Chapter 7

Microvesicles Released from Human Red Blood Cells: Properties and Potential Applications

Duc Bach Nguyen, Thi Bich Thuy Ly and Ingolf Bernhardt

Additional information is available at the end of the chapter

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Abstract

Microvesicles (MVs) are small spherical fragments of plasma membrane between 50 and 1000 nm in diameter. MVs arise through direct outward budding and fission of the plasma membrane. As almost all cells, human red blood cells (RBCs) are able to release MVs into extracellular environment under stimulating or storage conditions. Recently, it has been known that MVs not only play a role in homeostasis but also have diverse functions in cell-cell interactions and in the pathogenesis of diseases. In this chapter, the formation and release of MVs from human RBCs have been described. In addition, MVs have demonstrated to be potential vehicle for transport of nucleic acid and other molecules to the target cells. Although RBC-derived MVs are potential material for the development of delivery systems, it is still a great challenge to the clinical application. Future research should pay more attention to MVs as biological targets for diagnosis and practical therapeutics of cancer and other diseases.

Keywords: microvesicles, red blood cell, exosomes, nucleic acid delivery, THP-1 cells, endothelial cells, transfection

1. Introduction

Extracellular vesicles (EVs) are spherical fragments released from biological membranes of various cell types under both physiological and pathological conditions. So far, many terms have been used to describe EVs, such as exosomes, microvesicles (MVs), membrane microparticles, ectosomes, and apoptotic bodies. Recently, based on their size and origin, EVs are classified as exosomes, MVs, and apoptotic bodies. Under stimulating or storage conditions, human red blood cells (RBCs) release EVs. This chapter focuses on the formation and release of MVs

from human RBCs and considers the isolation and characterization of MVs in order to apply MVs as potential vehicles for nucleic acid delivery. Similar to EVs released from nucleated cells, MVs from human RBCs carry biomarkers originated from plasma membrane and also microRNAs but not DNA. These properties suggest that MVs can be used as potential vehicles to transport proteins, nucleic acids, or signal molecules. While the understanding of the biogenesis of MVs in human RBCs and their physiological role remains limited, accumulating data suggest that MVs may be applied in cancer therapy. This chapter reviews our current knowledge pertaining to MVs released from human RBCs. It describes the formation and biological properties of MVs and mentions the potential application of MVs as a molecular vehicle for drug and nucleic acid delivery. Furthermore, it gives an introduction in the application of MVs for cancer treatment. In addition, MVs and exosomes released from other cell types are also taken into consideration to provide findings of the nature of the membrane-derived vesicles, their mechanism of action, and their possible role in biological processes both under *in vitro* and *in vivo* conditions.

2. Microvesicles and their biological considerations

Under physiological and pathological conditions, various cell types release small spherical fragments called membrane vesicles or extracellular vehicles (EVs). So far, many different terms such as ectosomes, MVs, shedding vesicles, apoptosomes, membrane microparticles, or apoptotic bodies have been used in a vast number of reports on EVs [1–8]. Fifty years ago, in 1967, Wolf first identified small procoagulant structures deriving from activated platelets in human blood and created the initial term "platelet dust" [9]. Twenty years later, in 1987, Johnstone described the vesicle formation during maturation of sheep reticulocytes *in vitro* [10]. These findings were seen as a milestone in EV research allowing further studies on their function at various physiological conditions and in certain diseases. Since then, EVs have been detected in different body fluids such as peripheral blood, urine, saliva, semen, cerebrospinal fluid, synovial fluid, bronchoalveolar lavage, and bile. The mechanism of EV formation and the biochemical composition of EVs depend on cell types, physiological conditions, and the function of the cells from which they originate [11–16]. Recently, based on their size and biogenesis, EVs have been classified into exosomes, MVs, and apoptotic bodies. Exosomes are generally accepted to have size from 40 to 100 nm in diameter. They are secreted from endosomal compartments or multivesicular bodies of cells. In contrast, MVs including microparticles or membrane particles are larger in size varying from 50 to 1000 nm in diameter. The biogenesis of MVs arises through direct outward budding and fission of the plasma membrane following different kinds of cell activation or during early state of apoptosis [11, 17, 18]. Distinct from exosomes and MVs, apoptotic bodies are much larger with 1–5 μm in diameter. They are formed by cell-membrane blebbing when the cells undergo apoptosis [7, 11, 19–21]. Three subtypes of EVs, namely exosomes, MVs, and apoptotic bodies, are shown in **Figure 1**. In fact, it is still a challenge to separate one EV type from another because of their overlapping biophysical characteristics. Nevertheless, some discriminating markers have been reported [22]. In this chapter, the term MVs will be used for MVs, microparticles, or membrane microparticles (MPs) and EVs for both exosomes and MVs.

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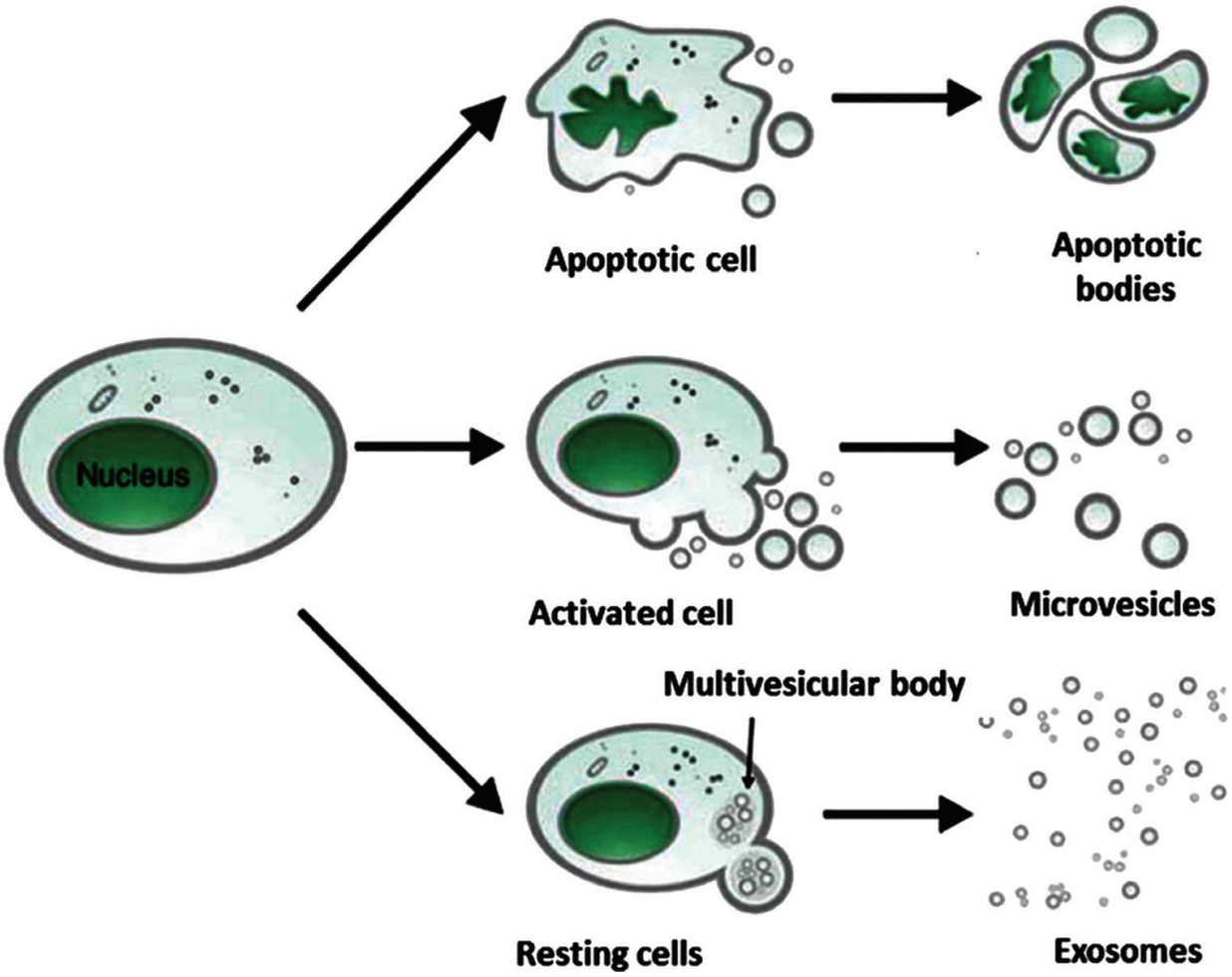

Figure 1. Potential vesicular structures of circulating DNA. Depending upon the mechanism of release, three subtypes of EVs, namely, exosomes, MVs, and apoptotic bodies, are described [28]. The figure is taken from Rykova et al. [29].

It has been reported that MVs are released from various types of activated or apoptotic cells including platelets, monocytes, endothelial cells (ECs), red blood cells, THP-1 monocytic cells, and granulocytes. MPs were also collected from the culture media, cell supernatants, and plasma by centrifugation at 20,000 g for 30 min. The average diameter of all types of MVs was varying much comparing different reports [19, 23–25]. The plasma MPs had the smallest size similar to MPs released from platelets and THP-1 cells, while MPs from monocytes were larger, and MPs from granulocytes and ECs were the largest ones. The data obtained from various reports indicate that the size of membrane MPs depends on the type of the cells from which they originate [23]. Although MVs have been discovered for years, the understanding of the mechanism of the formation as well as the biological roles of MVs is still a matter of debate. Recent reported findings led to advances of our understanding of the mechanism of formation and the role of MVs in many different diseases such as vascular diseases, cancer, infectious diseases, diabetes mellitus, diabetes, inflammation, and pathogen infection [24]. Inhibition of the production of MPs may serve as a novel therapeutic strategy for some diseases, especially for cancer treatment [11, 23, 26, 27]. In the next part of this chapter, the biogenesis, properties, and biological function of MVs released from human red blood cells (RBCs) are mainly addressed.

In the past decade, extracellular vesicles (EVs) have been recognized as potent vehicles of intercellular communication due to their capacity to transfer proteins, lipids, and nucleic acids, thereby influencing various physiological and pathological functions of both recipient and donor cells [30]. In addition, EVs also represent an important mode of intercellular communication by serving as vehicles for transfer between cells of membrane and cytosolic proteins, lipids, and RNA. Shortage of our knowledge of the molecular mechanisms for EV formation and lack of methods to interfere with the packaging of cargo or with vesicle release leads to a difficulty in identification of their physiological relevance *in vivo* [6]. EVs have been implicated in important biological processes such as surface-membrane trafficking and horizontal transfer of proteins and RNAs among neighboring cells, and distant tissues. Therefore, they play an important role in cell-to-cell communication under both physiological and disease conditions [11].

It is evident that direct investigation of the biological function of MVs *in vivo* is extremely complicated. Most of the studies regarding physiological roles of exosomes or MVs have to carry out *in vitro*, especially in the context of the immune system and cell-cell communication [31]. In 1996, a pioneering study by Raposo and colleagues demonstrated that exosomes derived from both human and mouse B-lymphocytes spread antigens bound to the class II major histocompatibility complex (MHC). These vesicle-associated complexes were capable of activating MHC class II leading to a restriction of T-cell responses. This finding suggests a role for exosomes in antigen presentation *in vivo* [32]. Furthermore, B cell–derived exosomes specifically interacted with the membrane of follicular dendritic cells derived from human tonsils. This finding is also an example for further supporting the idea of the active secretion of exosomes *in vivo* [33]. In addition, Montecalvo and colleagues demonstrated that different subsets of miRNAs are exchanged between follicular dendritic cells through exosomes at different maturation stages [34].

In a study, Wu showed that cancer cells release MVs and exosomes under both *in vivo* and *in vitro* conditions. MVs and exosomes carry different types of molecules on their surfaces, which are seen as biomarkers [24]. That is the reason why MVs or exosomes are used in cancer diagnosis. For example, circulating levels of MVs are elevated in gastric cancer patients. In these patients, MPs released from CD41a-positive platelets are significantly increased in stage IV compared with stage I or II/III [35]. It has been recently demonstrated that MVs released by cells represent another important mediator of cell-cell communication and are also an integral part of the intercellular microenvironment [3, 36, 37]. This opens a new scenario to understand signal and molecule transfers between cells even at long distances. For human RBCs, released MVs in both resting state (storage at 4°C) and stimulating conditions showed the ability to adhere together. It might be suggested that MVs are involved in the blot clot formation and also play a substantial role in the aggregation of stimulated RBCs [38, 39]. Further investigations have to be carried out to understand the role of MVs in both physiological and disease conditions.

It has been described that blood cells are able to generate a great variety of EVs. First identified in 1967, MVs are cell plasma membrane-derived small vesicles which are 0.1–1 mm in diameter. Later, the formation and release of EVs have been demonstrated in platelets, monocytes, endothelial cells, RBCs, and granulocytes [9]. EVs have been thought to serve as a disseminated storage pool of bio-effectors that circulate and play important roles in physiological homeostasis of the body under both physiological and disease conditions. Recent

functional assays and analysis of MVs by multicolor flow cytometry have shown that MPs possess a broad spectrum of biological activities and may play an important role in multiple cellular processes including intercellular communication, immunity, apoptosis, and homeostasis [24, 40]. In case of human RBCs, MVs have a phospholipid bilayer structure exposing coagulant-active phosphatidylserine and expressing various membrane receptors [40]. It should be mentioned that mature human RBCs do not contain DNA but RNAs including mRNA and other non-coding RNAs. Therefore, it suggests that MVs from human RBCs may not only be involved in thrombosis, amplifying systemic inflammation or cell adhesion, but also in cell-cell interactions in term of nucleic acid transfer [38, 39, 41, 42].

Recently, it has been reported that negatively charged membranes of erythrocyte-derived microparticles display procoagulant activity [38, 39]. However, relatively little is known about the possible fibrinolytic activity of such MVs. This issue becomes particularly important during RBC storage, which significantly increases the number of MVs [43]. Regarding the ability of carrying nucleic acid, recently, a novel system composed of MVs from RBCs was created for efficient delivery of ultra-small superparamagnetic iron oxide particles into human bone marrow mesenchymal stem cells for cellular magnetic resonance imaging *in vitro* and *in vivo*. It showed that MVs are highly bio-safe to their autologous (exosomes) as manifested by cell viability, differentiation, and gene microarray assays. The data suggest that MVs could be used as potential intracellular delivery vehicles for biomedical applications [44]. More recently, a study of the function of MVs from human RBCs infected with *Plasmodium falciparum* parasites showed that infected RBC-derived MVs contain miRNAs that can modulate target genes in recipient cells. In addition, multiple miRNA species in EVs have been identified. They are bound to Ago2 and form functional complexes. The infected RBC-derived MVs were transfected successfully into endothelial cells repressing miRNA target genes and changed endothelial barrier properties [45]. In addition, role of RBCs-derived MVs in malaria response showed that the development of MVs by *Plasmodium sp*. has a major impact in disease outcomes and serves as an integral part in controlling stage switching in its life cycle. Clinical studies have highlighted elevated levels of EVs in patients with severe malaria disease, and EVs have been linked to increased sequestration of infected RBCs to the endothelium [46].

3. Formation and release of MVs from human red blood cells

It has been known that during their 120-day of lifespan, RBCs lose approximately 20% of their volume through vesicle release, whereas their hemoglobin concentration increases by 14% [47]. Although a number of mechanisms explaining the formation of MVs have been proposed, the creation and the role of RBC microparticles are far from being completely understood. It has been pronounced that the formation of MVs involves the activity of certain components of the plasma membrane as well as cytoskeletal proteins [19]. Under physiological conditions, the phospholipids of the cell membrane are distributed asymmetrically. In particular, phosphatidylcholine (PC) and sphingomyelin (SM) are predominantly present in the outer membrane leaflet, while phosphatidylserine (PS) and phosphatidylethanolamine (PE) are located predominantly in the inner membrane leaflet. This asymmetric distribution is controlled by a group of enzymes, flippase, floppase, and scramblase [48–51]. The flippase

is responsible for the transfer of PE and PS from the outer to the inner leaflet of the cell membrane, while the floppase has been shown to have the opposite effect. Their activity is regulated by ABCC1 protein, also known as a multidrug-resistant protein 1 [19]. In contrast, the distribution of the phospholipid PS is determined by the activity of the scramblase. In human RBCs, the mechanism of the formation of MVs has been investigated and described by many research groups [50–54].

The integrity of RBC membrane is supported from many components of cytoskeleton structure, e.g., hexagonal actin–spectrin lattice anchoring with other proteins such as glycophorin A and band 3 protein [55]. It has been described that the vesiculation would be a mean for RBCs to get rid of specific harmful agents such as denatured hemoglobin, C5b-9 complement attack complex, band 3 neoantigen, and IgG that tend to accumulate in RBCS or on their membrane during their lifespan [22]. An influx of Ca^{2+} through nonspecific cation channels leads to the activation of several enzymes such as calpain or scramblase leading to the externalization of phosphatidylserine of the RBC membrane and degradation of cytoskeleton proteins and aggregation of band 3 leading to vesiculation [41, 56]. In our recent study, the kinetics of membrane blebbing and formation of MVs were characterized by using annexin V-FITC and fluorescence microscopy. The kinetics of budding and shedding of MVs were captured in every 30 s. Treatment of RBCs with a calcium inonophore (as positive control), lysophosphatidic acid (LPA), or phorbol-12-myristate-13-acetate (PMA) led to the externalization of PS at the outer membrane leaflet of RBCs as well released MVs. Moreover, it was interesting to see that a stimulation of RBCs by PMA in the absence of Ca^{2+} also led to the release of MVs [17, 41]. This suggests that the formation of MVs is also under the control of a calcium-independent pathway related to the activity of the PKC (**Figure 2**).

Based on the current understanding, a scheme with the interaction of protein components in the cells has been proposed. The proposed mechanism for the budding and shedding of MVs in human RBCs is shown in **Figure 3**.

Although many factors influence the formation and release of MVs, Ca^{2+} and PKC play essential roles in the process of MV formation [17, 19, 41]. An increase of intracellular calcium inactivates the flippase and activates the scramblase as well as the floppase leading to a reorganization of phospholipids in the cell membrane [21, 22, 41, 53, 54, 57]. The activation of calpain and degradation of actin filaments leads to breaking of bonds between the cytoskeleton filaments and the phospholipids. The weakening of the protein fibrils of the cytoskeleton initiates the budding and shedding of MVs [52, 58–60]. It has been demonstrated that reorganization or disruption of the cytoskeleton plays an important role in the release of MVs [36]. Another study showed that the activation of the scramblase requires a larger increase of the calcium concentration and therefore it is considered as being less important for the formation of MVs [19, 50]. By using a special compound R5421, a scramblase-specific inhibitor, it has been shown that vesicle shedding was attenuated in human RBCs [52, 61]. By adding ascorbic acid to RBCs during storage, a significant decrease in MVs formation was observed [62]. In our study, the MVs formation was observed within 1 hour when RBCs were treated with the PKC activator, phorbol-12-myristate-13-acetate (PMA), even in the absence of Ca^{2+}. In addition, the kinetics of the formation of MVs in human RBCs has recently investigated by real-time measurement using fluorescence microscopy [17].

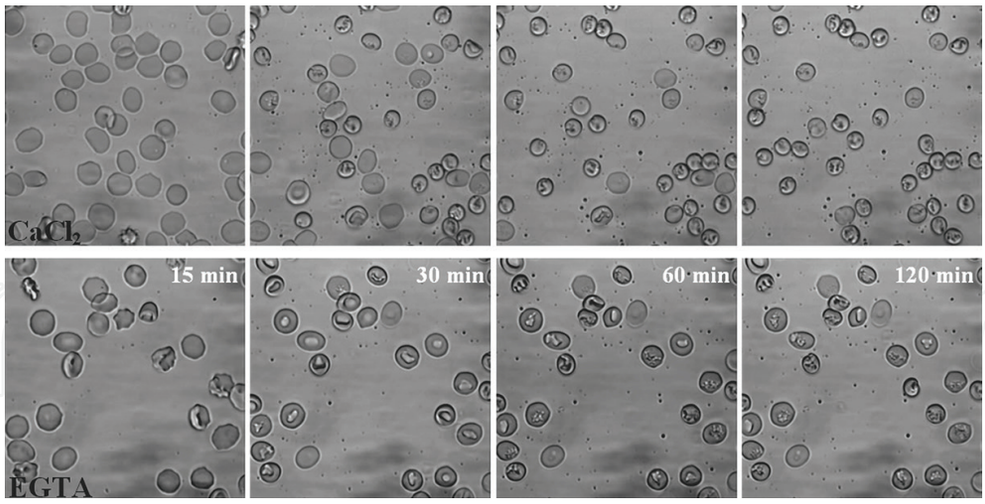

Figure 2. Bright field imaging of the formation of MVs in human RBCs depending on time (up to 120 min) stimulated by 6 μM PMA in the presence of 2 mM Ca^{2+} (upper row) and in the absence of Ca^{2+} and with 2 mM EGTA (lower row).

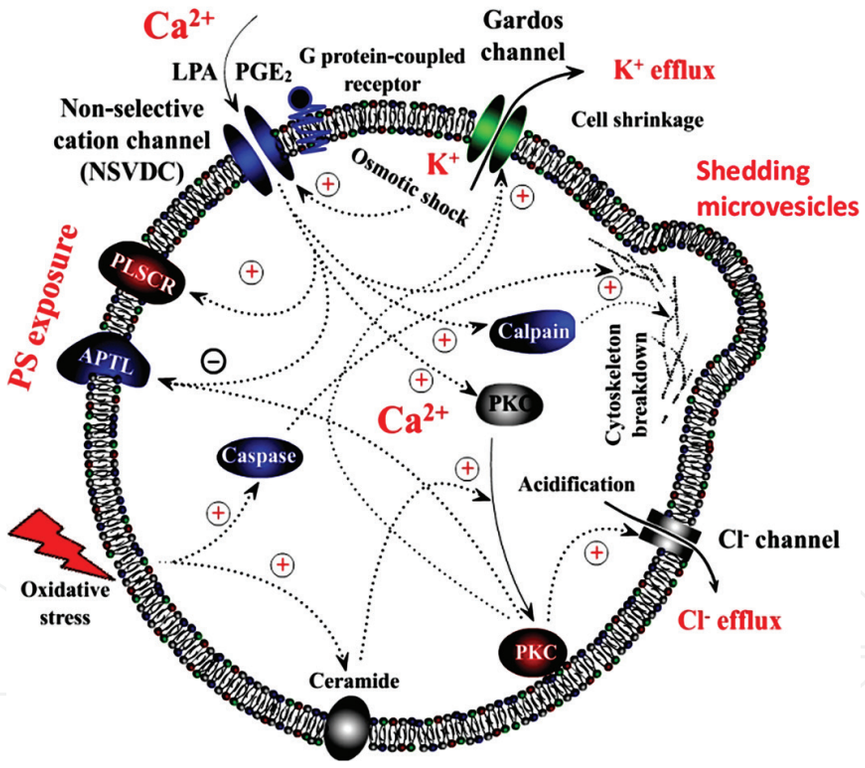

Figure 3. Proposed mechanisms of the formation of MVs in human RBCs. Lysophosphatidic acid (LPA) or prostaglandin E2 (PGE2), which are two typical substances released from activated platelets, activate a nonselective voltage-dependent cation (NSVDC) channel. The opening of this channel leads to an increase of the intracellular Ca^{2+} content. An increase of the intracellular Ca^{2+} level activates the phospholipid scramblase (PLSCR) and the protein kinase C (PKC). The activated PKC moves from the cytoplasma to the membrane. The amino-phospholipid translocase (APLT) is inhibited by high concentrations of intracellular Ca^{2+}, PKC, and ATP depletion. The PKC also activates and opens Cl^- channels leading to an efflux of Cl^-. The efflux of Cl^- leads to an intracellular acidification. Under stress conditions, ceramide is formed and caspases are activated. Calpains are a family of calcium-dependent non-lysosomal cysteine proteases activated by Ca^{2+}. When caspase and calpain are activated, they are able to break down the cytoskeleton by a proteolysis activity leading to membrane blebbing and vesicle formation [41].

4. Content and biomarkers of microvesicles

4.1. Content of microvesicles

In recent years, numerous works have focused on providing a comprehensive characterization of the content of exosomes and MVs. Recently, information about molecules including proteins, mRNAs, microRNAs, or lipids observed within these vesicles has been deposited in EVpedia and Vesiclepedia [48, 63, 64]. By the end of 2015, Vesiclepedia stores records for 92,897 proteins, 27,642 mRNAs, 4934 miRNAs, and 584 lipids from 538 studies in 33 different species [48]. These numbers suggest that exosomes and MVs contain an extremely broad and heterogeneous range of molecules. Although these databases are extremely valuable, it still needs more evidences to elucidate the biological role of MVs and exosomes because the processes of biogenesis and packing molecules into these vesicles are complicated. It should be also mentioned here that the interpretation of the content of exosomes and MVs may be influenced or interfered by artifacts in sample preparation, isolation procedures, and analysis methods [65]. In comparison to MVs, exosomes are vesicles secreted upon fusion of multivesicular endosomes with the cell surface. Thus, exosomes transfer not only membrane components but also nucleic acid among different cells. Therefore, in order to understand the function of exosomes, it is necessary to have more evidences at subcellular compartments and mechanisms involved in the biogenesis and secretion of these vesicles [66]. Moreover, for many years, it is commonly thought that human mature RBCs do not contain nucleic acids because they are terminally differentiated cells without nuclei and organelles. However, transcriptomic analysis of a purified population of human mature RBCs from individuals with normal hemoglobin (HbAA) and homozygous sickle cell disease (HbSS) showed that there was a significant difference in microRNA expression in HbAA in comparison with HbSS [67]. This finding is very important to understand that MVs released from human mature RBCs carry nucleic acid and are likely involved in the biological processes of cell-cell communication and nucleic acid delivery.

4.2. Biomarkers on microvesicles

It is known that the antigens occurring on MVs are typical for cells from which the MVs are released. Depending on the origin of formation, MVs contain numerous markers that determine their origin, e.g., CD41 for platelets, CD235a and Ter-119 for RBCs [55, 68], and CD11c for dendritic cells [69]. Additionally, MVs released from B cells, dendritic cells, and melanoma cell lines are richer in sphingomyelin, rather than in cholesterol which are also characteristics of their parental cells [70]. Some glycoproteins on the surface of RBCs expressed at low and variable levels protect RBCs from damage and elimination. These include complement inhibitors, such as DAF and CD59, and signaling molecules such as CD47 [71, 72] and SHPS-1, a multifunctional transmembrane glycoprotein [72]. These makers inhibit phagocytosis of RBCs by macrophages because CD47 prevents this elimination by binding to the inhibitory receptor signal regulatory protein alpha (SIRPα) [73]. Therefore, these markers also exist on the surfaces of MVs released from RBCs [11, 74, 75]. In human RBCs, if the released MVs carry CD47 on their surface, they may be avoided from the clearance by macrophages [76, 77].

Studies on proteomics of MVs released from human RBCs were first carried out by Bosman presenting pioneering investigations [78–80]. In these series of studies, membranes of intact RBCs and MVs were compared, allowing the identification of several proteins differentially expressed between the two types of samples. Together with further studies on the oxidation and the depletion of spectrins and cytoskeletal proteins such as proteins 4.1 and 4.2, band 3 followed by the time course of storage, it has been concluded that RBCs have the ability to get rid of harmful materials by vesiculation such as denatured hemoglobin, C5b-9 complement attack complex, and band 3 neoantigen [81, 82]. In human RBCs, the formation of MVs has been described as part of the RBC senescence process [47, 78] and also proposed as part of an apoptosis-like form of these cells [20, 21].

It should be also mentioned that due to the variation of the lateral composition of the cell membrane, MVs originated from the same cell may contain different proteins or lipid components. Proteomic analyses have revealed that the spectrum of proteins found in MVs released from cultured cells is influenced partly by the stimulating conditions, which were used to trigger the vesiculation [36]. A study on the components of proteins in human RBC-derived MVs by two-dimensional gel electrophoresis discovered that the protein components in MVs under various stimulating conditions (cold storage and increased intracellular calcium level) are different. This was especially the case for sorcin, grancalcin, PDCD6, and particularly annexins IV and V [83]. Therefore, the molecular pathways to form MVs are different under both *in vivo* and *in vitro* conditions. In addition, this finding suggests that MVs may be also classified based on the presence of proteins. Recently, a method has been reported using carboxyfluorescein diacetate succinimidyl ester, which allows to detect the phospholipid component PS in the outer membrane leaflet of MVs that fail to react with annexin V [84]. This study is very important for screening blood products during storage in blood bank because the formation of MVs with PS in the outer membrane leaflet may lead to thrombus formation or aggregation of RBCs or phagocytosis.

It seems relatively simple to isolate EVs from human plasma with available protocols described elsewhere. However, to isolate MVs from RBCs, it requires a step to separate only RBCs without contamination of platelets or white cells. Upon the purpose of study, MVs can be collected by differential centrifugation. Menck and colleagues isolated and distinguished MVs and exosome from human blood cells using Western blot analysis. The data revealed that MVs pelleted from EDTA-anticoagulated plasma samples by differential centrifugation were 100–600 nm in diameter. MVs can be distinguished from exosomes by detecting the presence of proteins tubulin, actinin-4, or mitofilin, while antibodies for CD9 and CD81 were used as markers for exosomes [85].

5. Stability of microvesicles

Jayachandran and colleagues isolated MVs from platelet-free plasma (PFP) and platelet poor plasmas (PPP) and stored the MVs at either −40 or −80°C for more than a year. No effect on MV counts irrespective of initial counts was observed after three freeze thaw cycles of PFP [86]. Another investigation on the stability of MVs after different times of storage at 4 and

−80°C by using flow cytometry analysis showed that there was no significant difference by counts and size distribution of MVs stored at 4°C for 3–4 days or 1 week and MVs frozen at −80°C for 1 or 4 weeks [87]. In another study, Gallart showed that plasma containing exosomes and MVs frozen at −150°C can keep vesicles intact for long time [88]. Investigation was carried on the effect of short-term storage and temperature on the stability of exosome by incubating at temperatures ranging from −70 to 90°C for 30 min. Immunoblot results showed that all exosome-associated proteins incubated at 90°C were mostly degraded for a short period of time. The effect of long-term storage was carried out by incubating isolated exosomes for 10 days at wide range of temperature from −70°C to room temperature (RT). It revealed that protein and RNA amounts were significantly reduced at RT compared with data obtained at −70 and 4°C. Incubation at 4°C and RT resulted in major loss of CD63, and decreasing level of HSP70 was shown only at RT. In addition, flow cytometry result showed that exosome population became more dispersed after RT incubation for 10 days compared with −70°C incubated or freshly isolated exosomes [88]. Study on exosomes isolated from urine defined that freezing at −20°C caused a major loss of the integrity of these exosomes. In contrast, storage at −80°C increased the recovery almost complete (86%). Vortexing after thawing resulted in a significantly increased recovery of exosomes in urine frozen at −20 or −80°C, even if it was frozen for 7 months [89]. A similar study has been done to evaluate the stability of MVs released in whole blood samples under the influence of different anticoagulants. Analysis of MVs stored at 4°C and RT using nanoparticle tracking analysis (NTA) showed that total MV counts increased after 24 hours in sodium citrated or heparinized blood. The presence of EDTA showed stable platelet-derived MVs and RBC-derived MV counts at RT over a period of 48 h [90].

6. Isolation and characterization of microvesicles

Currently, there is no standard protocol for isolation of EVs for either therapeutic application or basic research [91]. However, a conventional method to obtain EVs is ultrafiltration followed by differential centrifugation. Ultrafiltration and size-exclusion liquid chromatography is suitable for EV isolation at large scale [92]. In fact, many research groups use differential centrifugation combined with filtration to isolate and define the MVs or exosomes. For example, a centrifugation force from 10,000 to 20,000 g is commonly applied to pellet MVs and from 70,000 to 100,000 g or even higher for exosomes. Although the centrifuge force is indicated in a number of publications, it is still varying among research groups. Nevertheless, there is always an overlap in the size of collected MVs or exosomes when analyzed by using dynamic light scattering (DLS) method. Therefore, the procedure for sample preparation and also isolation of MVs should be simplified as much as possible with minimal steps. In general, four critical steps should be taken into consideration: (i) removal of intact cells and large cell debris by low-speed centrifugation of the extracellular fluid (200–1000 g for 3–15 min); (ii) pelleting of large, secreted vesicles from the cell-free supernatant by medium-speed centrifugation (10,000 g for 30 min, a minimum of 2 times); (iii) collection of small, secreted vesicles by ultracentrifugation at 70,000–100,000 g, and (iv) noting all other parameters and type of rotors used in experiments [7].

At present, there is still a lack of studies assessing EV products after periods of storage. However, our unpublished investigations showed that the polydispersity (PI) of MV increased proportionally with the storage time at −20°C in deionized water. Vortexing was useful to recover MVs after storage. Further studies have to be done investigating the stability and the polydispersity of MVs in different solvents or buffers. The results of such analyses will facilitate defining provisional shelf-life times of EV-based products. The materials used for sample preparation, isolation, and storage should also be taken into consideration, especially for human therapeutics because solvents and buffers have a strong influence on the stability of EVs, especially after storage [93]. There is a wide range of solvents from water, sodium chloride solution, to phosphate-buffered saline (PBS), Tris-HCl, HEPES, and glycerol. However, glycerol and dimethyl sulfoxide (DMSO) showed a significant influence to the stability of EVs [94]. For investigation of the function and physical properties of EVs, isotonic buffers are recommended to prevent pH shifts during storage as well as during freezing and thawing procedures. Although PBS or other phosphate-containing buffers are widely used, it has to be considered to avoid calcium even at a very low concentration due to the formation of calcium phosphate aggregated in the buffer as nanoparticles, which can interfere with EV quantification assays [93]. Storage vials can also affect the quality of EVs due to unexpected or irreversible binding to certain materials. Thus, vials should be carefully selected to eliminate the factors that influence the concentration or integrity of stored EVs [93, 95].

So far, a variety of techniques have been commonly used to study MVs released from human RBCs. Traditionally, nanoparticle analysis is available to analyze the particles at nanosize including flow cytometry, DLS, and electron microscopy. Most widespread is flow cytometry; however, commercial flow cytometry typically has a lower practical size limit (for polystyrene beads) of around 300 nm at which point the signal is hard to distinguish clearly from the baseline noise level or so-called "dust" [96]. Fluorescence labeling can be efficient to detect particles at lower sizes. DLS has also been used, but being an ensemble measurement, the results comprise either a simple z-average (intensity weighted) particle size and polydispersity (PI), or a very limited-resolution particle size distribution profile. Electron microscopy is a useful research tool for studying micro- and nanovesicles but at high running costs and extensive sample preparation [22]. Atomic force microscope (AFM) is also an applicable method to measure the size and also the morphology of MVs [17]. An alternative approach for measuring EVs is using the NTA method. In NTA, the size is derived from the measurement of Brownian motion of EVs in a liquid suspension [22].

In recent study, under stimulating conditions, MVs from RBCs were collected by differential centrifugation and characterized by using SEM, AFM, and DLS. Data from the measurement using a Zetasizer (Nano ZS) for both size and zeta potential showed that the sizes of two subpopulations of MVs were 125.6 ± 31.4 nm and 205.8 ± 51.4 nm. There was an overlapping in the size of the two populations in the region from 150 to 200 nm. Zeta potential of released MVs was measured in different solvents showing negative values from −40 to −10 mV depending on the solvent used [17]. The morphology and size of MVs released from human RBCs were also analyzed using AFM and SEM (**Figure 4**).

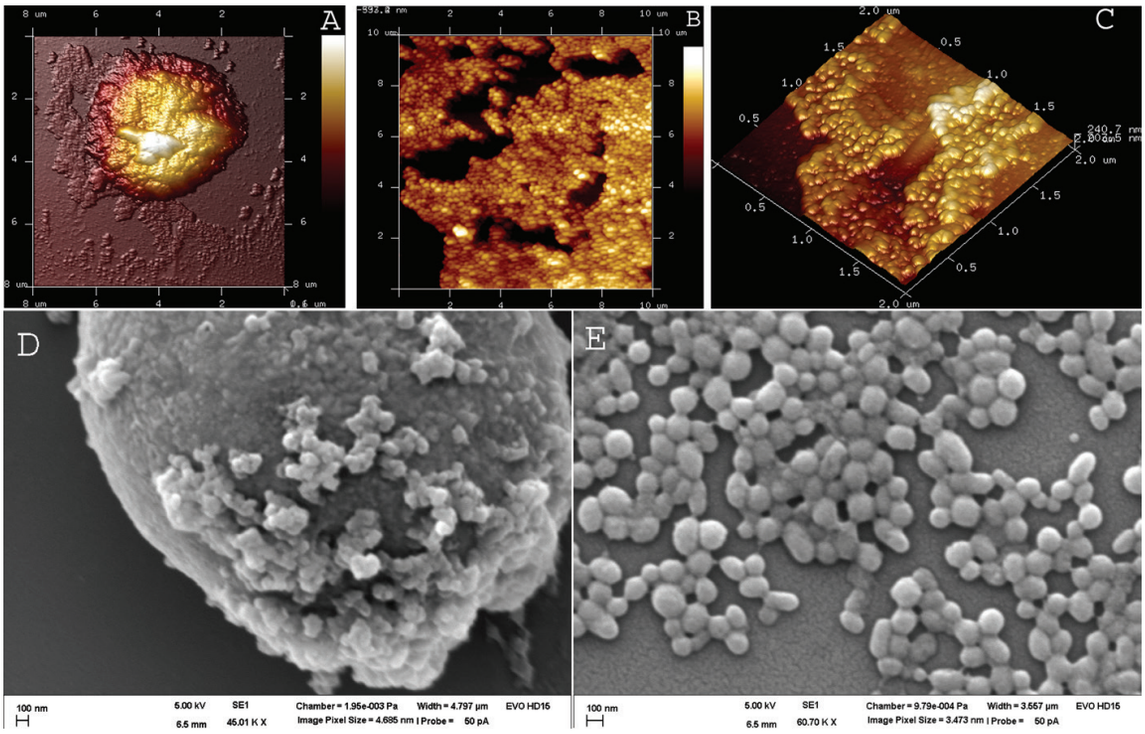

Figure 4. Topographical imaging of stimulated RBCs and released MVs. Glutaraldehyde-fixed samples of PMA-stimulated RBCs using AFM (A) and SEM (D); MVs scanning using AFM (B, C) and SEM (E) [17].

7. Potential applications of microvesicles

The structural feature that makes EVs especially attractive for drug delivery purpose is due to their analogy to liposomes. This means that EVs originated from an organism can be used as conventional liposome with an advantage when they are administered to the same organism *in vivo*. EVs are able to deliver molecules through hard-to-cross barriers like the blood-brain barrier. Therefore, EVs can be used for loading with drugs or other bioactive molecules and then work as efficient delivery systems. Several strategies are described for loading small molecule and genetic materials into liposomes; however, most of these strategies are not feasible for exosomes [97–99]. Two major strategies have been applied to load small molecules or drugs to EVs. The first possibility is the loading after EV isolation, and the second is the loading during EV biogenesis. In addition to loading, labeling of MVs is required to detect or investigate the efficiency of delivery to target cells and the expression of protein or function of miRNAs in recipient cells. So far, several techniques and methods have been applied to label MVs. Most common methods are incubation with fluorescence lipophilic dye, biotinylated radioisotope, substrate of luciferase (for *in vivo* trial), streptavidin-conjugated fluorescence dyes, or other modified proteins [100].

7.1. Microvesicles and nucleic acid transport

When nucleic acid (DNA, RNA) is directly introduced to the body, it will be rapidly removed out of the circulation via degradation by nucleases or by kidneys before reaching the target

tissues or cells of interest. Recent evidence has shown that different kinds of RNAs are transported by EVs during cell-cell communications. It has been shown that miRNAs are enriched in EVs in form of miRNA-RISC complexes and transferred from exosomes and MVs to many different cells. As such, EVs can be applied as a new attractive alternative approach for therapeutic miRNA delivery [14]. Recently, a study showed that embryonic stem cell MVs likely are useful therapeutic tools for transferring mRNA, microRNAs, protein, and siRNA to cells and also important mediators of signaling within stem cell niches [101]. It has been known that the lipids, proteins, mRNA, and microRNA (miRNA) delivered by these vesicles change the phenotype of the receiving cells [11, 102]. The ability to encapsulate and deliver different types of nucleic acid of both exosomes and MVs has been investigated. The results showed that MVs delivered functional plasmid DNA, but not RNA, whereas exosomes from the same source did not deliver functional nucleic acids. These results have significant implications for understanding the role of EVs in cellular communication and also the role of MVs for development of tools for nucleic acid delivery [11]. MVs from human RBCs infected with *P. falciparum* parasites contain miRNAs that can modulate target genes in recipient endothelial cells and serve as an integral part in controlling stage switching in the life cycle of the parasites [45, 46]. A typical example of application of EVs as vehicle for drug transport is the loading of curcumin, chemotherapeutic compounds paclitaxel and doxorubicin to EVs using electroporation. After transfecting loaded EVs to implanted breast tumor tissues, the results showed that the loaded EVs suppressed the growth of tumors without causing any toxicity [103]. As such, curcumin-loaded EVs have already made their way into the clinic to specifically suppress the activation of myeloid cells [93, 104].

7.2. Transfection of nucleic acid mediated by microvesicles

The strategy for cancer treatments is specifically killing malignant cells by vehicles, which carry appropriate substances or compounds to the target cells. Unfortunately, so far, it was not successful to cure the disease. The current concept in tumor treatment is to control the microenvironment of the tumor because the tumor is not only composed of malignant cells but also consists of other groups of cells that work together [105, 106]. Future research directions should draw more attention to EVs as biological targets for diagnosis, prognosis, and therapy of cancer. In addition, EVs participate and play a significant role in cell communication, and therefore they may become a valuable drug delivery system [107]. So far, a vast number of investigation on exosomes in carrying and transport of nucleic acid to target cells have been carried out; however, more information about using MVs to carry nucleic acid for transfection to cultured cells is required. Recently, an investigation of the capacity of MVs to deliver functional nucleic acids was carried out by using recipient HEK293FT cells cultured with exosomes and MVs derived from transfected donor cells with the fusion protein Luc–RFP as reporter. The data revealed that only loaded MVs led to Luc–RFP expression in the recipient HEK293FT cells, even though both MVs and exosomes encapsulated the reporter proteins. After the MV-mediated transfer, the bioluminescence signal increased over 3 days that was not observed in case of exosomes. The finding suggested that nucleic acids were delivered and led to a *de novo* expression of reporter proteins in recipient HEK293FT cells. By comparison with HEK293FT cells transfected by lipofectin with Luc-encoding pDNA, there was a different time course of Luc expression of the two methods. This observation suggested that the mechanism of MV-mediated delivery of nucleic acids and protein expression may be

different from that of cationic liposome-based delivery of pDNA, which is typically used for transfection to culture cells [11]. Although this finding was very important to confirm the ability of MVs in carrying nucleic acid and transfection to recipient KEK293FT cells, experiments with different cell types are required. Another example is the study using MVs shed from the monocytic cell line THP-1 enriched with miR-150 to transfect to endothelial cells promoting angiogenesis of these cells [108]. MicroRNA-223 delivered by platelet-derived MVs promotes lung cancer cell invasion via targeting tumor suppressor EPB41L3 [109]. Another example of using MVs in nucleic acid delivery was the work of Zhang to prove the inhibitory effect of TGF-β1 siRNA delivered by mouse fibroblast L929 cell-derived MVs (L929 MVs) on the growth and metastasis of murine sarcomas 180 cells both *in vitro* and *in vivo*. By comparing to the same concentration of free TGF-β1 siRNA, TGF-β1 siRNA delivered by L929 MVs efficiently decreased the level of TGF-β1 in the recipient tumor cells [110]. Other works dealing with miR-150 proved that MVs can be an excellent carrier for nucleic acid delivery [108, 110]. Taken all together, MVs carrying microRNAs can influence the recipient cell phenotypes.

7.3. Efficiency of nucleic acid transfection by microvesicles

Protein expression induced by MV-mediated pDNA delivery is a slower process than after transfection using cationic lipid complexes. It may be due to that fact that loaded MV need to fuse with the endosomal membrane before releasing nucleic acid contents into the cytosol. Studies on EVs from transiently transfected cells may be confounded by a predominance of pDNA transfer. Compare the efficiency of transfection of MVs loaded with pDNA or RNA, it revealed that MVs functionally deliver DNA much better than RNA. Further studies of the nature of this transfer are necessary to understand the specificity of pDNA loading pathways and delivery mechanisms [11]. So far, small RNAs have been successfully loaded into MVs for a variety of delivery applications; however, the potential use of MVs for DNA delivery has been abandoned. By using electroporation, Lamichhane investigated the ability of loading MVs with linear DNA. Loading efficiency and capacity of DNA in MVs were dependent on DNA size as well as on the conformation of DNA. By using this approach, linear DNA molecules with less than 1000 bp in length were more efficiently associated with MVs compared to larger linear DNAs and pDNA. In addition, MV size was also influencing the potential of DNA loading, as larger MVs encapsulated more linear and plasmid DNA than smaller vesicles and exosomes. These results demonstrated critical parameters that define the potential use of MVs for gene therapy [111]. Another example is the application of EVs isolated from media of cultured cardiomyocytes derived from adult mouse heart. These EVs, which were transfected to target fibroblasts, led to a change in the gene expression patterns in comparison with controls [112]. Recently, a study on delivery of a therapeutic mRNA or protein via MVs for treatment of cancer was carried out. Genetically engineered MVs by expressing high levels of the suicide gene mRNA and protein–cytosine deaminase (CD) fused to uracil phosphoribosyl transferase (UPRT) in MV from HEK-293T cells. Isolated MVs from these cells were used to treat pre-established nerve sheath tumors (schwannomas) in a mouse model. MV-mediated delivery of CD-UPRT mRNA or protein by direct injection into schwannomas led to regression of these tumors. This finding suggests that MVs can serve as novel cell-derived vehicle to effectively deliver therapeutic mRNA/proteins for treatment of diseases [113]. Taken all together, the results from these studies suggest that MVs can be used as new vehicles for nucleic acid transfer.

7.4. Development of microvesicle-based delivery systems

Although EVs were applied to humans already in the early 2000s for the treatment of cancer patients, no recommended standard techniques have been established for the production of EVs at clinical grade. Several manufacturing and safety considerations need to be addressed and appropriate quality controls have to be implemented and validated. It remains a challenge to set up platforms for the production of EVs at clinical grade that fulfill all necessary criteria for the successful approval of subsequent EV-based clinical trials [93]. The most relevant issue to be addressed at the various levels of the developmental processes is to bring MV-based therapeutics into the clinical application in treatment of diseases including cancers. It is obvious that MVs are part of parental plasma cells; therefore, their antigenicity is mainly determined by protein and lipid components, profile of miRNAs and mRNAs, and also other factors originated from the parent's cells. Similar to exosomes, MVs are able to overcome limitations of cell-based therapeutics including safety, manufacturing, and availability. With a capability of crossing the blood-brain barrier, which classically acts as a major hurdle in the administration of therapeutic agents for targeting cells and tissue, especially of the central nervous system, MVs can be applied for the transport of molecules to target cells or tissues [114, 115] The presence of biomarkers on the surface may drive the loaded MVs to the specific target and help them to protect their cargoes from degradation [65, 116]. The standard procedure for isolation, purification, and storage of EVs at large scale should be established for certain cell types for trials at both *in vitro* and *in vivo* levels.

Another important issue in application of MVs is how to load bioactive compounds into these vesicles. For example, in order to load MVs with therapeutic small RNA molecules, two encapsulation approaches commonly used are post-loading or pre-loading. Post-loading method is using a specific method to introduce RNA into EVs (e.g., electroporation) while pre-loading is carried out during the EV formation (it is also called endogenous method that exploits the cellular machinery for small RNA loading into EVs). This endogenous method has been successfully used for the packaging of both siRNA and miRNA in EVs [99, 117, 118]. Functional delivery into recipient cells has been shown in several reports [119–121]. Several recent reports have shown functional siRNA delivery into recipient cells using EVs loaded by electroporation. However, the efficacy of this exogenous method has not been fully demonstrated, and other research groups stated that the loading of EVs with miRNA by using this method was not successful [120, 122]. Therefore, further studies are needed to confirm the feasibility and efficiency of this method for EVs loading. Nevertheless, the feasibility of the method likely varies depending on the siRNA or miRNA species. Furthermore, the efficiency of the overexpression or the direct transfection of particular small RNA-loaded EVs to recipient cells is still the matter of concern.

8. Conclusion

MVs are able to carry macromolecules, especially nucleic acid, and play a key role in cellular communication. In near future, MVs may efficiently support for the conventional treatment of tumor or cancer, which are using chemotherapeutic drugs, radiation therapy, or surgery.

Recent findings suggest that released MVs from human RBCs can be applied as novel treatment for various diseases including cancer. Structurally, MVs contain various membrane receptors and also carry nucleic acids, proteins, or other molecules. With many advantages in overcoming many of the limitations of cell-based therapeutics including safety, manufacturing, and availability, MVs may serve as cell-to-cell shuttles for carrying bioactive molecules to target cells. Therefore, MVs involve biological processes, especially the interaction with tumors or cancers. Human RBCs, with a large number of cells in the human body, can be easily collected without requiring cell culturing or sophisticated instrumentation. In addition, MVs released from RBCs can move to almost all tissues in the body without being hindered by any biological barrier. Therefore, MVs from human RBCs are potential candidates for the transport of nucleic acid and other bioactive compounds to the target cells. However, to make MVs to become applicable and efficacious in therapeutic treatments, underlying functions of MVs still need to be better understood. Future research directions should pay more attention to MVs as biological targets for cancer diagnosis, prognosis, and therapy that enable MVs as new source and of new material and promising approach for practical therapeutics.

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Author details

Duc Bach Nguyen[1]*, Thi Bich Thuy Ly[2] and Ingolf Bernhardt[3]

*Address all correspondence to: ndbach@vnua.edu.vn

1 Department of Molecular Biology, Faculty of Biotechnology, Vietnam National University of Agriculture, Hanoi, Vietnam

2 Institute of Biotechnology, Vietnam Academy of Science and Technology, Hanoi, Vietnam

3 Laboratory of Biophysics, Saarland University, Saarbruecken, Germany

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