The Approaches to None-Invasive Detection of Cell-Derived Extracellular Vesicles

Alexander E. Berezin*
Internal Medicine Department, State Medical University of Zaporozhye, 26 Mayakovsky av., Zaporozhye, UA-69035, Ukraine
*Correspondence author

Richard E Mokhnach
Technical Department, Zaporozhye National Technical University 64 Zhukovsky str., Zaporozhye UA-69063, Zaporozhye, Ukraine

Copyright © 2016 Alexander E. Berezin and Richard E Mokhnach. This article is distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Abstract

Cell-derived extracellular vesicles (EVs) are heterogeneous population of phospholipid-based endogenously produced particles. EVs are detected in several biological fluids and tissues as biomarkers of diseases and target of medicines. The conventional approach for measuring the MPs is based on commonly used flow cytometry, fluorescent methods, and nano-particle tracking analysis that recognized as a gold standard, as well as Western blot analysis, dynamic light scattering, resistive pulse sensing, mass spectrometry-based proteomic methods and electron microscopy. However the definition of MPs using these techniques is yet under discussion. The aim of the review: to summarize the knowledge regarding detection and measurement of the EVs and define the balance between advantages and limitations of each contemporary analytical methods of EV assay.

Keywords: extracellular vesicles; flow cytometry, nano-particle tracking analysis; Western blot analysis; dynamic light scattering; resistive pulse sensing; electron microscopy
Introduction

Cell-derived extracellular vesicles (EVs) have been identified in several biological fluids and tissues [1-3]. EVs are recently recognized key regulators of cell function, cell-to-cell cooperation, inflammation, proliferation and tissue repair [4, 5]. Despite the exact molecular mechanisms regarding the autocrine and paracrine actions of EVs affecting several physiological and pathological processes are yet not completely clear [6], there is the progress in understanding the role of circulating EVs and their molecular contents (DNA, RNAs, active molecules, and proteins) taken directly from peripheral blood as biomarker of diseases and targeting in the treatment [7-9]. There is reason for optimizing of EV assay to increase utilization of single and serial measurements of number EV in routine clinical practice. By now, there is large body of evidences regarding perspectives to use of EVs as diagnostic tool with promising predictive value in several diseases, i.e. cancer, leukemia, cardiovascular and rheumatic disease, diabetes, autoimmune and renal diseases, thrombosis, infections, inflammation [10-16]. The aim of the review: to summarize the knowledge regarding detection and measurement of the EVs and define the balance between advantages and limitations of each contemporary analytical methods of EV assay.

Definition and classification of extracellular vesicles

Extracellular vesicles are heterogeneous population of phospholipid-based endogenously produced particles (30-1000 nm in diameter), which contain cell-specific collections of proteins, glycoproteins, lipids, nucleic acids and other molecules. Abundant cells including cardiomyocytes, blood cells, endothelial cells, immune cells, and even tumor cells are capable to secrete EVs of different size and compositions (Table 1). Depending on their origin EVs are graduated to follow subsets, i.e. the exosomes (30–100 nm in diameter), the microvesicles (50–1000 nm in diameter), ectosomes (100–350 nm in diameter), microparticles (100-1000 nm) small-size MPs (<50 nm in diameter) known as membrane particles and apoptotic bodies (1-5 μm in diameter) [17]. The exosomes are formed by inward budding of the endosomal membrane and they are released on the exocytosis of multivesicular bodies (MVBs) known as late endosomes, whereas the microvesicles are attributed via budding from plasma membranes. However, the exosomes have been predominantly labeled in the case of immune cells (macrophages, T cells, B cells and dendritic cells) and tumor cells. Unlike the exosomes, the ectosomes are ubiquitous microvesicles assembled at and released from the plasma membrane [18]. Microparticles (MPs) and microvesicles are released by cellular vesiculation and fission of the membrane of cells [19]. The mechanisms of vesiculation affect genome and may mediate by some triggers including inflammation [20], while in some cases there is a spontaneous release of MPs from stable cells or due to injury from necrotic cells or from mechanically damaged cells.
Biological function and regulation of extracellular vesicles

Recent investigations have been shown that EVs as derivate of cellular membrane are discussed powerful paracrine regulators of target cell functions [21-23]. Indeed, EVs possess a wide spectrum of biological effects on intercellular communication by transferring different molecules (autoantigens, cytokines, mRNA, iRNA, hormones, tissue coagulation factors, and surface receptors) able to modulate other cells affected growth of tissue, reparation, vasculogenesis, inflammation, apoptosis, infection, and malignancy. However, EVs are not only cargo for biological active substances. Growing evidence supports the idea that regarding association between immune pattern of MPs originated from different cells (endothelial cells, mononuclears, dendritic cells, platelets) and nature evolution of various diseases including CV diseases, cancer, sepsis, eclampsia, autoimmune and metabolic states, etc. [24-26].

In is well known that EVs appear to be found into circulation in response to many situational changes (physiological conditions, stress, laminar shear stress on endothelium) micro-environmental stimulation, coagulation / thrombosis, endotoxinemia, activated cells or those undergoing apoptosis, ischemic injury, hypoxia, and malignancy [27-29]. Optionally, it is well known that several haemodynamic conditions via laminar shear stress may stimulate a secretion of EVs from endothelial cells. There are some controversial in understanding of regulation in EVs’ secretion. There are data that confirm a close link between high endothelial shear stress and release of EVs from endothelial cells [30]. In opposite, an inverse association between number of endothelial cell-derived EVs in circulation and shear stress values was found [31]. Authors have suggested that increased release EVs following apoptosis of endothelial cells may be trigger of low laminar stress. Finally, EVs depending on their origin, structure and inducers secretion might possess both physiological (cell-to-cell cooperation, regulation of endogenous reparation, angiogenesis) and pathological effects (promoting oxidative stress, vascular inflammation, coagulation, neovascularization).

The methodology of detection of extracellular vesicles

The current stand of knowledge regarding morphology, transcriptomics, and proteomics of circulating EVs is still not fully [32, 33]. The difficulty of separating EVs realized from other types of cells limits or efforts to extend actual cognitions in features affected biogenesis, secretion, and subsequent biological role of EVs [34]. Up to date, the methods of isolation of EVs are crucial for accuracy of measurement and clinical utility of nano-particles. Indeed, there are several criticisms regarding impact of centrifugation-based methods including co-isolation of non-EV materials on further measurement of EVs. It might relate to damage of the EV's membrane structure and non-standardized parameters leading to qualitative and quantitative variability [35]. The commonly used methods for purifying EVs for post-isolation analyses may impact on quality and accuracy of EV measurement [36].
Many EVs are at the limits of detection of conventional methods, i.e. flow cytometry, and other biophysical methods that enable detection of particles of such small sizes do not readily allow assessment of their origin. Moreover, accurate assessment of EVs remains a serious challenge because of a lack of consensus regarding methodologies to measure EVs and the inability of most techniques to capture the entire size range of these vesicles [37]. Therefore, the EV detection methods used till date are costly and time consuming [38].

The conventional approach for measuring the MPs is based on commonly used flow cytometry, and nano-particle tracking analysis (NTA) that recognized as a gold standard, as well as Western blot analysis, dynamic light scattering, resistive pulse sensing and electron microscopy, mass spectrometry-based proteomic methods etc. However the definition of MPs using these techniques is still an area of great debate [39]. Unfortunately, all methods have crucial limitations regarding complicated assay and suffers from relatively low sensitivity and accuracy because of resolution problems occurring for the majority of commercially available flow cytometers [40, 41]. To date, the accurate measurement of EVs by these methods depends on EV size heterogeneity, refractive index, and the dynamic measurement range that could require a complementary use for most of the available technologies [42].

**Flow cytometry**

Flow cytometry is considered a well-standardized and optionally accepted analytical method for cell identification, phenotype detection and measurement, although the standard tool requires special attention when measuring EVs in diameter less 200 nm and especially less 50 nm [43]. Indeed, due to the small size of EVs, it is needed to prevent the frequently occurred signal noise for detection of fluorescently labelled EVs. Currently there are a number of solutions that might help to improve accuracy and merge reproducibility of the method. The first is careful titration of the probe before EV labelling [36]. The second is removal of unbound probe by washing using size-exclusion filter and / or high-speed centrifugation. To note, the carefully use of higher speed centrifugation is crucial step for detection of EVs even when probes are prepared correctly. The centrifugation may mechanically injure the cells and attenuate the occurring the cell fragments or debris in probe that activates aggregation and mediates artefactual release of EV in the samples [44]. However, there is serious limitation regarding ability to recognized small-size particles like MPs in diameter, i.e. low-density lipoproteins, using flow cytometry technique. The calibration in flow cytometry is essential to overcome the limitations regarding nano-particles’ identification using gating 1µm. Polystyrene microspheres (PMs) are often used in commercial flow cytometers to distinguish EV from cells by setting a 1-µm EV gate in a side-scatter (SSC) versus forward-scatter (FSC) dot plot because of PMs usually exhibit higher FSC and SSC than EVs of equal size. However, the flow cytometer provides the possibility to measure MPs directly in plasma samples and to analyze MP-subsets [45]. The novel approaches that could allow increasing an
ability of flow cytometry technique to detect small-size MPs is advanced cytofluorimetric method based on BD Horizon Violet Proliferation dye. However, the utilization of flow cytometers specifically designed for analysis of small-size MPs is probably to provide considerable methodological advantages and should be the preferable options [46].

**Atomic force microscopy**

Because of atomic force microscopy (AFM) is reliable method for analysis of samples containing very few target molecules; it is permitted the characterization of membrane vesicles as small as 30 nm in hydrodynamic diameter [47]. AFM lets to detect morphology, surface properties and surface antigen presentation in the target samples. Although AFM may exhibits a well agreement with transmission electron microscopy and X-ray diffraction in measurement of both the EV size and size-related parameters of the different EV fractions, it is noted that an accurate of results depends on pre-analytical preparation of samples (i.g. separation and isolation), methods of standardization using particles with appropriate sizes and the counting statistics [48]. However, the distinguishes in concentrations between the detected EVs are discussed a primarily cause in differences between the minimum detectable particle sizes [49]. In this context, the AFM could be promised method in identification of the size and concentration of EV, when dynamic light scattering is failed due to lower EV concentration [50]. Nevertheless, there are no reliable markers that might distinguish subsets of various EVs, i.e. exosomes and ectosomes. In this context, AFM is considered as a component prior nano-particle tracking analysis and global proteogenomics analysis [51].

**Nano-particle tracking analysis**

Nanoparticle tracking analysis (NTA) is a light-scattering technique that is useful for the rapid sizing and enumeration of EVs [52]. NTA is based on the Brownian motion of individual particles in solution (monodisperse and polydisperse samples) with further tracking identification using light scattering. The data analysis requires commercial NTA software. The minimum detectable EV sizes for NTA are 70-90 nm [50]. It is needed to take into consideration the NTA is measured NTA the hydrodynamic diameter of the particles only. It is suggested that NTA is able to have better sensitivity for EVs with diameter less 100 nm [53], whereas fluorescence technique exhibits better results in measurement of EV size ranged > 100 nm [54].

There are as least two limitations of NTA that should be taken into consideration for the analysis of EVs. The first limitation of NTA is lack of optionally calibration method of regarding EV measurements. However, there are several attempts to standardize this method using polydisperse nanosized particles [55]. Although most calibration of NTA measurement has been performed using polystyrene microspheres, silica microspheres may be better in estimation of MV
diameter [56, 57]. The next serious barrier created surmountable problems for NTA is sizing of small MPs (<50-100 nm). In addition, problems with concentration limits of NTA measurements might restrict the use of this method for clinical samples [58].

**Dynamic light scattering (DLS)**
Dynamic light scattering (DLS) recently known as photon correlation spectroscopy (PCS) and quasi-elastic light scattering is well-developed methods regarding measurements of intensity size distribution and on counting the number of different EV sizes less 1 nm [59]. There is a possibility to measure the full particle characteristic distribution including size, weigh, shape, and charge [60]. DLS can use to distinguish nano-particles depending on their size through detection of Brownian motion intensity, while the monogenity of sample and higher concentration of the EVs are critical requirement to perform this method. Contamination of the samples with other particles, i.e. low-density lipoproteins or aggregated microparticles / cell debris, may dramatically worse the data quality [61].

**Resistive pulse sensing**
The label-free technology of resistive pulse sensing uses the qNano system and implements to determine the concentration and size of EVs based on the Coulter principle [62]. Resistive pulse sensing is used a membrane with pores of size with a diameter less than 100 nm [63]. Indeed, the recently performed experiments have been demonstrated that this resistive-pulse sensor could be able to differentiate and count multiple particle solutions simultaneously through its four micro channels fabricated on polymer membranes. Thus, resistive pulse sensing may detect small-size EVs.

**Western blot analysis**
The conventionally methods regarding preparation and isolation of EVs based on higher-speed centrifugation associate with contamination of the samples with lipoproteins, cell debris and protein complexes [64]. Western blot analysis is well-developed methods of the study of target molecule characteristics that allows to optionally recognizing MPs depending on determination of different markers, represents a useful tool for examining particles. However, Western blot analysis requires subsequent technical efforts, needs to complementary methods, i.e. NTA, electron microscopy that optionally appears to be much expensive technology.

**Electron microscopy**
There are at least two types of electron microscopes named transmission electron microscopy and scanning electron microscopy. Transmission electron microscopy is the most commonly used in the real diagnostic practice and has the higher resolution when compared to scanning electron microscopy. Both electron micro-
Cell-derived extracellular vesicles in diseases

Scope

Techniques require preparing biomaterials via fixation and dehydration that may reduce EV size and size-related features of EV morphology. However, the electron microscopy applies to visualize EVs in size ranged from 20 nm to 100 nm. Therefore, complimentary to microscopy immuno-gold labeling attenuates to receive biochemical information regarding EVs’ surface [65].

There is cryo-electron microscopy that is applied at temperatures below −100°C to analyze form and structure of EVs [66]. This method does not require staining and fixing of sample prior to the analysis. Currently, digital technologies allow to create the 2D and 3D models that might improve recognizing of the EV structure. However, the useful of 2D and 3D cryo-electron microscopy in EV identification is required more investigations.

Field emission scanning electron microscopy (FESEM) analysis revealed marked disintegration and vesiculation of the plasma membrane, i.e., pseudopodia formation and cytoskeleton modification. These changes indicate loss of plasma membrane integrity rather than activation. The main advantages of the FESEM are ability to identify the presence of EVs without previously fixation and dehydration that preserve the structure of the particles and minimize the risk to hyperdignose the changes of inner structure of the EVs [67]. In contrast, the high concentration of EVs in the probe is essential to obtain the size distribution [67].

Nano-particles- surface plasmon resonance - based imaging microscopy

Alternatively, recently recognized as a method for quantification and sizing of biological nano-particles- surface plasmon resonance - based imaging microscopy (SPRi microscopy) might be significantly useful to resolve the majority problems affected MPs recognition. SPRi is discussed a highly sensitive label-free biochemical surface sensor measurement technique that has only recently been applied to the field of cell-biology. This method is based on phenomenon known as surface plasmon resonance that associates with a high resolute diffraction generated at a thin metal surface [68-72]. The high contrast in SPR signal between cell edges and substratum facilitates identification of cell edges and segmentation of cell areas [72]. Importantly that several cells, cellular components (i.e., focal adhesions, nucleus, and cellular secretions), viruses, bacteria, micro- and nano-particles have not just became visible, but they are able to be calculable [73, 74].

As expected, a quantitative interpretation of SPRi imaging might improve resolution of MP determination and allow investigators unprecedented to overcome flow cytometry limitations regarding low detectable small-size MPs [73]. Moreover, simultaneous application of a high-sensitive fluorescent microscopy and SPRi microscopy should enhance the sensitivity and selectivity of a created biosensor platform [71-73]. This might have a high value for identification of small-size MPs originated from different cells that were recently determined as debris [72]. Probably small-size MPs derived from apoptotic cells play a pivotal role in tissue injury, inversely MPs secreted activated cells, i.e., mononuclears, endothelial cells, dendritic cells, may have a positive effect on tissue repair and homeostasis [73].
Highly sensitive fluorescent microscopy

A highly sensitive fluorescent (HSF) microscopy also permits to detect individual sub-micro and nano-EVs. As compared with SPRi microscopy, this technique could provide higher detection sensitivity due to a large fluorescence excitation and a high effective quantum yield of fluorescence [75]. Indeed, analysis of highly concentrated EV samples resulted in an underestimation of the number of EVs and an interdependent overestimation of light scattering and fluorescence signals [76]. In this setting, highly sensitive fluorescent approach to capture and detect smaller EVs appears to be promising.

Novel methods of EV detection and measurement

Therefore, there are some methods that are not commercially available: surface-assisted laser desorption/ionization mass spectrometry, Raman micro-spectroscopy, micro nuclear magnetic resonance technique, small-angle X-ray scattering, and anomalous small-angle X-ray scattering [77].

Surface-assisted laser desorption/ionization mass spectrometry

Surface-assisted laser desorption/ionization mass spectrometry (SALDI-MS) is a high throughput analytical technique capable of detecting low molecular weight analysis, including EVs [78]. The main advantage of this novel approach is possibility for the analysis of EVs isolated from multiple biological fluids without sample preparation protocols. In this setting the effect of EV size, pore diameter, pore depth and “functionalization” on analytical performance might be studied. In fact, the method detection limits are 10-30 nm that opens novel perspectives in investigations of small-size MPs. However the role of SALDI-MS in biosensing of MPs is not fully investigated and requires scrutiny.

Raman micro-spectroscopy

Raman micro-spectroscopy is a spectroscopic method, based on inelastic scattering of monochromatic light using directly labelled fluorescent probes or of indirect labelling with mono- and polyclonal antibodies [79, 80]. The principle of the method based on interaction of photons with molecular vibrations that leads to shift of their energy. This signal strength presents important information about the vibrational transitions proportional to composition of the target molecules [80]. The main advantage of vibrational laser-based Raman spectroscopy in comparison to conventional biological assays is an ability rapid and non-invasive biochemical analysis of EVs beyond fixation or labeling [81]. Importantly, Raman micro-spectroscopy might complement NTA, transmission and scanning electron microscopies, DLS to improve data regarding size and morphology of EVs.

Micro-nuclear magnetic resonance technique

Highly sensitive detection of MP antigens by micro-nuclear magnetic resonance are currently introduced onto a microfluidic chip and labelled with target-specific magnetic nanoparticles [82]. Compared with current methods, this integrated
system has a much higher detection sensitivity and can differentiate MPs derived from tumor cells from non-tumor host cell-derived MPs [83].

Small-angle X-ray scattering
Small-angle X-ray scattering (SAXS) is a promising method that has implemented for determination of solid particles in suspension through traceable size detection [64]. The size (1–200 nm size range) and size-related features of EVs are capable to recognize by SAXS and are presented as traceable size distributions from the on-line measurements [84]. The method is based on the elastic scattering of X-ray photons on the electrons of the sample at low angles. As other methods based on analyzing of traceable size distributions, the highly concentrated EV fractions are needed to perform the measurement carefully. In this concerning, the monodispersity of the sample is essential to receive higher reliable results of the EV measurement. The heterogeneous sample meets several obstacles for interpretation of the scattering curve. In this context, the centrifugation as a method of preparing and isolation of EVs is not complementary to SAXS technique. The next main disadvantage of SAXS is the presence of plasma proteins in the sample analyzed that may not associate with the EVs.

Future perspectives

There are commercial platforms that offer massively parallel, label-free biosensing of EVs based on combining all-electrical detection with low-cost integrated circuits [85]. Despite these successes, bioelectronics has so far failed to deliver a broadly applicable EV biosensing platform in routine practice. All these methods are currently being explored to assay MPs, while an incorporation of these techniques into analytical care is probably addressed in the future. However, isolation, purification and content analysis of EVs remain to be technological challenged.

In conclusion, a standardization of the methods of EVs’ determination, isolation and characterization are extremely required, because are yet largely lacking. Commonly used procedures, such as flow cytometry with NTA, Western blot analysis, SALDI-MS, electron microscopy, and other methods might not have universal utility for EV determination; especially for small-size MPs. Novel techniques regarding identification of EVs based on real-time and label-free optical biosensors and principles of SPR phenomena appear to be promised and could sufficiently overcome limitation of option methods of EV determination.

Abbreviations
EVs - extracellular vesicles;
FSC - forward-scatter dot plot
HSF - highly sensitive fluorescent microscopy
ICAM - intracellular adhesion molecule;
MPs - microparticles;
MV - microvesicles;  
NTA - nano-particle tracking analysis;  
PMs - polystyrene microspheres;  
RBC - red blood cells;  
SALDI-MS - surface-assisted laser desorption/ionization mass spectrometry;  
SAXS - small-angle X-ray scattering;  
SPRi microscopy - nano-particles- surface plasmon resonance - based imaging microscopy;  
SSC - side-scatter dot plot;  
VCAM - vascular cell adhesion molecule.

**Funding.** This research received no specific grant from any funding agency in the public, commercial, or not-for-profit sectors.

**References**


Cell-derived extracellular vesicles in diseases


[83] A. E. Berezin, The development of biological molecular sensing techniques to detect microparticles: focus on clinical medicine benefits, *Journal of*
http://dx.doi.org/10.4172/1948-5948.1000214

http://dx.doi.org/10.1088/1742-6596/247/1/012027


Received: May 15, 2016; Published: June 7, 2016
Table 1: Classification and key features of extracellular vesicles

<table>
<thead>
<tr>
<th>Populations of vesicles</th>
<th>Diameter</th>
<th>Origin</th>
<th>Main contained components</th>
<th>Best characterized cellular sources</th>
<th>Markers</th>
<th>Detection</th>
</tr>
</thead>
<tbody>
<tr>
<td>EV</td>
<td>30-1000 nm</td>
<td>cell membranes</td>
<td>regulatory proteins (i.e., heat-shock proteins, tetraspanin), lipids, active molecules, nucleic acids (mRNA, miRNA), cytokines, growth factors, hormones, VCAM, ICAM, procoagulant phosphatidylserine, likely complement</td>
<td>All cell types</td>
<td>Annexin V binding, tissue factor and cell-specific markers</td>
<td>Flow cytometry, western blotting, mass spectrometry, electron microscopic technique, SPRi microscopy, NTA</td>
</tr>
<tr>
<td>MPs</td>
<td>100 -1000 nm</td>
<td>plasma membranes</td>
<td></td>
<td>Platelets, RBC and endothelial cells</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MV</td>
<td>50–1000 nm</td>
<td>plasma membranes</td>
<td></td>
<td>Platelets, RBC and endothelial cells</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Small-size MPs</td>
<td>&lt;50 nm</td>
<td>plasma membranes</td>
<td></td>
<td>Endothelial cells</td>
<td>CD133+, CD63-</td>
<td></td>
</tr>
<tr>
<td>Exosomes</td>
<td>30–100 nm</td>
<td>endosomal membranes</td>
<td></td>
<td>Immune cells and tumors</td>
<td>CD63, CD61, CD63, CD81, CD9, LAMP1, TSG101</td>
<td></td>
</tr>
<tr>
<td>Ectosomes</td>
<td>100–350 nm</td>
<td>plasma membranes</td>
<td></td>
<td>Platelets, RBC, activated neutrophils, and endothelial cells</td>
<td>TyA, C1q</td>
<td></td>
</tr>
</tbody>
</table>
Table 1: (Continued): Classification and key features of extracellular vesicles

<table>
<thead>
<tr>
<th>Classification</th>
<th>Size Range</th>
<th>Membrane Type</th>
<th>Key Features</th>
<th>Source Cells/Tissues</th>
<th>Detection Method</th>
</tr>
</thead>
<tbody>
<tr>
<td>Late endosomes</td>
<td>50–1000 nm</td>
<td>Endosomal membranes</td>
<td>Close-packed lumenal vesicles</td>
<td>Immune cells, dendritic cells and tumors</td>
<td>Annexin V binding, DNA content</td>
</tr>
<tr>
<td>Apoptotic bodies</td>
<td>0.5–3.0 µm</td>
<td>Plasma membranes</td>
<td>Pro-apoptotic molecules, oncogenic receptors</td>
<td>Cell lines</td>
<td>Flow cytometry</td>
</tr>
</tbody>
</table>

Abbreviations: EVs, extracellular vesicles; MPs, microparticles; MV, microvesicles; RBC, red blood cells; ICAM, intracellular adhesion molecule; VCAM, vascular cell adhesion molecule; SPRi microscopy, nano-particles- surface plasmon resonance - based imaging microscopy; NTA, nanoparticle tracking analysis.