Detection of extracellular vesicles: size does matter
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Abstract

Both eukaryotic and prokaryotic cells release small, phospholipid-enclosed vesicles into their environment. Why do cells release vesicles? Initial studies showed that eukaryotic vesicles are used to remove obsolete cellular molecules. Although this release of vesicles is beneficial to the cell, the vesicles can also be a danger to their environment, for instance in blood, where vesicles can provide a surface supporting coagulation. Evidence is accumulating that vesicles are cargo containers used by eukaryotic cells to exchange biomolecules as transmembrane receptors and genetic information. Because also bacteria communicate to each other via extracellular vesicles, the intercellular communication via extracellular cargo carriers seems to be conserved throughout evolution, and therefore vesicles are likely to be a highly efficient, robust, and economic manner of exchanging information between cells. Furthermore, vesicles protect cells from accumulation of waste or drugs, they contribute to physiology and pathology, and they have a myriad of potential clinical applications, ranging from biomarkers to anticancer therapy. Because vesicles may pass the blood-brain barrier, they can perhaps even be considered naturally occurring liposomes. Unfortunately, pathways of vesicle release and vesicles themselves are also being used by tumors and infectious diseases to facilitate spreading, and to escape from immune surveillance. In this chapter, the different types, nomenclature, functions, and clinical relevance of vesicles will be discussed.

Parts of this chapter have been published as:

Chapter 2. Classification, functions, and clinical relevance of extracellular vesicles

2.1 Introduction

2.1.1 Cell-derived vesicles

Both eukaryotic and prokaryotic cells release vesicles, which are spherical particles enclosed by a phospholipid bilayer. The diameter of vesicles typically ranges from 30 nm to 1 µm [69], the smallest being some 100-fold smaller than the smallest cells. Because cells release vesicles in their environment, body fluids such as blood and urine, but also conditioned culture media, contain numerous cell-derived vesicles, usually more than $10^{10}$ per mL [333]. It is becoming increasingly clear that most vesicles have specialized functions and play a key role in, for example, intercellular signaling, waste management, and coagulation. Consequently, there is a growing interest in the clinical applications of vesicles. Vesicles can potentially be used for therapy, prognosis, and biomarkers for health and disease. Nevertheless, because of the small size and heterogeneity of vesicles, their detection and classification is challenging [233]. Although different types of vesicles have been identified, widely used terms, such as “exosomes” and “microparticles,” are often inconsistent, especially in the older literature. Even if “purified exosomes” are claimed to be measured, it is prudent to remain cautious. Moreover, it should be emphasized that the extent to which vesicles really contribute to processes underlying physiology and pathology is virtually unexplored, and therefore one should also remain cautious to extrapolate results from in vitro studies on vesicles and their functions to the in vivo situation.

In this chapter, the focus will be on exosome-mediated signaling, although we will also outline recent developments on other vesicles within the relatively novel and rapidly expanding research field. A screening of the literature on cell-derived vesicles provided more than 6,000 publications, from which approximately 500 were selected for review, with emphasis on recent publications from high-impact journals.

2.1.2 History

The discovery of cell-derived vesicles dates back to 1940, when preliminary studies were performed, addressing the “biological significance of the thromboplastic protein of blood” [61]. Clotting times of plasma were determined after centrifugation at different speeds, and prolonged high-speed centrifugation (150 min at 31,000 g) was shown to significantly extend the clotting time of the supernatant. Furthermore, when the pellet containing “the clotting factor of which the plasma is deprived” was added to plasma, the clotting times shortened, indicating that cell-free plasma contains a subcellular factor that promotes clotting of blood [61]. More than 20 years later, in 1967, this subcellular fraction was identified by electron microscopy and was shown to consist of small vesicles, originating from platelets and termed “platelet dust” [327]. These vesicles were reported to have a diameter between 20 and 50 nm and had a density of 1.020 to 1.025 g mL$^{-1}$ [327]. One decade
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Later, fetal calf serum was also shown to contain “numerous microvesicles” ranging in diameter from 30 to 60 nm [78]. Meanwhile, within a completely different line of research, the term “exosomes” was introduced when vesicles were isolated from conditioned culture medium of sheep reticulocytes. These vesicles had several characteristic activities in common with the reticulocyte plasma membrane, including the presence of the transferrin receptor, whereas cytosolic enzyme activities were not detected. Therefore, it was concluded that “vesicle externalization could be a mechanism for shedding of specific membrane functions, which are known to diminish during maturation of reticulocytes to erythrocytes” [152]. Because these exosomes contained the transferrin receptor but expressed no lysosomal activities, it was also suggested that there may be a common mechanism to segregate and externalize specific plasma membrane proteins [153]. The formation of the transferrin receptor-containing exosomes proved to be a major route for removal of plasma membrane proteins. Because not only mammalian but also embryonic chicken reticulocytes were shown to produce transferrin receptor-containing exosomes, this may be a conserved and common pathway [154, 110]. It was then discovered that exosomes are formed within multivesicular endosomes (MVEs), also known as multivesicular bodies, and are being released when membranes of MVEs fuse with the plasma membrane. This pathway of protein sorting turned out to be highly selective, because other major transmembrane proteins, such as the anion transporter, are fully retained within the mature red cell and are absent within exosomes [151]. Taken together, these early studies revealed that exosomes might be essential in a sophisticated and specific mechanism to remove obsolete transmembrane proteins.

2.1.3 Nomenclature

Because of the detection difficulties, the multidisciplinary research field, and different ways of classification, there is currently no consensus about the nomenclature of cell-derived vesicles. For example, cell-derived vesicles have often been called after the cells or tissues from which they originate [e.g., dexosomes (dendritic cell-derived exosomes) [184], prostasomes (prostate-derived vesicles) [277], matrix vesicles (vesicles in bone, cartilage and atherosclerotic plaques) [284], and synaptic vesicles (vesicles from neurons) [313]]. However, such names do not provide a clue for classification with regard to the type of vesicles involved.

Types of vesicles in recent literature

In four recent reviews, vesicles were classified into between two and six major different types [66, 294, 32, 195]. Two common types were distinguished unanimously [i.e., exosomes and microvesicles (also called shedding vesicles, shedding microvesicles, or microparticles)], and in three of these reviews, apoptotic vesicles (also called apoptotic blebs, or apoptotic bodies) became a separate class [294, 32, 195]. In addition, “ectosomes,” “membrane particles,” and “exosome-like vesicles” were distinguished on the basis of the physicochemical characteristics of vesicles, in-
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Figure 2.1: Different types of eukaryotic cell-derived vesicles. Transmission electron micrographs of cell-derived vesicles isolated by differential centrifugation from plasma (A; exosomes, diameter < 100 nm, urine (B; microvesicles, diameter > 100 nm), or saliva (C; either exosomes or membrane particles, diameter < 100 nm) of a healthy human subject. A scanning electron micrograph (D) showing a human umbilical vein endothelial cell releasing apoptotic vesicles. Please notice the much larger size of apoptotic vesicles compared with the other types of vesicles, and the typical cup shape of vesicles.

Including size, density, appearance in microscopy, sedimentation, lipid composition, main protein markers, and subcellular origin [i.e., originating from intracellular compartments (exosomes) or plasma membranes] [294]. Although this classification is the best and most extensive so far, it is difficult to use in daily practice. For instance, a vesicle with a diameter of 50 nm can be classified either as an exosome, ectosome, membrane particle, exosome-like vesicle, or apoptotic vesicle according to this scheme, and, as correctly mentioned by the authors “in practice, all vesicles preparations are heterogeneous, with different protocols allowing the enrichment of one type over another.”

Types of vesicles in this chapter

In this chapter, we propose to distinguish four different types of eukaryotic cell-derived vesicles: (1) exosomes, (2) microvesicles (microparticles), (3) membrane particles, and (4) apoptotic vesicles, thereby omitting “ectosomes” and “exosome-like vesicles,” because there is insufficient evidence to support the existence of
Table 2.1: Overview of the main characteristics of different types of eukaryotic cell-derived vesicles

<table>
<thead>
<tr>
<th>Vesicle type</th>
<th>Diameter (nm)</th>
<th>Density (g mL(^{-1}))</th>
<th>Morphology</th>
<th>Cellular origin</th>
<th>Subcellular origin</th>
<th>Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Exosomes</td>
<td>50–100</td>
<td>1.13–1.19</td>
<td>Cup-shaped</td>
<td>Most cell types</td>
<td>Plasma membrane endosomes</td>
<td>Biochemical composition known, but most proteins and lipids not unique for exosomes</td>
</tr>
<tr>
<td></td>
<td>[93, 129]</td>
<td>[93, 129, 243, 298]</td>
<td></td>
<td></td>
<td></td>
<td>[93, 243]</td>
</tr>
<tr>
<td>Microvesicles</td>
<td>20–1,000</td>
<td>Unknown</td>
<td>Cup-shaped</td>
<td>Most cell types</td>
<td>Plasma membrane</td>
<td>Insufficiently known</td>
</tr>
<tr>
<td></td>
<td>129, 300</td>
<td>[129, 300, 90, 120, 327]</td>
<td></td>
<td></td>
<td></td>
<td>[14, 32, 75, 110, 111]</td>
</tr>
<tr>
<td>Membrane particles</td>
<td>50–80, 600</td>
<td>1.032–1.068</td>
<td>Cup-shaped</td>
<td>Epithelial cells only</td>
<td>Plasma membrane</td>
<td>CD133</td>
</tr>
<tr>
<td></td>
<td>138</td>
<td>[138, 194]</td>
<td></td>
<td></td>
<td></td>
<td>[138]</td>
</tr>
<tr>
<td>Apoptotic vesicles</td>
<td>1,000–5,000</td>
<td>1.16–1.28</td>
<td>Heterogeneous</td>
<td>All cell types</td>
<td>Plasma membrane endoplasmic reticulum</td>
<td>Histones, DNA</td>
</tr>
<tr>
<td></td>
<td>294, 300</td>
<td>[294, 300, 164]</td>
<td></td>
<td></td>
<td></td>
<td>[300, 138, 164, 35, 136]</td>
</tr>
</tbody>
</table>

Morphology is obtained by transmission electron microscopy.
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these types of vesicles [294]. The main characteristics of these types of vesicles are summarized in Table 2.1 and Fig. 2.1 illustrates the various types of vesicles.

“Ectosomes” have been omitted because (1) the reported size of these neutrophil-derived vesicles (50–200 nm) was based partially on flow cytometry using 200 nm beads, a procedure now known to lead to underestimation of the diameter and concentration of the vesicles as a result of differences in refractive indexes between the beads and the vesicles [233, 58, 232], (2) these vesicles were observed in vitro only, and (3) neutrophils also release microvesicles, which was not known when the term “ectosomes” was introduced [77].

The “exosome-like vesicles” were omitted because (1) these vesicles, which were shown to contain the 55 kDa full length from the tumor necrosis factor (TNF)-α receptor, were erroneously reported to be mainly present in the 175,000 g fraction of conditioned medium of human umbilical vein endothelial cells and bronchoalveolar lavage (BAL) fluid, whereas they are present predominantly in the 100,000 g fractions (i.e., the fraction in which also exosomes are usually isolated) [294]. (2) the transmission electron microscopy (TEM) micrographs show damaged and disrupted vesicles, which makes estimation of the real vesicle size difficult, and (3) endothelial-conditioned medium and BAL also contain exosomes [11, 236, 165, 316].

2.1.4 Current limitations of classification

Important criteria for classification with regard to the type of cell-derived vesicles are size, density, morphology, lipid composition, protein composition, and subcellular origin [294, 233], which are summarized in Table 2.1. In the near future, it is expected that also the refractive index, ζ-potential, and chemical composition will be accessible from individual vesicles to become novel relevant characteristics. It is important to point out the limitations and problems with the current criteria, which is necessary to fully understand and appreciate the literature about exosomes and other types of cell-derived vesicles.

Isolation

Because of the biological complexity of body fluids, isolation of vesicles has proven to be extremely difficult. For instance, isolation of vesicles from blood is affected by venipuncture, time between blood collection and handling, the anticoagulant, the applied separation process, the high viscosity of blood, and the presence of sticky proteins, including fibrinogen and albumin [331]. Because of their small size, vesicles are below the detection range of conventional detection methods. Consequently, recovery and contamination of the separation process cannot be reliably quantified, and isolation protocols have not been standardized. The interrelated difficulties of the detection and isolation of vesicles partly explains the differences in classification criteria and clearly exposes one of the main issues to be solved by the research field.

In most studies, vesicles are isolated by differential centrifugation. With cen-
trifugation, the centrifugal force is used for the sedimentation of particulate matter, such as vesicles in solution. Separation of the various sorts of vesicles present in a sample is based on size and density, larger and denser components migrating away from the axis of the centrifuge and smaller and less-dense components migrating toward the axis. Differential centrifugation involves multiple sequential centrifugation steps, each time removing the pellet and increasing the centrifugal force to separate smaller and less dense components than the previous step. Typically, applied centrifugal accelerations are approximately 200 to 1,500 \( g \) to remove cells and cellular debris, 10,000 to 20,000 \( g \) to pellet vesicles larger than 100 nm, and 100,000 to 200,000 \( g \) to pellet vesicles smaller than 100 nm.

Besides the size and density of vesicles, the efficiency to isolate vesicles will depend on the shape and volume fraction of the vesicles, the volume, viscosity, and temperature of the fluid in which the vesicles are present, the centrifugation time, and the type of rotor used (fixed angle or swing-out). Because vesicles are heterogeneous in all aspects involved in differential centrifugation, complete separation of vesicles with a certain diameter or density is still utopian. For example, we recently applied differential centrifugation to the best of our ability to separate vesicles smaller than 100 nm from larger vesicles, all present in human saliva, and observed substantial cross contamination (~10\%) in both fractions [30].

Centrifugation also raises other problems that have to be taken into account. The removal of all cells from biological fluids can be challenging, for instance in the case of blood, where small platelets and apoptotic bodies overlap in size with large vesicles. When the centrifugal force applied to remove cells is too high, cells may fragment or become activated. Washing of vesicle pellets will often result in the loss of vesicles, resulting in variable yields. For instance, approximately 40 to 60\% of platelet-derived vesicles are lost at every washing step, whereas vesicles from erythrocytes are unaffected (M.C.L. Schaap and R.J. Berckmans, personal communication). In addition, a high centrifugal acceleration of 100,000 to 200,000 \( g \) may result in vesicle fusion and contamination of the pellets with proteins, thus hampering TEM and proteomic studies [21, 254, 121]. Moreover, the functional properties of vesicles may change during isolation. For example, centrifugation of vesicles may increase their exposure of phosphatidylserine (PS), thereby enhancing the ability of vesicles to promote coagulation (R.J. Berckmans, personal communication).

Besides differential centrifugation, filtration can be applied to isolate vesicles. With filtration, the isolation of vesicles is based on differences in size, shape, and deformability between types of vesicles and other particles. Although most filters have a well-defined pore size, the filtrate may contain larger vesicles than the pore size due to the deformation of vesicles. In addition, filters may bind subpopulations of vesicles, and increasing forces have to be applied with decreasing pore size. One recent advance is the availability of nanofabricated filtration sieves, which have pores with well-defined diameters as small as 100 nm [72, 249, 299]. Fig. 2.2A shows a scanning electron micrograph of a filtration sieve containing pores with a diameter of 900 nm. Nanofabricated filters have the capability to separate plasma...
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Figure 2.2: Advanced methods to isolate vesicles. (A) Scanning electron micrograph of a filtration sieve containing pores with a diameter of 900 nm. Image courtesy of C.J.M. van Rijn. (B) Schematic of the underlying principle of FFFF. Since relatively small vesicles (purple) have a large diffusion coefficient compared to relatively large vesicles (green), small vesicles are on average located more centrally in the flow channel than larger vesicles. Consequently, due to the parabolic flow profile of the channel flow, small vesicles elute faster than larger vesicles.

from whole blood by capillary forces only, as was demonstrated using a planar filter with a thickness of 500 nm [76]. However, the total obtained volume of plasma from 5 µL blood was merely 45 nL. Further investigation is necessary to employ nanofabricated filters for the isolation of EVs.

Alternatively, vesicles can be isolated and fractionated by size using flow-field flow fractionation (FFFF). Fig. 2.2B shows a schematic of the underlying principle of FFFF. In an open flow channel a laminar flow with a parabolic stream profile is formed. This main flow transports the sample through the channel to which a cross flow is applied perpendicularly. The cross flow is directed through a semipermeable membrane that is located at the bottom wall. The membrane allows the fluid to exit the channel but prevents the vesicles to pass through. Under the influence of the Brownian motion of vesicles and the counteracting cross flow, different equilibrium layer heights are formed by different vesicle size fractions. Small vesicles with high diffusion coefficients are on average located more centrally in the flow channel in fast stream lines and elute first. Larger vesicles with lower diffusion coefficients are on average located in slow stream lines and elute later. This results in size-based fractionation with a resolution of up to 10 nm without exposing the vesicles to high shear stress [170]. Although FFFF is successfully applied to isolate exosomes from human neural stem cells [159], FFFF is not widely applied because it requires extensive optimization of the settings and is expensive relative to differential centrifugation.

Size

Because vesicles are assumed to be spherical in their natural state, the size of vesicles given is usually the diameter. The relation between the range of the diameter of vesicles and their concentration is the size distribution, preferably expressed
as number of vesicles per unit particle size and suspension volume. However, because most size determinations of vesicles are based on TEM, the original volume of the suspension from which the vesicles originate cannot be assessed, and a differential size distribution is used, indicating the number of vesicles per unit particle size only. Because TEM is performed in a vacuum, fixation and dehydration are essential preparation steps likely to affect the size and morphology of vesicles [233]. Novel methods have been explored to determine the size and concentration of vesicles directly in suspension, including atomic force microscopy [267, 333], nanoparticle tracking analysis [90], and resistive pulse sensing. Typically, size distributions obtained from vesicles have the shape of a Gaussian or log normal distribution, but to what extent this reflects the natural population of vesicles is unknown, because the results are strongly influenced by (pre)analytical variables such as the isolation procedure and the detection limit of the applied detection technique. The (pre)analytical variables together with the choice of statistical parameters used to describe the size distribution (e.g., minimum, maximum, full width at half-maximum, mean, median, and mode diameter) all give rise to a marked range in the reported size for the different types of vesicles.

Density
The determination of the density of vesicles is usually based on sucrose gradient centrifugation. Because vesicles are very heterogeneous in all aspects involved in centrifugation, and because observed differences in the densities between types of vesicles are very small, discriminating types of vesicles by density is difficult.

Morphology
The morphology of cell-derived vesicles can be assessed with detection methods having subnanometer resolution, such as TEM and atomic force microscopy. For example, the morphology of exosomes has been traditionally described as “typical cup-shaped” after fixation, adhesion, negative staining, and visualization by TEM. However, to what extent this feature is either an artifact due to extensive sample preparation or actually unique for exosomes and does not apply to other vesicles is unknown [194]. The cupshaped morphology may still be a useful feature to distinguish cell-derived vesicles from similar-sized particles. With atomic force microscopy, the morphology of vesicles adhered to a surface can be studied directly in solution [267, 333]. However, because of the adhesion of vesicles to the surface, their structure changes from spherical to hemispherical or flat, depending on the composition of the membrane [160], which may cause artifacts in the interpretation of both size and morphology.

Lipid composition, protein composition, and cellular origin
Studies on the cellular origin and intracellular versus plasma membrane origin of vesicles are often based on the measurement of the lipid and protein composition of the total population of vesicles that has been isolated (e.g., by Western blotting and
mass spectroscopy). Obviously, this approach does not provide information on the presence of contaminants, such as other types of vesicles or copurified proteins. For example, exosomes from malignant pleural effusions isolated by sucrose gradient centrifugation contain immunoglobulins [21]. By far the most widely used method to establish the cellular origin and phenotype of single vesicles is flow cytometry, which is based on the detection of light scattering and fluorescence from labeled vesicles. Nevertheless, the smallest detectable single vesicle by flow cytometry varies between 300 and 700 nm on older generation flow cytometers [232]. Modern flow cytometers using high collection angle optics are capable of detecting single polystyrene beads with a diameter as small as 100 nm [250], corresponding to vesicles with a diameter larger than 150 nm owing to refractive index differences [58]. Because most vesicles have a diameter smaller than 100 nm, it is not surprising that according to recent estimates only 1 to 2% of all vesicles present in biological fluids (e.g., plasma and urine) are actually detected [233, 333, 58]. One popular solution to this problem has been to use specific capture beads that are sufficiently large to be measurable. The capture beads bind multiple smaller vesicles, thus facilitating their phenotyping by flow cytometry.

In sum, although there is no doubt that different types of cell-derived vesicles do exist, the interrelated difficulties of the detection and isolation of vesicles hamper the development of criteria to distinguish them. Consequently, the classification of vesicles is clearly work in progress. Nonetheless, recent studies have shown that the detection of single vesicles smaller than 100 nm is becoming feasible. Therefore, new developments on the detection of vesicles are likely to improve the criteria for classification [233, 333, 90].

2.2 Types of cell-derived vesicles

2.2.1 Exosomes

Exosomes are cell-derived vesicles that are present in many and perhaps all biological fluids, including urine, blood, ascites, and cerebrospinal fluid [227, 53, 163, 309], fractions of body fluids such as serum and plasma, and cultured medium of cell cultures. The reported diameter of exosomes is between 30 and 100 nm and the density ranges between 1.13 and 1.19 g mL⁻¹. Exosomes are usually isolated by ultracentrifugation (100,000 to 200,000 g). The morphology of exosomes has been described as cup-shaped after fixation, adhesion, negative staining, and visualization by TEM. Regarding their biochemical composition, exosomes are surrounded by a phospholipid membrane containing relatively high levels of cholesterol, sphingomyelin, and ceramide and containing detergent-resistant membrane domains (lipid rafts) [329, 268, 294, 195]. The membrane proteins have the same orientation as the cell. Exosomes are characterized by the presence of proteins involved in membrane transport and fusion, such as Rab, GTPases, annexins, and flotillin, components of the endosomal sorting complex required for transport (ESCRT) complex such as Alix, tumor susceptibility gene 101 (TSG101), heat shock
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proteins (HSPs), integrins, and tetraspanins, including CD63, CD81, and CD82.

Although all of the aforementioned properties of exosomes are frequently reported and accepted, none of these properties is unique and identifies exosomes. There is increasing evidence that there is overlap between properties previously thought to be unique for exosomes and properties of other types of cell-derived vesicles, suggesting that there is a continuum of vesicle types with overlapping properties present in body fluids.

For example, the typically reported diameter of exosomes may be biased toward smaller particles for two reasons. First, exosomes are often isolated by differential centrifugation, which involves a loss of relatively large vesicles during removal of cells by centrifugation. Second, because in most studies only a limited number of exosomes are visualized by TEM, and because the size distribution of vesicles typically has the shape of a Gaussian or log normal distribution with a peak below 100 nm, as also confirmed by novel detection methods, such an analysis will easily overlook the presence of vesicles larger than 100 nm. Although these larger vesicles represent only a relatively small fraction of the total population, their total surface area or volume and thus their functional contribution may be relatively large.

Identification of exosomes based on their cup-shaped morphology after negative staining and visualization by TEM seems questionable. For example, exosomes and similar-sized vesicles, called membrane particles or prominosomes, both appear with a cup-shaped morphology on the same electron micrographs. Fig. 2.1 shows that not only exosomes but also vesicles larger than 100 nm may appear cup-shaped by TEM. Finally, recent evidence indicates that not all exosomes originate from intracellular MVEs, the “classic pathway” of exosome formation, thereby making identification of exosomes even more complex.

Classic pathway of exosome formation

The “classic pathway” of exosome formation is by far the best studied and involves the formation of intraluminal vesicles within MVEs (Fig. 2.3). In turn, MVEs can fuse with either lysosomes for cargo degradation or with the plasma membrane to secrete the intraluminal vesicles, which are then released as exosomes.

Different intracellular sorting pathways exist in directing proteins toward intraluminal vesicles destined for either degradation or secretion, thus implicating the existence of different types of MVEs. Redundant transmembrane receptors are sorted to intraluminal vesicles destined for lysosomal degradation after ubiquitination, a post-translational modification that is executed by ESCRT. On the other hand, there is no compelling evidence that the ESCRT is involved in the sorting of transmembrane receptors to intraluminal vesicles that are destined to become secreted as exosomes. Although several ESCRT proteins and ubiquitinated proteins are present in exosomes, the ubiquitinated proteins are soluble proteins and not transmembrane proteins, suggesting that ubiquitination may have occurred in the cytosol rather than by the ESCRT. Likewise, the sorting of the proteolipid protein to intralu-
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Figure 2.3: Pathways of exosome formation. Cells release exosomes via two mechanisms. The classic pathway (left) involves the formation of intraluminal vesicles (ILVs) within multivesicular endosomes (MVEs). In turn, the membrane of MVE fuses with the plasma membrane, resulting in the release of ILVs. When secreted, ILVs are called exosomes. Alternatively, the direct pathway (right) involves the release of vesicles, indistinguishable from exosomes, directly from the plasma membrane.

Minal vesicles predestined to become secreted as exosomes is independent from the ESCRT but depends on the sphingolipid ceramide \( \text{c}_{197} \). Further evidence for two pathways comes from studies showing that lysobisphosphatidic acid induces the formation of intraluminal vesicles predestined for lysosomal degradation but does not affect the formation of exosomes \( \text{c}_{196} \). This is confirmed by studies in which cholesterol is labeled with perfringolysin O, which reveals perfringolysin O-positive and -negative MVEs in B cells, of which only the cholesterol-containing MVEs fuse with the plasma membrane resulting in the release of exosomes \( \text{c}_{205} \). In addition, both epidermal growth factor and the epidermal growth factor receptor (EGFR) travel to exosomes via MVEs not containing the lipids bis(monoacylglycerol)phosphate/lysobisphosphatidic acid, whereas the bis(monoacylglycerol) phosphate/lysobisphosphatidic acid-containing vesicles are degraded by lysosomes \( \text{c}_{322} \). Thus, clearly two intracellular pathways of MVE sorting exist.

Cytosolic domains of proteins \( \text{c}_{291} \) or lipid domains enriched in the tetraspanins CD9 or CD63 are thought to play a role in the sorting of transmembrane proteins toward intraluminal vesicles \( \text{c}_{52} \). Several different types of small GTPases from the Rab family play a role in the intracellular trafficking of MVEs toward either the plasma membrane \( \text{c}_{258} \) or to lysosomes for degradation \( \text{c}_{278} \), as well as cytosolic calcium levels \( \text{c}_{259} \), citron kinase, \( \text{c}_{187} \), and a still unidentified combination of soluble N-ethylmaleimidesensitive factor attachment protein receptors, which is involved in the final fusion of the MVE membrane with the plasma membrane \( \text{c}_{242} \).
Direct pathway of exosome formation

Next to the “classic pathway” of exosome biogenesis, there is a second and much more immediate route of exosome formation (Fig. 2.3). T cells and erythroleukemia cell lines release exosomes directly from their plasma membrane, both spontaneously as well as upon expression of HIV Gag or Nef, or after cross-linking of surface receptors [43, 96, 185]. These vesicles are indistinguishable from exosomes formed by the classic endosomal pathway because they are enriched in classic exosome markers such as CD63 and CD81 and have a similar diameter and density. The extent to which such exosomes are also released from other cells or in vivo (e.g., in biological fluids) is unknown.

2.2.2 Microvesicles

Microvesicles, often called microparticles, is a term used for vesicles that are released from the plasma membrane during cell stress. This term is also often used to describe total populations of vesicles isolated from biological fluids [213, 129, 32]. In addition, microvesicles are present in most if not all biological fluids, atherosclerotic plaques and conditioned culture medium [190, 29, 30, 55, 208]. Although microvesicles are believed to be larger than exosomes and are usually reported to range in size between 100 nm and 1.0 µm in diameter [294], there is much confusion on this matter. For instance, with regard to the diameter of vesicles in plasma from healthy human individuals, the following size ranges have been reported: 20 to 50 nm by TEM [327], 200 to 800 nm by TEM [300], 180 nm (mean) by TEM and atomic force microscopy [120], and 80 nm (mean) by nanoparticle tracking analysis [90]. Consequently, the size ranges of microvesicles and exosomes may overlap, especially when body fluids are used as a source for isolation of vesicles. In vitro, activated platelets release two clearly distinct populations of vesicles, small (< 100 nm) vesicles exposing CD63, exosomes, and large (100–1,000 nm) microvesicles exposing typical platelet receptors such as glycoprotein Ib [129]. Therefore, when only a single cell type is studied in vitro, both types of vesicles may be distinguishable. The density of microvesicles is unknown, and microvesicles are usually isolated by centrifugation at 10,000 to 20,000 g [294]. The term “microparticles”, however, has also been used for total populations of vesicles isolated from human plasma at 100,000 g [265] and such populations will contain exosomes [129]. Exosomes have a typical cup shape when studied by TEM (Fig. 2.1A), but larger vesicles (Fig. 2.1B) also show this morphological feature. Although exposure of PS is often mentioned as a typical marker for microvesicles [294, 32], this seems questionable at best, because a substantial number of microvesicles do not expose PS [156, 70], and because exposure of PS is markedly increased by centrifugation and freeze-thaw procedures [70]. The mechanisms underlying the formation of microparticles have been recently summarized elsewhere [208]. Taken together, although exosomes and microvesicles are distinct types of vesicles, neither size, morphology, nor exposure of PS is a sufficient criterion to distinguish both types of vesicles from each other.
2.2.3 Membrane particles

The lumen of the neural tube of embryonic mouse brain contains ventricular fluid, in which two types of prominin-1 (CD133)-exposing vesicles are present \[194\], called prominosomes or membrane particles \[194, 294\]. One type of membrane particle has a diameter of approximately 600 nm, whereas the other type has a diameter between 50 and 80 nm as determined by TEM. Although the small type of membrane particles is precisely in the size range of exosomes, they showed a slightly lower density \((1.032 - 1.068 \text{ g mL}^{-1})\) than exosomes on sucrose gradients, and they do not expose CD63. Because these CD133\(^+\) vesicles (1) originate from the plasma membrane of epithelial cells, (2) occur in human body fluids that contact the epithelium, such as saliva, urine, and seminal fluid, and (3) coexist with exosomes in saliva \[194, 30\], we assume these CD133\(^+\)/CD63\(^-\) vesicles to be different from exosomes. The extent to which the larger type of membrane particle is different from microvesicles, however, will need additional studies.

2.2.4 Apoptotic vesicles

When cells are undergoing apoptosis, they release PS-exposing vesicles, often called apoptotic bodies or vesicles. The major difference between apoptotic vesicles and other cell-derived vesicles is their size. In all studies so far, the diameter of apoptotic vesicles is reported to range between 1 and 5 \(\mu\text{m}\) \[164, 293, 138, 300\]. This is in the precise size range of platelets in the human blood. Because activated platelets or platelets undergoing a process resembling apoptosis also express PS, it may be impossible to resolve apoptotic bodies from platelets based upon size and a PS-positive phenotype. The density of apoptotic vesicles is 1.16 to 1.28 g mL\(^{-1}\), which is partly overlapping with the density of exosomes, and their morphology is typically more heterogeneous than other cell-derived vesicles when visualized by TEM.

The inappropriate clearance of apoptotic vesicles is considered to be the primary cause of developing systemic autoimmune disease. Apoptotic cells release at least two immunologically distinct types of apoptotic vesicles. Apoptotic vesicles originating from the plasma membrane contain DNA and histones, whereas apoptotic vesicles originating from the endoplasmic reticulum do not contain DNA and histones but expose immature glycoepitopes \[35\]. In general, the process of “membrane blebbing” is thought to precede the release of apoptotic vesicles and microvesicles. The extent to which this assumption is true, however, is unclear. Membrane blebbing requires phosphorylation of myosin light chain and Rho-associated coiled coil kinase I activity, which becomes constitutively active upon cleavage by caspase 3, induces a net increase in myosin light chain phosphorylation and subsequent membrane blebbing \[260\]. The key role of caspase 3, one of the executioner enzymes of apoptosis, in membrane blebbing is confirmed by the observation that a human breast cancer cell line, MCF7, which is deficient of functional caspase 3, lacks membrane blebbing \[140\]. Alternatively, granzyme B cleaves Rho-associated coiled coil kinase II, which produces
a constitutively active enzyme, and increases myosin light chain phosphorylation as well as membrane blebbing \[273\].

2.3 Functions and clinical applications of cell-derived vesicles

An extensive description of the functions and clinical applications of cell-derived vesicles is beyond the scope of this thesis and can be found elsewhere \[229\]. In short, vesicles play a role in intercellular signaling (e.g. immune suppression, antigen presentation, inflammation), cell adhesion, waste management, coagulation, and protection against extracellular and intracellular stress. Vesicles have potential clinical applications spanning therapy, prognosis and biomarkers for disease.

2.4 Conclusions and future directions

The research on cell-derived vesicles from eukaryotic cells is a fast-growing and exciting new field. Although major progress has been made during the last decade, our understanding of the molecular mechanisms underlying the release of vesicles and the sorting of compounds into these vesicles are still incompletely understood, the criteria to identify different types of vesicle have only been partially elucidated so far, and, most importantly, the biological relevance of vesicles in health and disease is poorly understood, especially in vivo.

The finding that vesicles enable the intercellular exchange of biomolecules suggests a new level of communication that may increase our understanding of disease development and progression. Moreover, vesicles may be useful as clinical instruments for prognosis and biomarkers, and they are promising as autologous drug vehicles capable of passing pharmacological barriers. To increase their clinical usefulness, however, novel and dedicated instruments will have to be developed to detect vesicles and to standardize vesicle measurements between laboratories.