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The review briefly summarizes main features of extracellular vesicles, a joint terminology for exosomes, microvesicles, and apoptotic vesicles. These vesicles are in the center of interest in biology and medical sciences, and form a very active field of research. Mass spectrometry (MS), with its specificity and sensitivity, has the potential to identify and characterize molecular composition of these vesicles; but as yet there are only a limited, but fast-growing, number of publications that use MS workflows in this field. MS is the major tool to assess protein composition of extracellular vesicles: qualitative and quantitative proteomics approaches are both reviewed. Beside proteins, lipid and metabolite composition of vesicles might also be best assessed by MS techniques; however there are few applications as yet in this respect. The role of alternative analytical approaches, like gel-based proteomics and antibody-based immunoassays, are also mentioned. The objective of the review is to give an overview of this fast-growing field to help orient MS-based research on extracellular vesicles. © 2015 Wiley Periodicals, Inc. Mass Spec Rev 35:3–21, 2016.

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I. INTRODUCTION

In recent years, advanced mass spectrometry (MS)-driven “omics” has matured into a fundamental technique for cell biology and biomarker discovery. Isolation of cellular organelles with various fractionation methods and the systematic study of their protein, lipid and, metabolite contents provide unique insights into the molecular mechanisms that govern cell functions in health and disease. Here, we review MS-based analysis of extracellular vesicles (EVs), which are important secreted, cell-derived structures. Interest in EVs has grown tremendously in recent years; as shown by the number of publications which has increased tenfold in the last decade (Fig. 1). The numbers of papers that use MS-based analysis

show a similar, fast-growing trend. The major interest is due to the observation that these vesicles provide a novel means of communication among distant cells. In this respect, DNA and RNA content of these vesicles have a major role, but other molecular components, like proteins and lipids, are also of interest (Fig. 2). Here, we build on several reviews (Thery et al., 2002; Bobrie et al., 2011; Gyorgy et al., 2011; Raimondo et al., 2011; Deatherage & Cookson, 2012; Raj et al., 2012b; van der Pol et al., 2012; Akers et al., 2013; Fang et al., 2013; Momen-Heravi et al., 2013; Tissot et al., 2013; Beach et al., 2014; Buzas et al., 2014; Vader et al., 2014) and recently published results to provide an up-to-date overview on the secretion, structure, and function of EVs. The review will highlight contemporary applications of MS-based analyses for the identification (Raimondo et al., 2011) and quantitative characterization of main EVs’ constituents (Choi et al., 2012; de Jong et al., 2012; Del Boccio et al., 2012; Li et al., 2012; Raj et al., 2012a; Brouwers et al., 2013; Choi et al., 2013; Fraser et al., 2013; Cypryk et al., 2014; Jeppesen et al., 2014; Rodríguez-Suárez et al., 2014) in comparison with other analytical techniques (Graner et al., 2009; Choi et al., 2013; Jorgensen et al., 2013).

II. BIOPHYSICAL PROPERTIES, FORMATION, AND FUNCTIONAL CHARACTERISTICS OF EXTRACELLULAR VESICLES

A. Nomenclature, Classification, and Formation of Various Extracellular Vesicles

Prokaryotic and eukaryotic cells release different vesicles into the extracellular microenvironment (Perez-Hernandez et al., 2013; Reinhardt et al., 2013; Im et al., 2014). These vesicles are classified into three major types: exosomes, microvesicles (also called ectosomes, shedding vesicles, shedding microvesicles, or microparticles), and apoptotic vesicles (also called apoptotic blebs or apoptotic bodies or apoptosomes) based on their origin, physical properties, and functions (Akers et al., 2013; Raposo & Stoorvogel, 2013; Comelli et al., 2014; Cypryk et al., 2014; Rodríguez-Suárez et al., 2014) (Fig. 3). Within a specific field, cell-derived vesicles have often been called after the cells or tissues such as dexosomes (dendritic cell-derived exosomes), oncosomes (derived from tumor cells), prostasomes (prostate-derived vesicles), matrix vesicles (vesicles in bone, cartilage,

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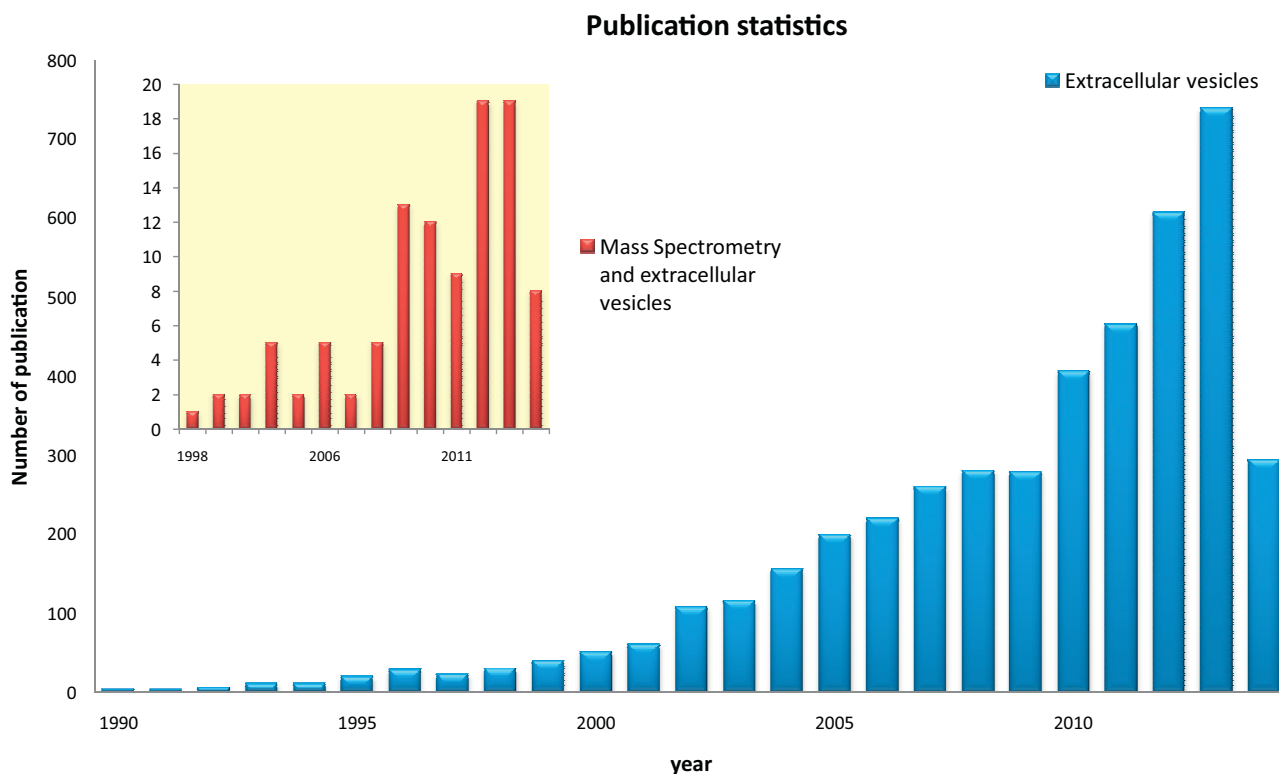


FIGURE 1. Publication statistics in the last 15 years on extracellular vesicles showing the number of publications which has used mass spectrometry methods. (Topic search was performed using terms: exosome or microvesicle or platelet or “apoptotic body” or “extracellular vesicle” in Web of Science database (execution date October 2014). References found in the search were exported into EndNote and re-searched with the term of Mass Spectrometry (see insert).

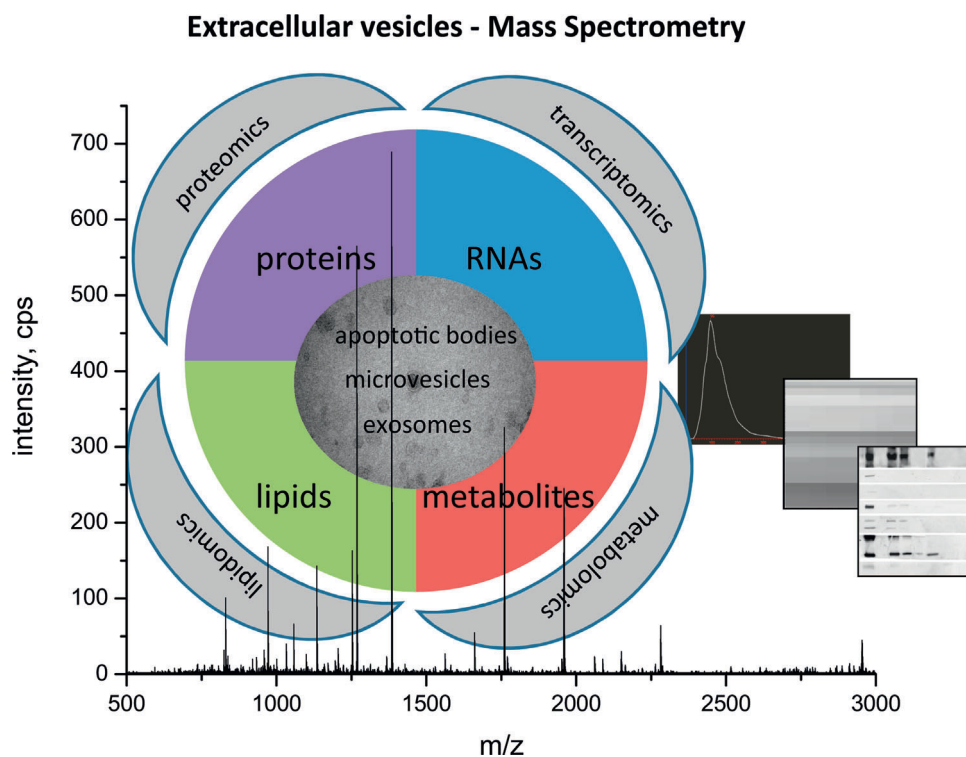


FIGURE 2. Mass spectrometry has an important role in the characterization of extracellular vesicles. The increasing quantitative data will likely increase our knowledge in their pathophysiological roles.

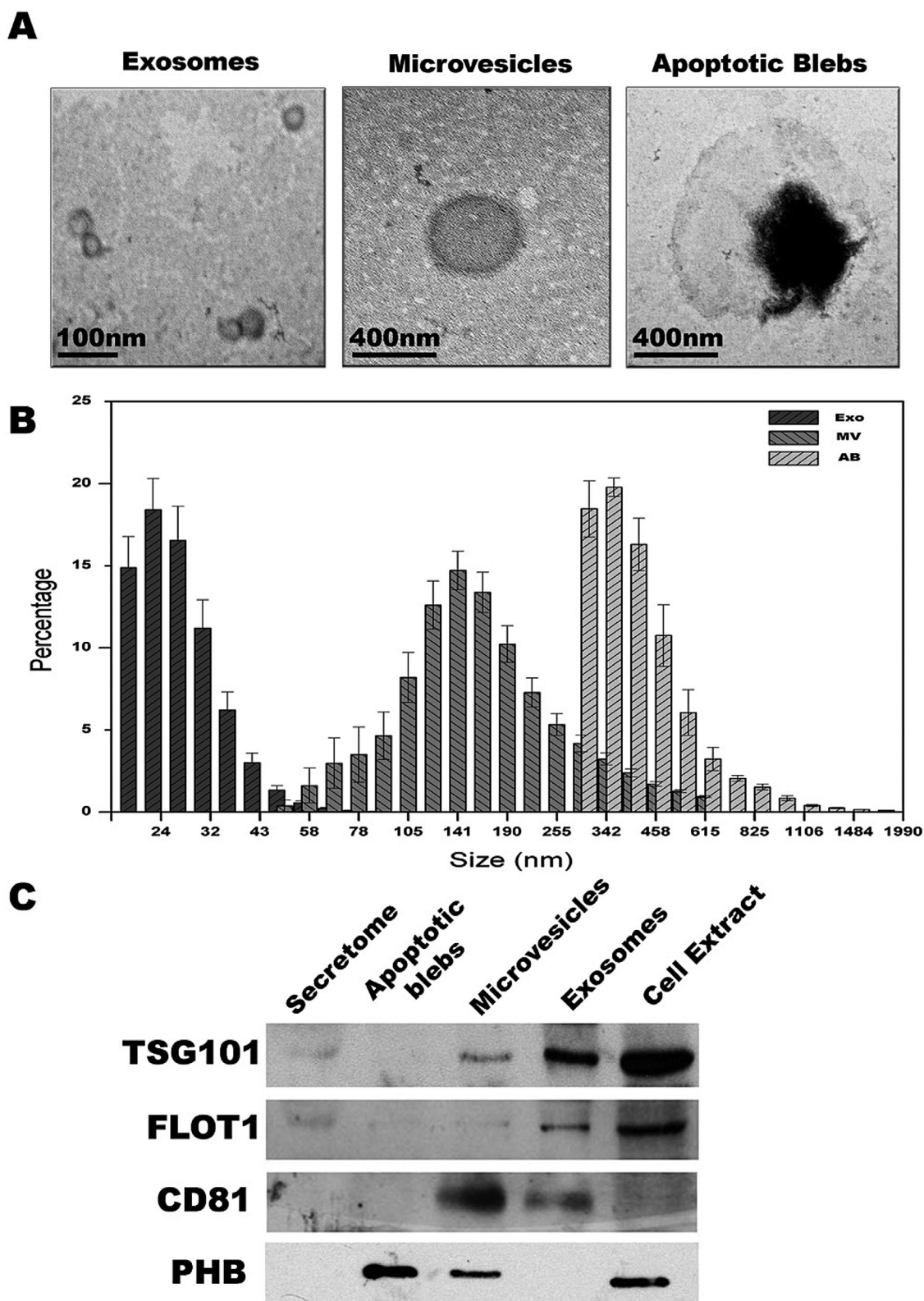


FIGURE 3. Representative (A) transmission electron micrographs, (B) dynamic light scattering analysis, and (C) Western blots showing isolated exosomes, microvesicles and apoptotic vesicles. Reproduced from (Comelli et al., 2014) with permission of The Royal Society of Chemistry.

and atherosclerotic plaques), synaptic vesicles (vesicles from neurons), tolerosomes (vesicles purified from serum of antigen-fed mice), cardiosomes (vesicles secreted by cardiomyocytes), and vexosomes (vesicles associated with adeno-associated virus vectors) (Forterre et al., 2014; Im et al., 2014; Jeppesen et al., 2014). In fact, the nomenclature and classification of vesicles is very complex and still a major point of debate within the research community because a common consensus is difficult to reach (Gould & Raposo, 2013; Im et al., 2014). Of note, at present there is no universal molecular marker that would distinguish the different types of extracellular vesicles. In this review, we use extracellular vesicles (EVs) as a collective term for the different cell-secreted extracellular vesicles as it was suggested in the workshop of the International Society for Extracellular Society held in October 2012.

1. Exosomes

The vesicles to receive most attention over the last few years are the exosomes (Palmisano et al., 2012). The physicochemical properties and biological function of exosomes are well-documented in contrast to other types of vesicles. Exosomes are nano-sized structures (30–100 nm) surrounded by a lipid bilayer and they have an endosomal compartment origin. Exosomes have been isolated from blood, urine, saliva, synovial fluid, breast milk, cerebrospinal fluid, and various other body fluids, and are also secreted by most cell types in culture (Im et al., 2014). Although they were initially considered to be “garbage bags”, where the cells deposit all their unwanted components, now there is plenty of evidence to prove that they have specific biological functions. Exosomes contain a variety of cell-specific proteins (membrane and cytosolic), lipids, metabolites, and nucleic acids. Surface markers, like tetraspanins, Alix, flotillin, TSG101, and Rab5b are detected with Western blotting (WB) (Forterre et al., 2014; Jeppesen et al., 2014) or with ELISA (Akers et al., 2013; Perez-Hernandez et al., 2013) to show the presence of exosomes.

The genesis of exosomes is within the endosomal network, a membranous compartment that sorts the various intraluminal vesicles and directs them to their destinations including lysosome and cell surface membranes (Lee et al., 2012; Raj et al., 2012b; Akers et al., 2013; El Andaloussi et al., 2013; Robbins and Morelli, 2014; Sluijter et al., 2014). Early endosomes initially fuse with endocytic vesicles and incorporate their content into those destined for recycling, degradation, or exocytosis. The content to be recycled are sorted into recycling endosomes. The remainder of early endosomes undergoes a series of maturation steps to become late endosomes. During this transformation, contents are sorted into 30–100 nm vesicles that bud into the lumen of MVBs (Beach et al., 2014). The formation of exosomes involves the endosomal-sorting complexes required for transport (ESCRT) and other associated proteins such as Alix (Lee et al., 2012; Kowal et al., 2014). MVBs either fuse with lysosomes (lysosomal pathway) or the plasma membrane (secretory pathway) (Robbins and Morelli, 2014). Fusion of the endocytic vesicles with the lysosomes will result in the degradation of the contents, whereas fusion with the plasma membrane will result in the release of exosomes into the extracellular micro-environment (Lee et al., 2012; Akers et al., 2013). The mechanisms of the release of exosomes into the extracellular space by exocytosis (i.e., fusion of the multi-

vesicular bodies [MVBs] with the plasma membrane of the cell) have recently been the object of substantial attention and research (Bobbie et al., 2011; Palmisano et al., 2012; Akers et al., 2013; Alvarez et al., 2013; Jeppesen et al., 2014). ESCRT-independent sorting of cargo into exosomes has also been proposed (Trajkovic et al., 2008). Different lipids and lipid-related enzymes control secretion of the exosomes. Another theory emphasizes the translocation of a mainly cytosolic protein, galectin-5, into the endosome lumen and its release into exosomes (Barres et al., 2010). Changes in proteomic and lipidomic signatures of exosomes released by reticulocytes that undergo differentiation have been compared, and significant differences were observed during the 7-day differentiation protocol; that data suggest that indeed different mechanisms might be involved in the biogenesis of exosomes (Carayon et al., 2011). The biogenesis of exosomes has been reviewed recently by Colombo et al. (Colombo et al., 2014).

2. Microvesicles

The next major type of vesicle that is secreted into the extracellular space are microvesicles (MVs). MVs incline to be larger in size (100–1000 nm) than exosomes, although the size ranges overlap between the two types of vesicles. Unlike exosomes, which are secreted by the fusion of MVBs with the plasma membrane, MVs arise through direct outward budding and fission of the plasma membrane during cell stress (Lee et al., 2012; Turola et al., 2012; Akers et al., 2013; Forterre et al., 2014; Im et al., 2014; Jeppesen et al., 2014; Sluijter et al., 2014). Various cellular constituents are accumulated and packaged into the MVs in the course of their formation (Turola et al., 2012). MVs are heterogeneous structures (Lee et al., 2012); those that originate from distinct cell types are molecularly different from each other (Turola et al., 2012). MVs have been predominantly classified as products of platelets, red blood cells, and endothelial cells (Sluijter et al., 2014). They are formed as a result of dynamic interaction between phospholipid redistribution and cytoskeletal protein contraction. Vesicle formation is induced by translocation of phospholipids to the outer-membrane leaflet. The budding process is completed through contraction of cytoskeletal structures by actin-myosin interactions (Akers et al., 2013).

MVs are often characterized by exposure of phosphatidylserine (PS) residues on their outer surface (Akers et al., 2013; Perez-Hernandez et al., 2013) and they are also highly enriched for a characteristic subset of proteins. Depending on the cell type, membrane of MVs can be rich in cellular lineage markers, β 1 integrin, matrix metalloproteases and their activators and P-selectin glycoprotein ligand 1 (Jeppesen et al., 2014). Though the primary distinction between exosomes and MVs is the difference in their biogenesis (Akers et al., 2013), their larger size, surface PS, and presence of specific molecules are also used to distinguish them. The mechanisms of the biogenesis of exosome and MVs have recently been reviewed (Colombo et al., 2014).

3. Apoptotic Vesicles

Cells that undergo programmed cell death or apoptosis release Apoptotic Vesicles (AVs) or apoptotic bodies (Akers et al., 2013; Forterre et al., 2014; Jeppesen et al., 2014). These vesicles can be distinguished from exosomes and MVs because they

have larger size (up to 5 μm) and by the presence of intact organelles, histones, and genomic DNA within the vesicles (Akers et al., 2013; Perez-Hernandez et al., 2013; Im et al., 2014). Note that exosomes and MVs are secreted during normal cellular processes, whereas AVs are secreted only during apoptosis. Apoptosis is the major mechanism for immunological silent clearing of damaged cells which is common for normal and cancerous cells (Sluijter et al., 2014). Apoptosis begins with condensation of the nuclear chromatin, followed by membrane blebbing and packaging of cellular contents into AVs. Release of AVs is dependent on Rho-associated kinase I and myosin ATPase activity (Palmisano et al., 2012; van der Pol et al., 2012; Sluijter et al., 2014). The inappropriate clearance of AVs is considered to be the primary cause of developing systemic autoimmune diseases (Im et al., 2014). Thrombospondin (Tsp) and the complement protein C3b have been suggested as possible markers for AVs (Akers et al., 2013).

B. Biological Roles of Extracellular Vesicles

The release of EVs represents a special type of secretion. EVs secretion might serve the purpose of several cell-autonomous functions; e.g., elimination of membrane segments with attached molecules that threaten cellular integrity (e.g., releasing of Membrane Attack Complex that contains parts of the plasma membrane by EVs (Pilzer et al., 2005). Furthermore, accumulating data support the role of the EV-associated removal of signaling and regulatory molecules such as: (i) transferrin receptors (Harding et al., 1983); (ii) G-protein coupled receptors (Guescini et al., 2012); (iii) molecules involved in *Wnt* signaling (Gross et al., 2012); and (iv) miRNAs (Valadi et al., 2007).

EVs are best known for their multiplex roles in intercellular communication (Simons & Raposo, 2009; Thery, 2011; Ludwig & Giebel, 2012; Fang et al., 2013; Schneider & Simons, 2013). Because EVs are present at high local concentrations at specific intimate cell-cell contacts and can travel to distant sites in the body via circulation, EVs mediate horizontal transfer of membrane molecules (lipids, glycolipids, proteins, and glycoproteins) as well as cargo molecules (proteins, nucleic acids [mRNA, ncRNAs, miRNA, and DNA], and metabolites).

The evolutionarily conserved nature of vesicle secretion strongly argues for essential physiological roles of EVs. Indeed, EVs have been implicated in a number of homeostatic processes that include the role of MVs/microparticles in blood clotting (Howard et al., 1988) due to the presence of tissue factor and PS on the surface of EVs (Yu & Rak, 2004). Furthermore, placental (trophoblast-derived) EVs have been shown to contribute to maternal immune tolerance to the semi-allogeneic fetus during pregnancy (Tannetta et al., 2014). EVs have been shown to play a role in angiogenesis (Kim et al., 2002), tissue repair (Anthony & Shiels, 2013), and bone calcification (Anderson et al., 1975). Importantly, dendritic cell-derived EVs are capable of cell-free antigen presentation (Raposo & Stoorvogel, 2013) and neutrophil-derived EVs exhibit bacteriostatic functions (Timar et al., 2013).

EVs regulate immune responses; however, depending on the status of particular immune cells, they might activate adaptive immune responses or suppress inflammation. (El Andaloussi et al., 2013) EVs confer immune suppression by several mechanisms: they can enhance the function of regulatory T

cells, suppress natural killer (NK) cells and CD8⁺ cell activity, and also inhibit monocyte differentiation (El Andaloussi et al., 2013; Raposo & Stoorvogel, 2013). Tumor cells, as well as other cells in the tumor microenvironment also secrete EVs (Raposo & Stoorvogel, 2013). Although tumor-derived extracellular vesicles can suppress tumor-specific immune responses, vesicles also facilitate tumor invasion and metastasis. They also transfer native antigen to antigen-presenting cells (APCs) (van der Pol et al., 2012; Robbins and Morelli, 2014). The presence of functional major histocompatibility complex (MHC) class II molecules in immune cell-derived exosomes highlights their role in antigen presentation (Fang et al., 2013). The ability of EVs, especially those derived from APCs, to regulate the immune response can be enhanced through pharmacological and biological treatments including gene transfer of APCs.

Numerous studies demonstrated the presence of tumor-derived EVs in the circulation that raise the possibility that EVs might serve as minimally invasive tumor biomarkers (D'Souza-Schorey & Clancy, 2012). Importantly, circulating EVs have been suggested to play a role in cancer-associated thrombosis (Unlu & Versteeg, 2014). Furthermore, the role of EVs are recently recognized in cardiovascular diseases (Gaceb et al., 2014). EVs have been also implicated in a broad variety of inflammatory diseases (Buzas et al., 2014). Infectious agent-derived EVs carry pathogen-associated molecular patterns, and host cell-derived EVs may carry damage associated molecular pattern molecules. Moreover, host cell-derived EVs (in particular apoptotic vesicles) have been implicated as complex autoantigens in autoimmune diseases, and EV-associated enzymes were suggested to play an important role in tissue damage associated with inflammation (Buzas et al., 2014).

Taken together, although yet not complete, our understanding of EVs functions strongly suggest very important roles of these previously neglected structures in homeostatic and pathological processes of the body.

C. Molecular Constituents of Extracellular Vesicles

A large number of studies have been focused towards the characterization of the molecular architecture of EVs over the past few years (Simons and Raposo, 2009; Muralidharan-Chari et al., 2010; Raimondo et al., 2011). These complex extracellular organelles contain proteins, lipids, RNA, DNA, and various metabolites. Studies on lipid composition and the presence of metabolites in EVs are underrepresented compared to proteomics. The proteins present in EVs can be divided into two main groups. The first group consists of cytosolic and membrane proteins that are commonly identified by different studies, and can be considered as conserved among exosomes of different origin like cytoskeletal proteins (actins, tubulins, ezrin, moesin, etc.), metabolic enzymes (GAPDH, enolase1, PGK1, PKM2, etc.), ribosomal proteins, transmembrane proteins (PIGR, LAMP1, and CD59), annexins, tetraspanins, heat shock proteins, adhesion proteins (integrins and MFGE8), etc. These proteins can clarify the biogenesis of vesicles. A recent review (Choi et al., 2014) provides a list of the top 100 mammalian EVs proteins reported by most studies. The second group is a set of proteins that are specific to cell type-associated functions (e.g., cell-surface receptors). This second group is of great interest, because identification of these proteins might clarify the role of

EVs under different physiological or pathophysiological conditions and enhance their usefulness as potential biomarkers.

One of the major breakthroughs in the EVs field was the demonstration that exosomes mediate transfer of mRNA and miRNA among cells, and that EV-associated mRNAs could be translated into proteins by target cells (Ratajczak et al., 2006; Valadi et al., 2007). Different analytical techniques, including high-throughput proteomics and transcriptomics, have been employed to identify respectively the protein and RNA contents of EVs derived from various cell types and body fluids (Choi et al., 2013). EVs with features of exosomes released by immune cells selectively incorporate miRNA that can be functionally transferred as a consequence of fusion with recipient cells (Mittelbrunn et al., 2011; Montecalvo et al., 2012). Recently, analysis of RNA from EVs by unbiased deep sequencing approaches demonstrated that, in addition to mRNA and miRNA, EVs also contain a large variety of other small noncoding RNA species, including RNA transcripts, that overlap with protein-coding regions, repeat sequences, structural RNAs, tRNA fragments, vault RNA, Y RNA, and small interfering RNAs (Bellingham et al., 2012; Nolte-'t Hoen et al., 2012). Enzymatic (dsDNA-specific shrimp DNase methods) and physical/structural studies (atomic force microscopy) also provide evidence that tumor-derived exosomes carry double-stranded DNA (dsDNA). Recently, it was shown that exosomal DNA (exoDNA) represents the entire genome and reflects the mutational status of parental tumor cells (Thakur et al., 2014). Analysis of genetic material in EVs is outside the scope of the present review, because these studies are not based on mass spectrometry.

III. QUALITATIVE MASS SPECTROMETRY ANALYSIS TO IDENTIFY EXTRACELLULAR VESICLE MOLECULAR CONSTITUENTS

A. Proteins and Protein Identification

Beside nucleic acids, the best-characterized compounds of EVs are proteins. Proteomics data of EVs and MVs are underrepresented in the literature compared to the number of publications on exosomes. There is a steep increase in the number of publications in the past 10 years when one searches the literature for the keywords “exosome” or “microvesicle” or “platelet” or “apoptotic body” or “extracellular vesicle” and the results searched with the term “mass spectrometry” (Fig. 1). This increase is mainly due to rapid improvements in proteomics methodologies. Current state-of-the-art methods allow routine identification and quantification of several hundred proteins present in various EVs derived from cell culture or body fluids. However, routine characterization was not the case 15 years ago, when the first proteomics dataset of EVs secreted by dendritic cells (Thery et al., 1999) appeared followed by several similar studies on mainly immune cell-derived EVs (Skokos et al., 2001; Thery et al., 2001). These studies used gel electrophoresis prior to MALDI-MS detection and could identify only the most-abundant proteins in vesicle preparations (20 on average).

In order to obtain reliable proteomics data on EVs, the first requirement is the availability of homogeneous and contamination-free vesicle preparations (i.e., free of viruses, aggregates, etc.). Isolation of vesicles can be performed using ultracentrifugation (Welton et al., 2010), immunoaffinity isolation (Clayton

et al., 2001; Tauro et al., 2012), gel filtration (Ogawa et al., 2008), or microfluidics-based (Chen et al., 2010) methods. Recently, Kalra *et al.* (Kalra et al., 2013) compared three different exosome isolation techniques from human blood plasma. These techniques included differential ultracentrifugation, immunoaffinity pull-down, and density gradient separation. Purity of the preparations was assessed with MS, WB, and microscopy. The purest EV preparations were obtained with the density gradient method. Effects of storage conditions were also assessed, and EVs were found to be stable and biologically active in plasma up to 90 days when stored at -20°C . Another study that assessed influence of centrifugation time on EVs RNA and protein yield found that prolonged centrifugation times were beneficial (Cvjetkovic et al., 2014).

The current mainstream proteomics workflow is based on proteolytic (tryptic) digestion of the protein mixture, followed by nano-HPLC combined on-line with tandem mass spectrometry (nanoHPLC-MS/MS). This technique can identify hundreds or even thousands of proteins in a mixture. Nevertheless, it is often beneficial to reduce complexity of EV preparations prior to mass spectrometry analysis. Current proteomics technologies generally employ one of the following sample-preparation steps: (i) SDS-PAGE separation of proteins prior to tryptic digestion (Pisitkun et al., 2004; Mathivanan et al., 2010); (ii) isoelectric focusing-based fractionation (Choi et al., 2012); or (iii) on-line two-dimensional nano-HPLC separation of the tryptic peptides (Gonzalez-Begne et al., 2009; Raj et al., 2012a). In the latter case, generally strong cation-exchange chromatography is the first dimension, followed by reverse phase separation. Other analytical techniques can also be used to study proteins present in EVs including WB, flow cytometry, and immunoelectron microscopy.

A recent review (Raimondo et al., 2011) grouped proteins identified in EVs based on their cellular origin. According to that review, cytoplasmic proteins were most-abundant in cases of EVs isolated from biological fluids (47%) and cell culture media (43%). Membrane proteins were also present in high amounts (28% and 34%), respectively. Nuclear and mitochondrial proteins were present in much lower amounts. Most cytosolic and membrane proteins are conserved among EVs of various origin, and are commonly detected in the different studies. A lower number of proteins have been identified in EVs from cell-culture media than from biological fluids because generally they show a more homogeneous vesicle distribution (Raimondo et al., 2011). EVs from various body fluids have been characterized with MS-based proteomics techniques. Perhaps the most well-defined are urinary EVs (Pisitkun et al., 2004; Hoorn et al., 2005; Zhou et al., 2006; Dimov et al., 2009; Moon et al., 2011a; Wang et al., 2012) which can contain useful biomarkers related to different pathophysiological conditions of the kidney. Detailed reviews on urinary EVs are available (Pocsfalvi et al., 2014; Wang et al., 2014) that summarize results of different findings. Other EV preparations that have been isolated and analyzed from body fluids include cerebrospinal fluid (Chiasserini et al., 2014), saliva (Gonzalez-Begne et al., 2009; Xiao & Wong, 2012), semen (Poliakov et al., 2009), breast milk (Admyre et al., 2007), amniotic fluid (Keller et al., 2007), plasma (Looze et al., 2009), pleural effusion (Bard et al., 2004), and synovial fluid (Skriner et al., 2006) etc. EVs present in different body fluids can reflect pathophysiological alterations and express important biomarkers. EVs isolated from

cerebrospinal fluid (Chiasserini et al., 2014) were enriched in brain-derived proteins and biomarkers of neurodegenerative diseases. Proteins of EVs isolated from human parotid saliva reflected the physiological status of the gland and putative protein markers useful in the diagnosis and treatment of systemic diseases (Gonzalez-Begne et al., 2009). EVs present in seminal fluid (prostatosomes) contained biomarker candidates of prostate cancer (Poliakov et al., 2009). Human breast milk EVs express specific molecules with immune modulatory properties (Admyre et al., 2007).

Many studies have described EVs from conditioned media of cell cultures. Among these studies, a great number of tumor cell-derived EVs have been characterized (van Niel et al., 2001; Hegmans et al., 2004; Choi et al., 2007; Keller et al., 2009; Qu et al., 2009; Mathivanan et al., 2010; Welton et al., 2010). Several proteins have been identified from tumor cell derived EVs that play roles in the progression and development of cancer. HSP90 alpha increases cell motility (McCready et al., 2010), EGFR identified in pancreatic and brain cancer EVs is responsible for proliferation (Al-Nedawi et al., 2008; Graner et al., 2009; Adamczyk et al., 2011) and amphiregulin identified in breast and colorectal cancer EVs for invasion (Higginbotham et al., 2011). Evidence that the oncogenic proteins indeed influence recipient cells have been demonstrated in the case of latent membrane protein 1 (LMP1) (Meckes et al., 2010). This protein activates several signaling pathways.

Proteomic content of MVs and AVs have been analyzed to a less extent. MVs have mainly been characterized from platelets (Garcia et al., 2005; Dean et al., 2009), endothelial cells (Banfi et al., 2005), and tumor cells (Baj-Krzyworzeka et al., 2007; Antonyak et al., 2011). Dean et al. (2009) divided platelet MVs into four size classes with gel filtration chromatography to facilitate identification of proteins and lipids and their functional effects on platelets and endothelial cells. Significant differences were detected in the content of plasma membrane receptors and adhesion molecules, chemokines, growth factors, and protease inhibitors among the different size classes of platelet MVs. Amount of circulating platelet MVs increases in different disease states; therefore, these types of studies can be very useful to discover the roles of these EVs. Joshi *et al.* (Joshi et al., 2014) tested the hypothesis whether MVs released by reactive microglia contribute to degeneration in Alzheimer's disease (AD). These MVs were toxic for cultured neurons and emphasized their involvement in AD degeneration. This discovery might pave new strategies to treat that disease. Comparative shotgun analysis has been performed on AVs isolated from primary cultures of human intrahepatic biliary epithelial cells, human renal proximal tubular epithelial cells, human bronchial epithelial cells, isolated intrahepatic biliary epithelial cells from explanted primary biliary cirrhosis PBC, and control liver (Lleo et al., 2014). The authors discovered unique pathways associated with induction of a signature inflammatory cytokine response against biliary epithelial cells. Proteomic composition of thymocyte-derived MVs and AVs were compared in BALB/c mice (Turiak et al., 2011). The significant overlap detected in the proteins between the two populations suggests complex functions for both in regulating thymus.

It is also possible to analyze posttranslational modifications (PTMs) of proteins present in extracellular vesicles. Despite their importance, PTMs have received little attention so far. Glycosylation is a very important PTM because it plays roles in

several biological processes. Glycosylation analysis of EVs from ovarian cancer cells has been performed recently with lectin blotting, NP-HPLC and MS (Escrevente et al., 2013). The study found that galectin-3-binding protein was enriched in EVs. Furthermore, complex and high mannose glycans were both detected in the vesicle preparations. Apart from MS, lectin microarray technology can also be used to characterize glycosylation patterns of EVs (Batista et al., 2011; Gerlach et al., 2013). Glycome of microvesicles and their parent cell membranes expressed differences in glycan epitopes in cell culture- and body fluid-derived microvesicles (Batista et al., 2011). Surface glycosylation profile of urinary EVs has been characterized with lectin microarrays and flow cytometry (Gerlach et al., 2013). Differences were observed in the binding properties of six lectins when urinary EVs from autosomal dominant polycystic kidney disease and age-matched controls are compared; those results suggested modifications specific to the disease. A shift from high-mannose-type N-glycans to complex-type has been identified with MALDI-TOF/TOF MS in urinary EVs of patients with galactosemia (Staubach et al., 2012). All these data on changes in glycosylation with regards to disease states indicate the importance of their analysis. Phosphorylation is another PTM with high importance in cellular signaling. Fourteen phosphoproteins have been identified in urinary EVs by Gonzales *et al.* (Gonzales et al., 2009). Another study aimed to understand shedding of specific proteins in the vitreous humor of patients affected by age-related macular degeneration (Bia-sutto et al., 2013). Phosphoprotein and protein content of EVs released by ARPE-19 cells upon oxidative stress were analyzed with reverse phase protein arrays. Alterations in some phosphoproteins were observed after oxidative stress. Besides glycosylation and phosphorylation, the presence of other PTMs, like ubiquitination, sumoylation, methylation, oxidation, nitrosylation, palmitoylation, myristoylation, farnesylation, etc. has also been reported in EV proteins, and an up-to-date view of the current state of scientific knowledge in this aspect has recently been published (Moreno-Gonzalo et al., 2014). Next steps, however, are required to address the emerging roles of each of the identified PTMs in vesicle biogenesis and protein sorting, intercellular communication, protein localization, function, stability and pathophysiology.

Over the past decade, the large number of proteomics studies performed on EVs clarifies the diverse roles of EVs. Large scale proteomics datasets and protein-interaction networks can clarify EVs and establish the relationship between the vesicular proteins, increasing thus our knowledge about vesicle biogenesis, pathophysiological roles, etc. Currently, relatively less is known about the PTMs of proteins present in these vesicles. PTMs might greatly contribute to the role of EVs to regulate their environment and can be the focus of future research in the field.

B. Lipid Analysis

Compared to the central role of MS in EVs proteomics, at present MS-based workflows are less commonly used for lipidomic investigations of EVs (Trajkovic et al., 2008; Del Boccio et al., 2012; Vallejo et al., 2012; Llorente et al., 2013). Most publications identify different lipid classes with thin layer chromatography (Johnstone et al., 1987; Vidal et al., 1989; Weerheim et al., 2002; Wubbolts et al., 2003; Subra et al., 2007;

Parolini et al., 2009; Carayon et al., 2011). Some investigators have used techniques such as HPLC (Laulagnier et al., 2004b; Vallejo et al., 2012) or Gas-liquid chromatography (Laulagnier et al., 2004b). LC-MS platforms for lipid characterization have been applied only in two recent articles (Del Boccio et al., 2012; Llorente et al., 2013).

Lipidomics analyses have been performed on EVs that originate from several cell types from different species such as reticulocytes (*Ovis aries*, *Cavia porcellus*, *Rattus norvegicus*) (Johnstone et al., 1987; Vidal et al., 1989; Carayon et al., 2011), human B cells (Wubbolts et al., 2003), dendritic cells (Laulagnier et al., 2004b) melanoma cells (Parolini et al., 2009), and prostate-cancer cells (Llorente et al., 2013); rat mast cells (Laulagnier et al., 2004a), and mouse oligodendroglial precursor cells (Trajkovic et al., 2008). Lipids of EVs present in body fluids plasma (Weerheim et al., 2002) and urine (Del Boccio et al., 2012) have also been investigated. A general observation of these studies is that some lipids are enriched in vesicles when compared to parent cells, whereas the amount of others is decreased. Exosomes typically seem to be enriched in cholesterol, sphingomyelin (SM), and ceramide; meanwhile lower amounts of phosphatidylcholine (PC) have been reported (Wubbolts et al., 2003; Laulagnier et al., 2004b; Trajkovic et al., 2008). Generally, the ratio of different lipids is not consistent in EVs of various origin (Kooijmans et al., 2012). In another study, Brouwers et al. found differences in lipid composition of two distinct populations of prostasomes (Brouwers et al., 2013). The two types differed in the contribution of SM and hexosylceramides. The observed alterations could be associated with differences in their formation and/or function. Saturation levels of fatty acids have also been analyzed in different exosomes and were mainly found to be saturated or monounsaturated (Laulagnier et al., 2004b; Subra et al., 2007; Trajkovic et al., 2008; Brouwers et al., 2013).

One of the aims of lipidomics experiments is to discover the different routes of EVs sorting mostly by a comparison of lipid compositions of parent cells and released EVs. Several sorting mechanisms have been proposed emphasizing the importance of different key compounds such as lysobisphosphatic acid (Matsuo et al., 2004) ceramide (Wubbolts et al., 2003; Trajkovic et al., 2008), and lectins (Barres et al., 2010). Another important aspect of lipidomics experiments is comparison of lipid composition of EVs under different pathophysiological conditions. With an LC-MS platform, differences have been detected between lipid profiles of urinary EVs released by renal cell carcinoma and healthy patients that can be potentially used as biomarkers (Del Boccio et al., 2012). A further feature of EV lipids that has been investigated is their involvement in cell death. The hypothesis of Beloribi *et al.* was that exosome interactions with tumor cells occur at cholesterol-rich membrane micro-domains (Beloribi et al., 2012). First, they determined the lipid composition of human pancreatic tumor cells, and based on this information, they designed Synthetic Exosome-Like Nanoparticles (SELN). Tumor cell survival was decreased by SELN due to the inhibition of the Notch signaling pathway that drove target cells towards mitochondria-dependent apoptosis.

Importance in characteristics of EVs microenvironment has also been addressed. Composition of the lipid bilayer

that surrounds EVs has been found to change according to the pH of the physiological environment. Higher concentrations of SM and GM3 have been found in exosomes if they were released in an acidic environment (Parolini et al., 2009). These two lipids interact with cholesterol, and increase exosome rigidity. Another study found EVs rigidity to increase between pH 5 and 7 (Laulagnier et al., 2004b) to possibly enhance the membrane fusion with target cells at physiological pH. All these data suggest that lipid composition of EVs is well-regulated and can adapt according to environmental factors.

Currently, quantitative lipidomics of EVs is still in its infancy. Quantification of nearly 300 lipid species has been achieved with lipidomic assays in a recent study that analyzed metastatic prostate cancer cell line exosomes (Llorente et al., 2013). So far this dataset provides the most-detailed information about exosomal lipids and provide more insight to understand exosome formation, release, and function. Compared to parent cells, an 8.4-fold enrichment of lipids/mg protein could be observed in exosomes. Furthermore, specific lipid species found selectively enriched in exosomes and could potentially be used as biomarkers.

Lipidomics data are available typically for exosomes, whereas lipids associated with MVs and AVs have been poorly characterized. Changes in surface expression of PS have been analyzed in MVs that originate from erythrocytes following lysophosphatic acid (LPA) treatment (Chung et al., 2007). LPA treatment resulted in the generation of PS-bearing MVs in a calcium-independent manner that are procoagulant and can induce thrombogenic activity, those data emphasize the importance of lipids in this extracellular vesicle population as well. PS also plays an important role in case of AVs (Chaurio et al., 2009). Externalization of PS in apoptotic cells allows their recognition by phagocytes.

To summarize, lipidomics of EVs has not yet reached its full potential, but holds great promise to discover more about EVs biogenesis and function as well as lipid biomarkers useful in clinical diagnostics. Detailed reviews are available on this topic (Subra et al., 2007; Kooijmans et al., 2012).

C. Other Molecular Components of Extracellular Vesicles

Among other molecular components of EVs, various nucleic acids form a functionally very important group. Their analysis is based on genetic techniques, so their discussion is outside the scope of the present review. There is little information available on the metabolic fraction of EVs. Metabolites represent an important class of cargos that, together with proteins, lipids, and nucleic acids, drive the biological function of EVs. Several metabolites were found in microparticles derived from human atherosclerotic plaques (Mayr et al., 2009). In that study, NMR spectroscopy revealed that these particles were enriched in low molecular weight molecules such as taurine, lactate, alanine, acetate, glutamate, succinate, aspartate, while choline, glycerophosphocholine, and creatine were major metabolites in the control samples. Thus far, MS, mainly devoted to the elucidation of EVs proteins and lipids composition, and no study has been reported on the analysis of metabolites. Metabolites will certainly be an objective for future MS-

based investigations due to the excellent specificity and sensitivity of the technique. State-of-the-art mass spectrometers can detect analytes routinely in the femtomolar to attomolar range. In addition, coupling MS with LC or GC enables measurement of hundreds of individual species within a single sample. Finally, the combination of mass accuracy and real-time tandem MS available with many mass spectrometers, along with increasingly comprehensive databases, makes the identification of these metabolites more routine (Veenstra, 2012).

D. Databases

Advances in high-throughput and high-content technologies have generated an exponential increase in molecular data that pertain to EVs and the need of a systematic classification of the data acquired. Currently, there are two integrated manually curated web-based databases publicly available: EVpedia (Kim et al., 2013) and Vesiclepedia (Kalra et al., 2012).

EVpedia 2.0 (<http://evpedia.info>) is an integrated and comprehensive proteome, transcriptome, and lipidome database of EVs derived from archaea, bacteria, and eukarya, including human. Among the 264 dataset, 234 derived from eukaryotes and 30 from prokaryotes. This database is updated every six months. Today, a total of 172,080 vesicular components from 6,879 high throughput studies were collected in EVpedia database. So far, 75,696 proteins, 74,430 mRNAs, 18,119 miRNAs, and 534 lipids identified in eukaryotic and mammalian EVs have been compiled. In addition, each dataset provides details on the isolation method used in the study. Moreover, EVpedia also provides an array of analytic tools, including: (i) Gene Ontology (i.e., Gene Ontology biological process, molecular function, and cellular component) enrichment; (ii) network; and (iii) set analysis (Kim et al., 2013).

Vesiclepedia 2.1 (<http://www.microvesicles.org>) is a manually curated compendium of molecular data (lipid, RNA, and protein) identified in different classes of EVs. It catalogs information from published non-mammalian eukaryotic and mammalian EVs. Currently Vesiclepedia comprises 43,731 proteins, 20,196 mRNA, 2400 miRNA, and 342 lipid entries encompassed from 358 independent studies that were published over the past several years. Vesiclepedia allows one to browse a molecule of interest within a dataset by Organism, Vesicle Type, Content Type, or Sample Material. For each Vesiclepedia dataset, an experimental description and isolation method is provided, together with the vesicle type in which they have been found, the organism, and the PubMed ID associated number (Kalra et al., 2012).

These free web-based databases are valuable resources to stimulate advancement in EVs studies and to elucidate fundamental roles or novel functions of these complex structures. Although due to the limitations in EVs isolation methods (Alvarez et al., 2012; Kalra et al., 2013; Momen-Heravi et al., 2013; Pocsfalvi et al., 2014) cautions is needed to employ current database data. It is, therefore, crucial to establish unified criteria for inclusion of high-quality EVs datasets, and a consensus for the isolation/purification method of different EVs from different sources (i.e., cell line and different body fluids, etc.) and purposes (i.e., biomarker discovery, clinical use and research use, etc.) is reached.

IV. QUANTITATIVE MASS SPECTROMETRY-BASED PROTEOMICS

Rather than providing only protein identification, quantitative proteomic analysis aims to provide information on the absolute or relative quantities of the proteins in the samples. In this section, we review the different mass spectrometry-based quantitative proteomics strategies and their applications to EVs to provide an overview on the current state, advantages, and potential pitfalls in respect to other techniques. Relative quantitation is the method of choice in current EVs-based protein biomarker discovery schemes (Hoorn et al., 2005; Nilsson et al., 2009; Conde-Vancells et al., 2010; Mathivanan et al., 2010; Moon et al., 2011b; Chen et al., 2012; Choi et al., 2012; Raj et al., 2012a,b; Dear et al., 2013; Raimondo et al., 2013; Tissot et al., 2013). Relative quantitation of EV proteins is useful to clarify the effects of a specific stimulus on the: (i) secretion mechanism; (ii) protein content; and (iii) cellular uptake of EVs. For these studies, EVs are typically isolated from cultured cell lines (de Jong et al., 2012; Palmisano et al., 2012; Shai et al., 2012; Alves et al., 2013; Perez-Hernandez et al., 2013; Forterre et al., 2014). The main technical advantages to employ isolated EVs as a biomarker source, compared to the commonly used biofluids (plasma, serum, urine, milk, Cerebrospinal fluid, saliva, etc.) are the reduced level of high-abundance proteins in the sample and their higher stability. In addition, vesicles carry cell-specific information and mediate events associated with disease progression; thus, they are an attractive starting material for biomarker research. Drawbacks of EV-based biomarkers, on the other hand, are the complex workflow, the low vesicle yield, and the difficulties to substantiate the relationship between the measured molecular alterations and pathophysiological processes (Raj et al., 2012b). Table 1 shows various quantitative proteomics studies performed to date on EVs isolated from different cell cultures and biofluids.

Quantitative proteomics can be classified as either gel-based or (MS)-based. The later includes various stable isotopic-labeling strategies as well as label-free approaches.

A. Quantitative Proteomics With Two-Dimensional Polyacrylamide Gel Electrophoresis

Two-dimensional polyacrylamide gel electrophoresis (2-DE) is a well-established technique and due to its high resolving power until recently it was the favored technology for quantitative proteomics. Traditional 2-DE separates proteins, followed by visualization and alignment of individual gel spots in the map with imaging software. In this workflow, proteins are quantified with optical density readouts of the visualized spots and identified with in-gel digestion mass spectrometry. As an example, Shai *et al.* carried out a high-resolution 2-DE-based proteomic analysis of platelet-derived MVs, and showed that activated platelets shed different amount of EVs with quantitatively different protein signature upon activation with thrombin (Shai et al., 2012). However, there are only a few studies in the EVs field that have used 2-DE-based quantitative proteomics (Zubiri et al., 2013) which can be explained by the known limitations of the technique, such as issues on quantitative reproducibility, low dynamic concentration range, difficulties in automation, and difficulties to analyze certain protein classes. Difference gel electrophoresis (DIGE) is an advanced version of

TABLE 1. Quantitative proteomics studies performed on extracellular vesicles of different origin

	Biological Source of EVs	Stimuli/disease	Type of EVs isolated	Method used for protein quantitation	Number of identified proteins	Number of differentially expr. proteins	Ref.
Cell or microorganism culture	Human bladder carcinoma cell lines (T24, FL3 and SLT4)	metastatic (FL3 and SLT4) versus non-metastatic (T24) cells	EVs lumen and membrane fractions	4plex-iTRAQ	1040, 1333 (membrane replicate) 547, 523 (lumen replicate)	58 (FL3 and SLT4 versus T24)	(Jeppesen, et al., 2014)
	Human primary (SW480) and metastatic colorectal cancer cells (SW620)	Primary and metastatic cancer	EVs	Label-free SC	803 (SW480) 787 (SW620)	368 (SW480) 359 (SW620)	(Choi, et al., 2012)
	Human prostate cancer cells (PC3)	expression of polymerase I and transcript release factor in the caveolin-1 positive cell line PC-3	EVs (prostasome)	SILAC	369 (Prostasome) 372 (Secretome)	123 (Prostasome) 125 (Secretome)	(Inder, et al., 2014; Inder, et al., 2012)
	Brain tumor cells (murine) and Sera (human)	Human versus murine brain tumor exosomes	EVs (density gradient)	2D-DIGE	45	36	(Graner, et al., 2009)
	Human microvascular endothelial cells (HMEC-1)	hypoxia, TNF- α -induced activation, high glucose and mannose concentrations	Exosomes (gradient centrifugation)	8plex-iTRAQ	1354	19	(de Jong, et al., 2012)
	Mouse myoblasts (C2C12)	Myogenesis (myoblast transformation to myotubes)	Exosomes	Label-free SC	455	31 (myoblast) 78 (myotubes)	(Forterre, et al., 2014)
	B-cells	Infection with EBV, Kaposi sarcoma-associated (KSHV) or with Human gamma herpes virus	Exosomes (sucrose cushion)	2D-DIGE and Label free SC	871 (B-cells) 360 (viral)	187	(Meckes, et al., 2013)
	β -cells	cytokine induced apoptosis	Microvesicles and Exosomes	SILAC	401 (Microvesicles) 191 (Exosomes)	366 (Microvesicles) 164 (Exosomes)	(Palmisano, et al., 2012)
	Human mesenchymal stem cells	Activin-A stimulus during <i>in vitro</i> osteogenic differentiation of human mesenchymal stem cells	Extracellular matrix and EVs	SILAC	293 (Extracellular matrix) 27 (EVs)	104 (Extracellular matrix) 12 (EVs)	(Alves, et al., 2013)
	Primary lymphoblast cells	WT and CD81-deficient animals	EVs	18O labeling and LC-MS/MS	692	25	(Perez-Hernandez, et al., 2013)
	Lymphocytic cells (H9)	HIV-1 infection	EVs	SILAC	770	14	(Li, et al., 2012)
	Trypanosoma cruzi culture	Secretion mechanism	EVs	Label-free SC	367	149	(Bayer-Santos, et al., 2012)
	Human monocyte-derived macrophages	fungal infections: β -glucan-stimulation	Microvesicles	4plex-iTRAQ	540	145	(Cypriak, et al., 2014)
	Human platelet	Thrombin, Shear	Microvesicles	2-DE-PAGE MS	2307 2271	26	(Shai, et al., 2012)
	Primary hepatocytes and sera (rat)	liver toxins (galactosamine and Escherichia coli-derived lipopolysaccharide)	EVs (differential centrifugation)	Label-free 1D-LC-DIA-MS and 2D-LC-DIA-MS	412 557	88	(Rodríguez-Suárez, et al., 2014)
Biofluids	Milk (bovine)	Staphylococcus aureus infected cows	Exosomes	iTRAQ	300	94	(Reinhardt, et al., 2013)
	Urine (human)	Hernia and bladder cancer	EVs (differential centrifugation)	Isotopic dimethylation labeling	2964	107	(Chen, et al., 2012)
	Urine (human)	Pooled sample compared to single sample, two different age groups	EVs (differential centrifugation and double-cushion)	4plex-iTRAQ	378	21 6	(Raj, et al., 2012a)
	Urine (human)	Diabetic nephropathy	Mixed EVs population	Label-free SC	562	25	(Zubiri, et al., 2014)
	Urine (rat and human)	Acute kidney injury, cisplatin treatment	EVs (differential centrifugation)	2D-DIGE-MALDI-TOF/TOF and LC-ESI-MS/MS	40	27	(Zhou, et al., 2006)

the classical two-dimensional (2D) polyacrylamide gel electrophoresis. In DIGE, samples are labeled with different fluorescent dyes, mixed together, and proteins separated within the same gel to limit quantitation errors. 2D-DIGE analysis was successfully applied to the evaluation of immune regulatory roles of EVs in brain tumors (Graner et al., 2009) and, more

recently, to the alteration of B-cell exosome proteins with gamma herpes virus infection (Meckes et al., 2013). However, the use of 2D-DIGE is also limited in the EVs field, due to complications with validation, like changes in spot positions due to PTM and the presence of multiple proteins per spot (Zhou et al., 2006).

B. Label-Free, MS-Based Quantitation Strategies In Extracellular Vesicles Research

During the last decade, high-resolution (HR) MS has become established as the method of choice for quantitative proteomics. HR-MS is typically performed by acquiring tandem (MS/MS) mass spectra with a mass spectrometer on-line coupled to a nano-HPLC. Improvements in sensitivity, resolution, speed, and statistical analysis have greatly expanded the use of MS for integrated qualitative in quantitative protein analysis (Meissner & Mann, 2014). There are two main approaches to make MS quantitative: the stable isotope-based (Cox & Mann, 2011) and the label-free method (Zhu et al., 2010).

Label-free quantitation relies on spectral counting (SC), or spectral peak intensities (TIC-based approach) (Neilson et al., 2011). The main assumption in label-free approaches is that the ion signal measured in the mass spectrometer linearly correlates with analyte concentration. MS-based label-free strategies applied to EVs are performed in four basic steps: (i) isolation and purification of intact EVs; (ii) vesicle lysis, protein extraction, in-solution digestion; (iii) peptide separation and analysis with LC-MS/MS; and (iv) data analysis, including protein identification, quantification, and statistical analysis (Fig. 4). A considerable advantage of label-free methods over stable isotope labeling is flexibility: each sample is processed separately, so there is no limit in the size of the sample cohort. In addition, it is much simpler, does not require special sample processing, provides higher dynamic range of quantification, and can be applied retroactively to existing data sets.

SC is based on the empirical observation that in the data-dependent acquisition (DDA) mode more-abundant peptides are selected for fragmentation with a higher frequency and produce

MS/MS spectra at higher abundance. SC estimates relative protein abundance based on the number of MS/MS spectra acquired for peptides derived from a given protein, and is performed with multiple HPLC-MS/MS datasets. The quantitative dimension is established through the comparison of a normalized count of peptide identifications for a given protein. Advantages and limitations of SC have recently been reviewed (Neilson et al., 2011). SC has been the most extensively used label-free quantitative method in the EV field (Bayer-Santos et al., 2012; Choi et al., 2012; Forterre et al., 2014; Zubiri et al., 2014). In a study of the role of EVs in metastasis, Choi *et al.* performed an SC-based quantitative analysis for the first time to compare primary and metastatic colorectal cancer cell lines derived exosomes and individuated potentially useful biomarkers for cancer metastasis (Choi et al., 2012). SC was also used to show the coexistence of at least two different mechanisms for vesicle secretion (Bayer-Santos et al., 2012). More recently, SC turned out to be very useful in the study of myogenesis by a comparison of the expression patterns of myoblast and myotube secreted nano-sized vesicles (Forterre et al., 2014).

The other label-free quantification approach is MS ion intensity (or peak area) measurement. Relative quantitation with ion peak intensities might rely on: (i) HPLC-MS only or on; (ii) HPLC-MS/MS data-independent analysis (Neilson et al., 2011; Xie et al., 2011). An example of the LC-MS only approach is the accurate mass and time tag strategy (Pasa-Tolic et al., 2004). This approach uses HR high-mass accuracy HPLC-MS to analyze each sample individually by matching accurate mass and normalized elution time to those stored in a previously established reference database of peptides. Following accurate alignment of detected HPLC-MS features across different analyses, the areas under chromatographic elution profiles of the

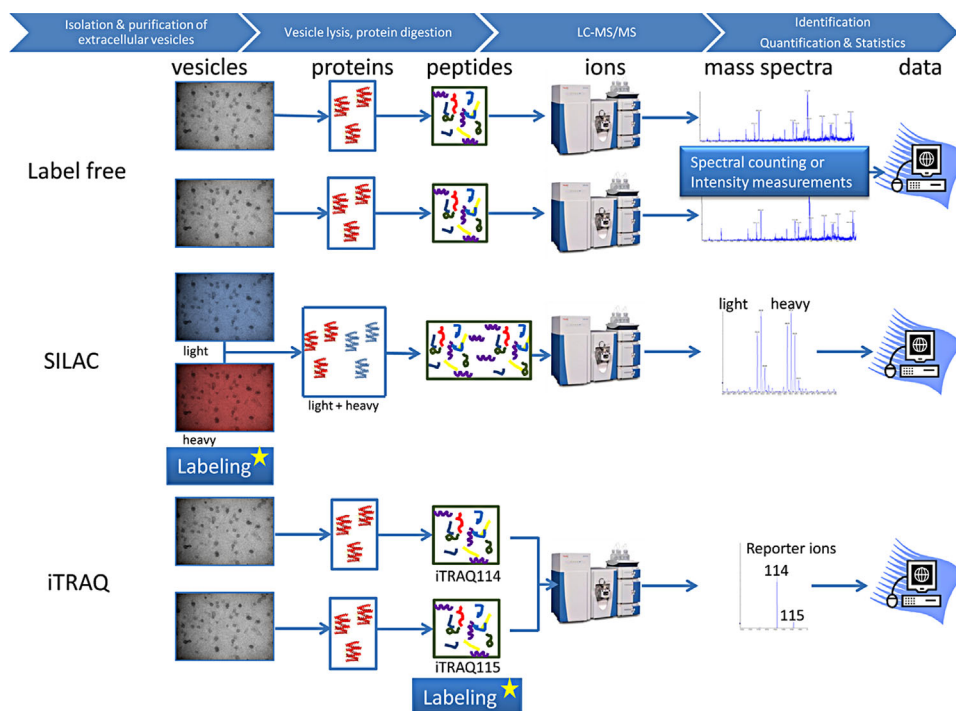


FIGURE 4. Workflows for mass spectrometry-based quantitative proteomics used in extracellular vesicles studies.

identified peptides can be compared among different samples for relative quantification. A different version of the technique is based on intensity quantitation of the precursor ions; based on the principle that the area of the peak in the MS1 ion chromatogram provides a better measure of the relative abundance of the corresponding peptide in the sample, than intensities or the number of scans in MS/MS. Peptides are identified based on their MS/MS spectra, and corresponding MS1 peaks are identified in each HPLC-MS/MS analysis.

One- and two-dimensional HPLC combined with data-independent MS/MS was applied to identify potential biomarkers of liver toxicity by analyzing hepatocyte-secreted EVs after exposure to well-characterized liver toxins (Rodríguez-Suárez et al., 2014). Identified biomarker candidates for acute liver injury (CES3, FRL1, clusterin, CPS1, MAT) were validated in EVs isolated from sera in animal mode. It is important to emphasize that the performance of the mass spectrometer is fundamental to the quality of results obtained with label-free MS-based quantification. A HR mass spectrometer is required to ensure optimum ion extraction at the MS1 level, whereas a sensitive mass spectrometer with high MS/MS sampling rate is necessary for reliable MS2 detection.

C. Stable Isotope-Labeled, MS-Based Quantitation Strategies in Extracellular Vesicles Research

Mass spectrometry is not inherently quantitative. Differences in proton- and ion-affinities and ion suppression effects have been reported to influence the measured abundance of peptides in mass spectra. Deviation from linearity and relatively poor accuracy are the main criticisms of label-free methods. To overcome these problems, samples to be compared should be uniquely labeled, typically with stable isotopes. Various labeling methods have been developed, including chemical, proteolytic, isobaric, and metabolic-labeling techniques. Biological samples to be compared are labeled differently (e.g., one unlabeled, the other contains an ^{18}O label); the labeled samples are combined and the mixture is subjected to MS analysis. MS will measure the peak intensity ratio between heavy and light peptides. Relative change in protein abundance is then calculated with robust statistical analysis. The most popular labeling methods used so far in the field of vesicle research are stable isotope-labeling with amino acids in cell culture (SILAC) (Inder et al., 2012; Li et al., 2012; Palmisano et al., 2012; Alves et al., 2013; Inder et al., 2014), and multiplexed isobaric tagging technology for relative quantitation (iTRAQ) (de Jong et al., 2012; Raj et al., 2012a; Reinhardt et al., 2013; Cypryk et al., 2014; Jeppesen et al., 2014). A simplified workflow for iTRAQ and SILAC labeling and quantitation are shown in Figure 4. Whereas iTRAQ is the favored method for analysis of EVs extracted from biofluids, SILAC is mainly used in cell cultures. iTRAQ is a multiplex isobaric protein labeling, available as 4-plex and 8-plex formats. Four or eight different isotope labels are available for the same compound; therefore, four (or respectively eight) different biological samples might be compared. The labeled peptides from the various samples have the same molecular mass, and therefore, are selected in the same MS/MS analysis, whereas the product ("reporter") ions contain the different labels used for discrimination. Quantification is performed via mea-

surement of reporter ions in the tandem (MS/MS) mass spectra. A multiplex biomarker discovery workflow based on iTRAQ technology and multidimensional protein identification technology (MuDPIT) has been recently proposed for urinary EVs. (Raj et al., 2012a,b) In another study, with iTRAQ methodology, 2971 milk proteins were identified in healthy and *Staphylococcus aureus*-infected cows' milk samples that were fractionated into whey, milk fat globule membrane, and milk exosome sub-proteomes (Reinhardt et al., 2013). In that study ninety-four significantly regulated proteins associated with host-defense proteins were found; these were differentially partitioned in the different fractions. The SILAC-based workflow typically compares protein expression in two (2-plex) or three different (3-plex) experimental conditions, where one set of cells is cultured in the presence of light-labeled lysine and arginine, whereas the other is labeled with heavy lysine and arginine. SILAC was used to compare protein expression pattern of EVs from HIV-1 infected and non-infected lymphocytic cells (Li et al., 2012). HIV-1 infected cells harbor EVs with a unique and quantitatively different protein pattern expressing regulatory molecules of cellular apoptosis and proliferation.

Though there have been relatively few quantitative proteomics studies on EVs (Table 1), the current contribution of MS-based quantitative proteomics to EVs biology is expected to grow in the near future. Qualitative proteomic analysis of EVs will likely to be replaced by quantitative approaches; to compare EVs with cellular secretomes or the biological fluids. Considering the difficulties to isolate EVs with high purity, qualitative studies risk adding background proteins from impurities co-isolated with the vesicle fraction, to thereby compromise the EVs proteome. Exploitation of HR quantitative MS will substantially contribute to the better understanding of biogenesis and biological role of EVs, and also might result in next-generation biomarkers.

D. Antibody-Based Quantitative Assays of Extracellular Vesicles

Besides gel- and MS-based quantitative proteomics, another well-established analytic technique for individual protein quantification is antibody-based assays. Their advantages are high-throughput and high sensitivity; even if detection is limited to a relatively few number proteins. Phenotyping and semi-quantifying circulating vesicles with protein microarrays using antibodies against 21 known vesicular surface antigens has been developed and used in culture supernatants and blood plasma (Jorgensen et al., 2013). Sensitive detection is achieved with biotinylated antibody cocktail against tetraspanins CD9, CD63, and CD81 and fluorescently labeled streptavidin. Advantage of the method is that it does not require vesicle enrichment, but is directly applicable to the samples. High interpersonal variability has been detected by the method in the protein profiles of blood plasma-derived EVs.

More recently, an antibody functionalized array for label-free, high-throughput analysis, called nano-plasmonic exosome (nPLEX) assay, has been shown to be useful to detect cancer-related EVs from biological samples (Im et al., 2014). The assay relies on surface plasmon sensor based on the enhanced light transmission through arrays of nanoholes in gold films, and

is integrated with a multichannel microfluidic cell. Specific binding of tumor exosomes that express CD24 and EpCA in ascites samples from ovarian cancer patients to the nPLEX sensor changes the local refractive index, which can be monitored as wavelength shifts in light spectrum and/or intensity changes at fixed wavelength. Next-generation methods for massive parallel high throughput detection of biomarkers in various other biofluids could be extremely valuable once cancer cell specific biomarker candidates are identified and specific antibodies are available. Whereas antibody-based multiplexed immunoassays are very attractive solutions for EVs analysis, they are not without limitations: the shelf life of antibodies, cross reactivity, limited range of linearity, and limited analytical specificity makes set-up and validation of this assay extremely challenging.

V. CONCLUSIONS

Characterization of molecular composition of EVs, with the exception of nucleic acids, is still in its infancy. The number of publications in this field increased tenfold in the last decade. These vesicles contain a variety of physiologically important molecules; are very small; and difficult to isolate, especially in a well purified form. Analysis, therefore, requires sensitive and selective techniques, and in the opinion of the authors, mass spectrometry seems to be the best choice. Having said that, other techniques, such as gel-electrophoresis and immunoassay, also have their role in certain aspects.

Among EV molecular components, proteins are the most-studied. Up to now, most publications dealt with qualitative proteomics, and listed the various proteins, protein groups found in EVs. Quantitative proteomics of EVs is fast gaining ground, and identification of the role of PTMs might provide breakthrough to better understand the role and mechanism of function of EVs. Preliminary results in this field support the idea that beside nucleic acids, EV proteins might also have a significant role in communication among distant cells. Because EVs carry specific biological information, beside their pathophysiological importance, they might provide us with a better (more specific) source for biomarkers than the conventionally used biological fluids. Besides nucleic acids and proteins, lipids and various small molecules (metabolites) are the main components of EVs. There are very few studies on the latter, but this situation may change in the near future.

The mainstream mass spectrometry platform for EV proteomics is centered around nano-HPLC coupled to HR-MS/MS. For quantitative studies, label free and stable-isotope labeled MS workflows are both used—each with its advantages and disadvantages. Sample preparation is important; possibly even more than in other proteomics research, primarily because isolation and purification of EVs is a major challenge. In addition, evidence is emerging that several proteins co-purify with EVs not only as “contaminants” due to the imperfection of EV isolation methods, but also because they clearly interact with EVs in biofluids.

MS is a prime tool to identify and characterize molecular components of EVs, but it has not yet reached its potential. The authors believe that there will be a fast growth in this field in the next few years. This review is intended to summarize present results and trends, and help orient the reader in this challenging area.

A. PERSPECTIVES

At present, limitations of the use of MS in EV analysis are largely due to imperfections of EV isolation techniques. There is increasing awareness that the currently available EV isolation methods do not enable purification of EVs without co-isolation of different other types of vesicles and molecules (such as lipoproteins). Importantly, it is quantitative MS that holds promise of identification of characteristic EV and EV subtype-specific markers in the future. Once available, these validated markers will enable reliable affinity purification of EVs from biological fluids in various disease conditions. MS will be essential also for the analysis of the affinity captured EVs to identify novel disease biomarkers. Thus, MS has an outstanding potential in the future discovery of novel EV-based prognostic and diagnostic biomarkers.

VI. ABBREVIATIONS

APCs	antigen presenting cells
AVs	apoptotic vesicles
DIGE	difference gel electrophoresis
ESCRT	endosomal sorting complexes required for transport
ELISA	enzyme-linked immunosorbent assay
EVs	extracellular vesicles
GC	gas chromatography
HPLC	high pressure liquid chromatography
HR	high resolution
iTRAQ	isobaric tags for relative and absolute quantitation
LC	liquid chromatography
MHC	major histocompatibility complex
MS	mass spectrometry
MALDI-TOF	matrix assisted laser desorption ionization - time of flight
MVs	microvesicles
MuDPIT	multidimensional protein identification technology
MVBs	multivesicular bodies
nPLEX	nano-plasmonic exosome
PC	phosphatidylcholine
PS	phosphatidylserine
PTMs	posttranslational modifications
SC	spectral counting
SM	sphingomyelin
SILAC	stable isotope labeling by/with amino acids in cell culture
2-DE	two-dimensional gel electrophoresis

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