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Chapter

The Biology and Therapeutic Applications of Red Blood Cell Extracellular Vesicles

Daniel Xin Zhang, Theodoros Kiomourtzis, Chun Kuen Lam and Minh T.N. Le

Abstract

This chapter focuses on the biology of red blood cell extracellular vesicles (RBCEVs) in normal and diseased conditions, and the potential application of RBCEVs in treatment. Extracellular vesicles (EVs) refer to membranous vesicles secreted by cells into the extracellular environment. EV biology belongs to a rapidly developing field in biomedical sciences. EVs represent a natural mode of cell-to-cell communication, which makes them suitable for delivery of therapeutic agents, such as nucleic acids and proteins, in the body. In particular, RBCEVs feature a wide range of benefits in drug delivery as compared to extracellular vesicles derived from other cell types. In comparison to other delivery systems currently available, RBCEVs are nontoxic, low immunogenic, conveniently obtainable, and easy to use and store. Therefore, RBCEVs boast promising and exceptional advantages in overcoming various limitations of conventional therapeutics.

Keywords: extracellular vesicles, red blood cells, therapy, drug delivery

1. Introduction

In the last decade, we observed a massive upsurge of studies in the field of extracellular vesicles (EVs) [1]. As it is known now, EVs can be loaded with different therapeutic molecules and transport them to recipient cells with little interrogation by the immune system. This property of EVs prompts new possibilities for treatment in various clinical settings [2–4]. In this chapter, we review the biology of EVs as a universal cellular component from a broader perspective, and afterward provide an updated view on red blood cell extracellular vesicles (RBCEVs), their merits and potential applications in therapeutics [5].

2. Overview of extracellular vesicles

2.1 History of extracellular vesicles

Wolf was the first to discover small procoagulant structures derived from activated platelets in human blood and named them "platelet dust" in 1967. He separated the small structures by ultracentrifugation and further characterized them using an

electron microscopy [6]. In 1987, Johnstone further studied the formation of such vesicles in the duration of sheep reticulocytes maturation *in vitro*. He was able to identify more activities and characteristics of the vesicles. However, he did not name the small vesicles or discover how they were generated in detail [7]. Both of these findings were important milestones in the field, which allowed for further studies on the function of these small vesicles. Today, we call these small vesicles as EVs. Valadi and colleagues were the first who discovered the natural delivery of microRNAs and mRNAs in EVs in mast cells. Later on, nucleic acid transport via EVs was also observed in many other cell types as an essential manner of intercellular communication [8–10]. We now have a much more profound understanding in the field of EVs due to the continuous efforts of various scientists throughout many decades.

2.2 Biogenesis and compositions of extracellular vesicles

EVs are a heterogeneous class of cell-derived structures with a lipid bilayer membrane, which comprise exosomes, microvesicles, and apoptotic bodies. They are either of the endosomal origins or are shed from the plasma membrane under physiological and pathological conditions. Additionally, they are present in almost all biological fluids, such as blood, urine, breast milk, cerebrospinal fluid, saliva, semen, etc. [11–17]. Further characterizations are based on the different sizes and biogenesis of EVs. Exosomes generally range from 50 to 150 nm in diameter and are secreted from endosomal multivesicular bodies, whereas microvesicles are larger vesicles ranging from 100 to 500 nm in diameter and are formed through a budding or exocytosis process of the plasma membrane [11, 18–23]. Apoptotic bodies are much larger, ranging from 800 to 5000 nm in diameter, and are generated by blebbing of plasma membrane from cells undergoing apoptosis. Hence, apoptotic bodies represent the fragments of dying cells and differ from exosomes and microvesicles in property (**Figure 1**) [17–22]. In this chapter, we will collectively term both exosomes and microvesicles as EVs with apoptotic bodies excluded.

The components of EVs are mainly proteins, lipids, and nucleic acids. However, due to different biogenesis mechanisms, the compositions of exosomes and microvesicles do vary slightly [11, 24–26]. Proteins that are associated with endocytic pathways can be usually found in EVs, such as flotillin and annexin. Some of the biogenesis-associated proteins, such as Tsg101 and Alix, and common tetraspanins, such as CD9 and CD81, are commonly used as EVs markers with CD63 which is mostly regarded as a marker of exosomes. However, currently, there lack well-defined protein markers to distinguish exosomes and microvesicles [11, 24–26]. Lipid components of EVs include phosphatidylcholine, phosphatidylserine, phosphatidylethanolamine, sphingomyelin, cholesterol, and so on, which can be found in plasma membrane as well. As microvesicles are formed by budding from plasma membrame, the lipid composition of microvesicles resembles that of plasma membrane of the cells more while exosomes are of higher levels in sphingomyelin, cholesterol, and phosphatidylserine [27–29]. It is noteworthy that many nucleic acid species are highly enriched in EVs. The lipid bilayer structure of EVs acts as a natural shelter against degrading nucleases in the extracellular environment and protects the nucleic acid cargo under adverse conditions such as long-term storage and multiple freeze-thaw cycles. In the recent decade, reports have it that many mRNAs, microRNAs, and other non-coding RNAs are discovered in EVs (**Figure 1**) [30–32].

2.3 Intercellular communication mediated by extracellular vesicles

As EVs are abundant and widely distributed in biological fluids and carry bioactive cargo, they influence various biological processes of the donor and recipient

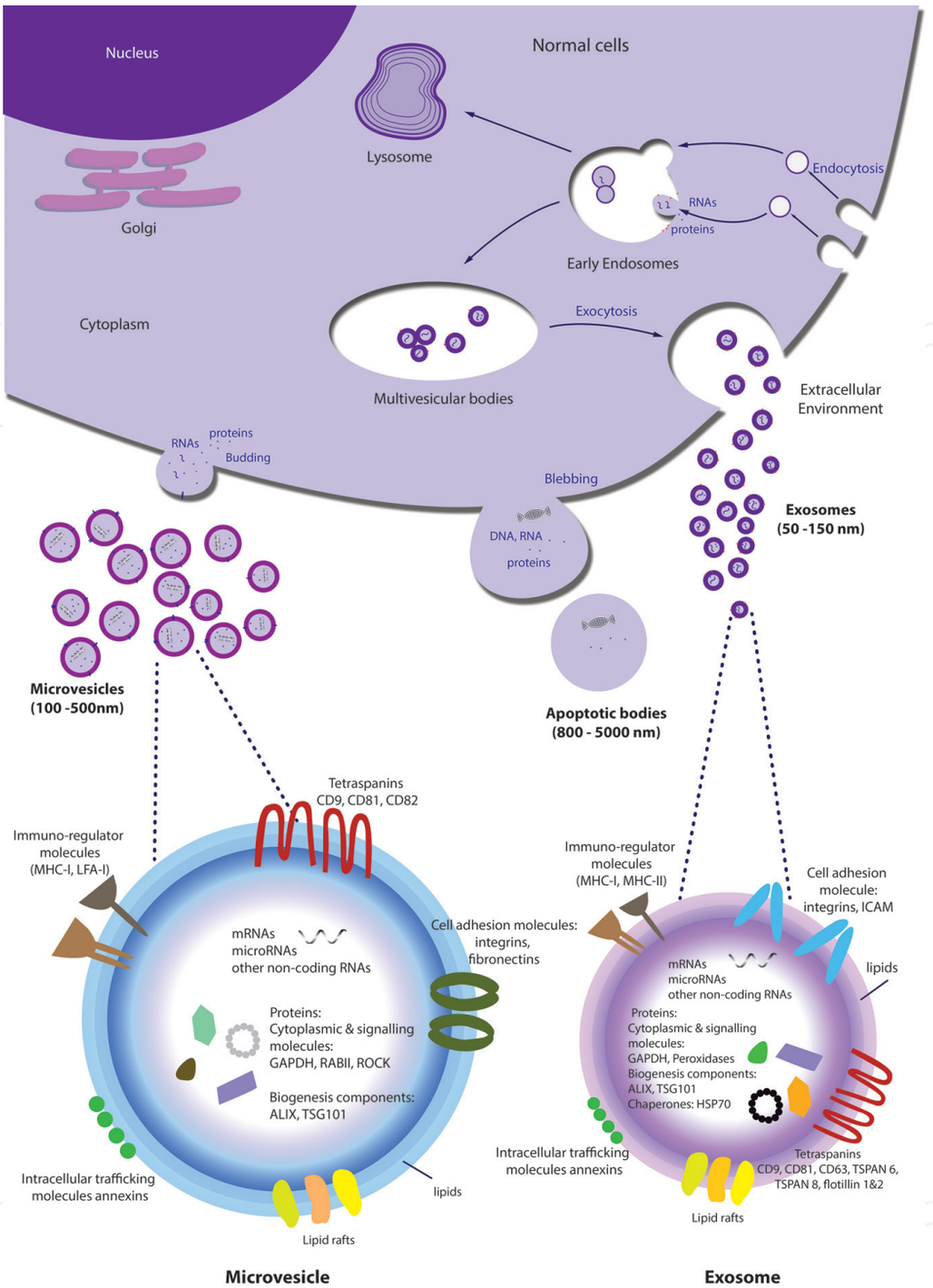

Figure 1.
Biogenesis and composition of extracellular vesicles. Extracellular vesicles (EVs) are composed of exosomes, microvesicles, and apoptotic bodies. Exosomes are typically of endosomal origins and are the smallest among them with 50 to 150 nm in diameter. Microvesicles are larger in size from 100 to 500 nm in diameter and are generated through an outward budding or exocytosis of the plasma membrane. Apoptotic bodies are usually the largest ranging from 800 to 5,000 nm in diameter and are generated by blebbing of plasma membrane from cells undergoing apoptosis. Major components of EVs are lipids, proteins, and nucleic acids. Due to different biogenesis mechanisms, the compositions of exosomes and microvesicles do vary.

cells [33]. The intercellular communication can occur between cells by transferring EVs that act as an exchange mediator of proteins, lipids, and RNAs. Thus, EVs have a fundamental role to play in important biological processes such as the exchange of surface membrane and horizontal RNA transport between neighboring and remote cells [18]. This aspect is being extensively investigated in cancers [34], neurodegenerative diseases [35], autoimmune disorders [36], aging [37], and so on. The bioactive cargo encapsulated by EVs contain valuable information from the source

of diseases, which can serve as robust biomarkers in diagnostics and status snapshots in treatment monitoring [38, 39]. The endogenous property of transporting molecules by EVs inspires researchers to utilize them as a superb delivery platform of therapeutic agents as well [40].

3. Concentrations on red blood cell extracellular vesicles

3.1 Red blood cell extracellular vesicles under physiological and pathological conditions

Similar to EVs released from other cells, EVs in the circulation carry biomarkers originated from the donor cells [41]. Usually EVs contain various markers which indicate their origins, e.g., CD235a (also called GPA) for RBCs, CD41 for platelets, and CD11c for dendritic cells [42–44]. RBCs express classes of CD59 and DAF, known as complement inhibitors, and signaling of CD47 and SHPS-1 molecules on the cell surface to protect themselves against endogenous elimination [45, 46]. For instance, the RBCs membrane protein CD47 inhibits RBCs phagocytosis via macrophages by binding to the inhibitory receptor signal regulatory protein alpha (SIRPα). The presence of such proteins on the surfaces of RBCEVs may help RBCEVs to escape from the clearance by macrophages if they carry CD47 on their surfaces [47–49]. Mature RBCs lack nuclei and most of the intracellular membrane structures; hence, EVs released from mature RBCs are microvesicles derived from the plasma membrane (**Figure 2**). Even with the same cell origins, the protein or lipid compositions of EVs may differ on account of the lateral cell membrane variation. Further proteomic assays have illustrated that to some extent, the proteomic spectrum difference of EVs and the releasing cells can be attributed to the stimulating conditions during EVs biogenesis [50]. Microvesicles derived from RBCs are reported to be different in protein contents when produced naturally *in vivo*, *ex vivo* released during cold storage of RBCs, or *in vitro* by treatment with EVs' release-inducing chemicals such as calcium ionophore, even though they seem homogeneous merely based on their size and/or surface markers. The most distinctively different proteins are stomatin and flotillin-2 [51].

There are many studies of RBCEVs under diseased conditions with malaria being frequently reported. Mantel and colleagues illustrated that EVs from human RBCs infected with *Plasmodium falciparum* parasites contain microRNAs that are able to moderate target genes in recipient cells [52]. The infected RBC-derived EVs in malaria were internalized by endothelial cells and the EVs-encapsulated miRNA-Argonaute 2 complexes repressed miRNA target genes and changed endothelial barrier properties. Furthermore, multiple miRNA species in such EVs were identified [52]. Ankarklev and colleagues reviewed the role of RBCEVs in malaria and found that the development of EVs by *Plasmodium* sp. is associated with clinical outcomes [53]. Studies have pointed out that elevated EVs levels were detected in patients with severe malaria cases, and increased EV excretion to the endothelium has been linked to infected RBCs [53].

3.2 Red blood cell extracellular vesicles for therapeutic purposes

Chang and colleagues demonstrated the ability of RBCEVs to efficiently deliver ultra-small superparamagnetic iron oxide particles into human bone marrow mesenchymal stem cells for cellular magnetic resonance imaging *in vitro* and *in vivo* in order to develop successful stem cell therapies [54]. The novel method overcomes the difficulty of relatively low intracellular labeling efficiency and addresses biosafety issues associated in comparison with traditional approaches. RBCEVs were

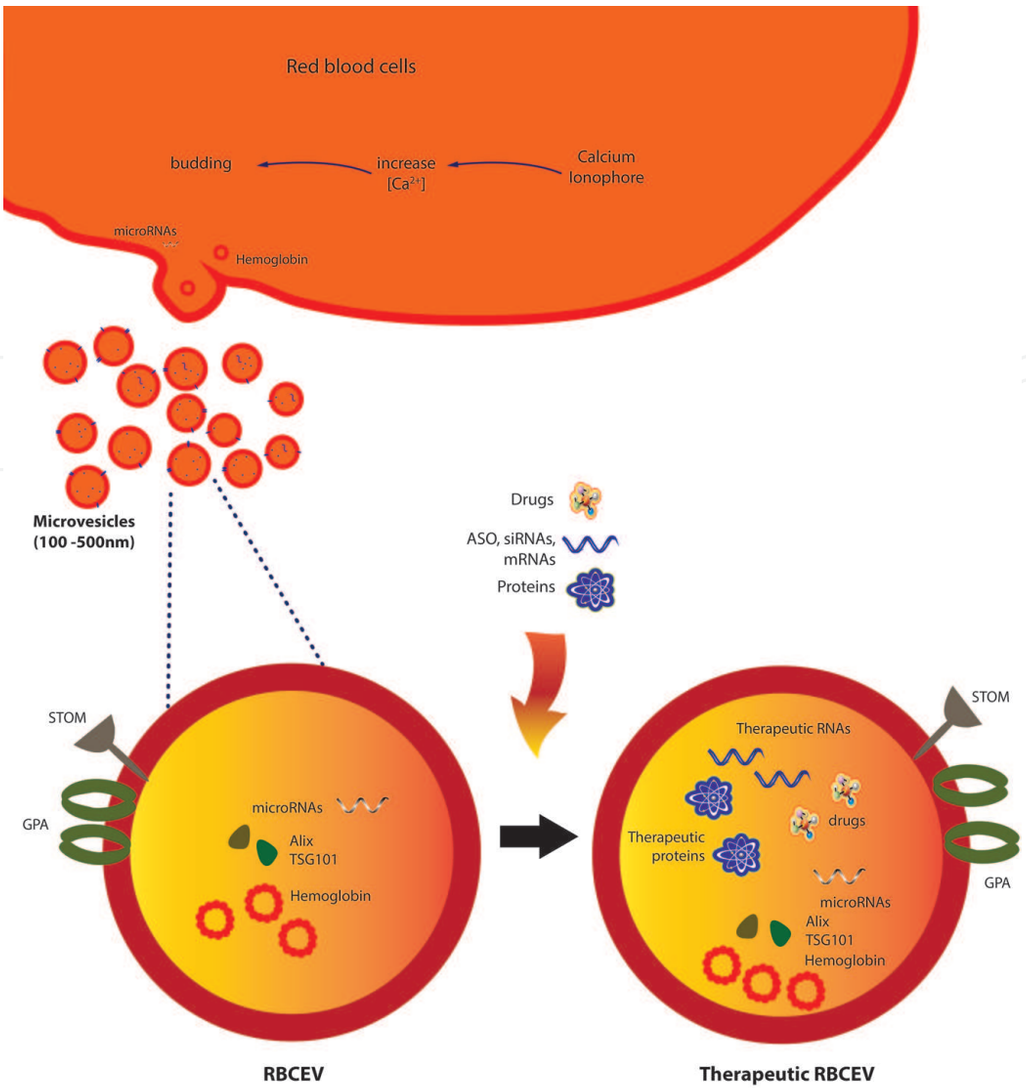

Figure 2.
Using red blood cell extracellular vesicles (RBCEVs) for therapeutic delivery. Calcium ionophore is added to RBCs which simulates the release of microvesicles, the only type of RBCEVs. Naturally, RBCEVs contain hemoglobin, Alix, TSG101, and some microRNAs in their lumen. They also display stomatin (STOM) and glycophorin A (GPA) on their membrane. RBCEVs can be loaded with therapeutic molecules including RNAs, proteins, and chemical drugs for delivery of these molecules to other cell types.

shown to be ultra biosafe and can be used as potential delivery vehicles for clinical applications due to their autologous property to human bone marrow mesenchymal stem cells [54]. Usman and colleagues developed a robust delivery system for RNA-based therapeutics using RBCEVs [55]. Using the novel RBCEVs delivery platform, both small RNA, e.g., antisense oligonucleotides (ASOs), and large RNA, such as Cas9 mRNA, can be electroporated into RBCEVs and transported to target cells in both solid and liquid tumors. In the study, microRNA-125b-ASO-loaded RBCEVs significantly dampened breast tumor growth by intratumoral injection and suppressed acute myeloid leukemia (AML) progression by systemic administration. Genome editing effects were also observed when RBCEVs were loaded with Cas9 mRNA with guide RNAs. The delivery efficiency was higher and far less cytotoxicity was observed as compared to other commercial transfection reagents [55].

3.3 Isolation and loading of red blood cell extracellular vesicles for therapeutic purposes

Up to now, standardized protocols for EVs isolation for either scientific research or clinical application are lacking [56]. One of the commonly used

methods to obtain EVs is ultrafiltration with subsequent differential ultracentrifugation. Ultrafiltration followed by liquid size exclusion chromatography suits the large-scale demand of isolating EV for therapeutics as the method results in, on the one hand, a significantly higher EV yield and, on the other hand, the well-preserved biophysical properties of the purified EVs [57]. Usman and colleagues provided a lab-based approach to purify RBCEVs using ultracentrifugation with sucrose cushion. To begin with, RBCs in whole blood were separated from white blood cells and plasma by low centrifugation and using leukodepletion filters. Then, the isolated RBCs were diluted in PBS and treated with 10 mM calcium ionophore overnight which can stimulate the release of RBCEVs and significantly increase the yield. In order to purify RBCEVs, RBCs and cell debris were removed by several rounds of low-speed centrifugation. Later, the resulting supernatants were passed through 0.45 μm syringe filters. Afterward, the RBCEVs were concentrated using ultracentrifugation at 100,000 × *g* for 70 min. Subsequently, RBCEVs were resuspended in cold PBS and layered above frozen 60% sucrose cushion and centrifuged at 100,000 × *g* for 16 h. The red layer of RBCEVs above the sucrose was collected and washed again with cold PBS by ultracentrifugation [55]. The approach is cost-effective, features high RBCEVs yield, and can be adopted in most laboratory settings. The use of sucrose cushion is also a highlight as it helps remove the protein contaminants outside RBCEVs, which might trigger unnecessary immune response and protects the integrity and biophysical properties of RBCEVs.

For therapeutic agents to be loaded into EVs, two major strategies currently have been applied. The first option is to load the therapeutic molecules, such as RNAs, into the EVs after EVs isolation, while the second one is conducted during EV biogenesis. These methods are also known as post-loading and pre-loading, respectively. The pre-loading encapsulation approach is also referred to as the endogenous method as it uses the cellular machinery in order to load small RNA into EVs. The pre-loading approach has been shown to work for the packaging of both siRNA and miRNA into EVs. The post-loading method artificially introduces RNAs into EVs, whereas pre-loading is performed in the EVs biogenesis. Post-loading can be subdivided into passive loading, such as by physical incubation, and active loading with instances of electroporation or sonication. Furthermore, the functional small RNAs delivery using electroporated EVs has been shown to be a success in several reports but it depends on the small RNA species [58–62]. Usman and colleagues used the electroporation method for post-loading of RNAs into RBCEVs [55]. Ideally, various therapeutic molecules including ASOs, siRNAs, gRNAs, mRNAs, plasmid DNA, proteins, peptides, and chemical drug compounds can be loaded into RBCEVs using electroporation (**Figure 2**). Other post-loading methods such as mild sonication and physical incubation may be applicable to RBCEVs as tested for other types of EVs. Labeling of EVs is then required to examine the efficiency of delivery to target cells. Various methods and techniques have been applied to label EVs, with most common methods being incubation with biotinylated radioisotope, substrate of luciferase, fluorescence lipophilic dye, streptavidin-conjugated fluorescence dyes, or the use of other modified proteins [55, 64–66].

3.4 Advantages of red blood cell extracellular vesicles in therapeutics

Due to their innate function on cell-cell communication, EVs can be used effectively for drug delivery [12, 67–69]. The biggest advantage of EVs drug delivery is probably that EVs can be taken from an organism and returned to the same organism *in vivo* after being loaded with therapeutic agents, which are thought to be nonimmunogenic. Another advantage to deliver nucleic acids with the help of EVs is

that EVs can carry molecules through physiological barriers, such as the blood-brain barrier, which are hard to cross using conventional delivery methods. Normally, when exogenous RNAs are introduced into the body directly, they will be cleared before reaching the target tissues or cells of interest through degradation by nucleases, or they will be filtered in the kidneys. Both coding and noncoding RNAs were shown to be transferred by EVs intercellular crosstalks. Additionally, it has been shown that microRNAs can be transported in EVs to various cell types. Thus, EVs can be used as a promising vehicle for delivery of RNA-based drugs. Potential fields for therapeutic use include gene therapy, targeted therapy, vaccination, improvement of pregnancy outcome, newborn medicine, kidney disease, and treatment of autoimmune disease [12, 67–69].

It has been reported that diversified types of cells, including RBCs, endothelial cells, monocytes, granulocytes, and platelets, release EVs. Additionally, EVs can be isolated by various methods from cell culture media, plasma, and other biofluids [23, 41, 70, 71]. Although several research groups have demonstrated the advantages of using EVs for RNA delivery, there are still issues with EVs generated from fibroblasts and dendritic cells being not permanently available from all subjects [69, 72]. RBCs are readily obtainable from any human subject and easy to store, and blood transfusion has been a relatively safe, well-established, and routine medical procedure for decades which makes RBCEVs easy to obtain and safe to use. Thus, EVs from whole plasma are easily accessible and substantially present, but these EVs are derived from various cell types, e.g., nucleated cells which represent a risk for horizontal gene transfer [63]. Therefore, obtaining ultrapure RBCEVs solely derived from RBCs is highly preferred as RBCs lack both nuclear and mitochondrial DNA, which means that RBCEVs for pharmaceutical purpose avoid the risk of horizontal gene transfer. RBCEVs formation has been extensively investigated and described in the recent years. Therefore, with such knowledge, RBCEVs are safer and less complicated to use [73–76].

Consequently, RBCEVs possess several features which make them better suitable for clinical applications than EVs from other cell types. First of all, blood units are easily accessible from existing blood banks. A large scale of RBCEVs can be produced at low cost as RBCs are the most abundant cell type in the body (84% of all cells) and, during their 120-day lifespan, RBCs continue to release RBCEVs, leading to an approximate 20% loss in RBCs volume and an increase of around 14% in hemoglobin concentration [23, 77–79]. Additionally, RBCEVs are obtainable for allogeneic and autologous transfusion from the patients' own blood. A large number of RBCs (~10^{12} cells/L) are obtainable from each blood unit. Thus, there exists no need to expand cells in culture and no risk of the emergence of mutations *in vitro*. In addition, no cGMP-qualified media or supplements are required, which are financially desirable. Large-scale amounts (10^{13}–10^{14}) of RBCEVs can be isolated from RBCs (per unit) when treated with calcium ionophore, which is a scalable strategy to obtain EVs. Secondly, RBCEVs are safer compared to EVs from other cell types, because the enucleated RBCs contain no DNA, unlike EVs from nucleated cell types which represent a potential risk for horizontal gene transfer. As plasma EVs are heterogeneous with unpredictable contents, RBCEVs are safer than plasma EVs for allogeneic treatments of cancer because cancer cells and immune cells are known to release large amounts of cancer-promoting EVs into their environment [80, 81]. Thirdly, RBCEVs are nontoxic; hence, they are safer as compared to the toxic synthetic transfection reagents which are typically used. As mentioned before, RNAs in RBCEVs are stable and completely functional *in vitro* and in *vivo* for both liquid and solid cancers. Fourthly, RBCEVs are presumably nonimmunogenic via blood type matching, unlike adenoviruses, adeno-associated viruses, lentiviruses, nanoparticles, and various synthetic

transfection reagents. Last but not least, RBCEVs can be frozen and thawed many cycles without affecting their integrity or efficacy. This fact suggests that RBCEVS can be developed into stable pharmaceutical products in the future, but further research needs to be done. Compared to most other current methods for programmable RNA drug therapies, which are unsuitable for the clinical use because of the low uptake efficiency and high cytotoxicity, RBCEVs show promising future prospects [55].

4. Conclusion

EVs are shed from the plasma membrane or released by endosomal pathways under both physiological and diseased conditions. Intercellular communication is one of the best known functions of EVs by far, which provides the possibility to utilize the EVs natural vehicle property of transporting nucleic acids, proteins, and lipids for drug delivery. Recent studies demonstrate that human RBCEVs can be developed as robust delivery platform for multiple therapeutic RNAs in cancer treatment. RBCEVs feature multiple benefits as compared to EVs from other cell types. They are easily obtainable in large amounts, can be frozen and thawed multiple times without significant compromise, are nontoxic and nonimmunogenic, can reach remote tissues in the body with minimal hindrance by physiological barriers, and do not contain DNA or other unpredictable contents which could result in horizontal gene transfer. By obtaining RBCEVs directly from the patient, they are safe to use allogeneic treatments and possess no risk of emerging mutations during expansion by cell culture. Thus, RBCEVs show promising advantages in overcoming various limitations of cell-based therapeutics. All in all, RBCEVs need further research in order to establish them as a new source and promising approach for practical therapeutics in clinical use.

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Conflict of interest

The authors declare no conflict of interest.

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