growth. In a recent study, ovalbumin antigen was encapsulated into erythrocytes whose surface had been modified with specific antibodies in order to induce direct phagocytosis by macrophages and DCs. In this way, an immune response toward cell expressing ovalbumin was obtained, with the observed cell lysis induced by the activation of CD4+ and CD8+ T cells [97].

In other conditions, an immune tolerance towards a specific antigen is needed in order to prevent the attack of the immune system against its own antigens, a condition which can occur in autoimmune diseases or during a transplant. In this case, RBCs employed as a carrier can offer great opportunities. One potential application concerns enzyme replacement therapy—loading the enzyme alglucosidase alfa (AGA) to treat glycogen storage disease caused by alfa-glucosidase deficiency. The targeted delivery of the therapeutic agent to the antigen-presenting cells of the liver and spleen has led to an elimination of the humoral response toward AGA—which is otherwise caused by administration of the free drug. This occurs because liver DCs have an immature phenotype and there are not able to elicit an Ag-specific T-cell response; however, they can induce the development of T-cell tolerance [98].

## 6. Conclusions

Red blood cells (RBCs) represent an optimal platform for engineering novel delivery carriers, thanks to their targeting capacity (with respect to macrophages, the spleen, and the liver), their ability to avoid immune recognition and their long-life in the bloodstream. Several applications employing RBCs have been reported, including applications in which the whole cell is loaded with drugs or NPs, applications in which nano-RBCs transport drugs, and applications in which engineered NPs have been shown to affect various components of RBCs and their membranes.

In order to improve and broaden the application of RBCs in new therapeutic systems, it is crucial to preserve their functionality by developing specific storage and manufacturing processes that preserve their biocompatibility and prolonged survival in the circulatory system. Another crucial point to consider for the successful production of RBC-based therapeutic nano-systems regards the improvement of the effectiveness of delivery. Due to their ability to circulate in the bloodstream, erythrocytes are often limited in crossing tissue barriers such as the blood-brain barrier or endothelial barriers. Although targeted delivery systems consisting of RBCs conjugated with NPs are a challenging task (due to the difficulty of movement across physiological barriers, as well as uptake), new methods, e.g., in terms of masking, will be useful in targeting tumors. The methods represent a new paradigm of thinking.

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