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The Promises, Methodological Discrepancies and Pitfalls in Measurement of Cell-Derived Extracellular Vesicles in Diseases

Alexander E Berezin[1]* and Richard E Mokhnach[2]

[1]*Internal Medicine Department, Medical University, Zaporozhye, Ukraine*
[2]*Technical Department, National Technical University, Zaporozhye, Ukraine*

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

Abbreviations: AFM: Atomic Force Microscopy; DLS: Dynamic Light Scattering; EVs: Extracellular Vesicles; FESEM: Field Emission Scanning Electron Microscopy; FSC: Forward-Scatter Dot Plot; FTIR: Fourier Transform Infrared Spectroscopy; HSF: Highly Sensitive Fluorescent Microscopy; ICAM: Intracellular Adhesion Molecule; miRNA: Micro RNA; MPs: Microparticles; MVs: Microvesicles; NTA: Nanoparticle Tracking Analysis; PCS: Photon Correlation Spectroscopy; PMs: Polystyrene Microspheres; RBC: Red Blood Cells; SALDI-MS: Surface-Assisted Laser Desorption/Ionization Mass Spectrometry; SAXS: Small-Angle X-Ray Scattering; SEM: Scanning Electron Microscope; SPRi microscopy: Nano Particles Surface Plasmon Resonance based Imaging Microscopy; SSC: Side-Scatter Dot Plot; VCAM: Vascular Cell Adhesion Molecule

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 defined as heterogeneous population of particles with variable sizes ranging from 30 to 1000 nm in diameter, which are produced by broad spectrum of cells (Table 1). By now, EVs are classified to several subsets depending origin, sizes, and specifically presented on their surfaces biochemical marker. There are follow EV subsets: exosomes (30–100 nm in diameter), the microvesicles (50–1000 nm in diameter), ectosomes (100–350 nm in diameter), and microparticles (100-1000 nm). So called the "small-size MPs" (<50 nm in diameter) and various apoptotic bodies (1-5 μm in diameter) are graduated by some investigators additionally to the main groups of EVs [17]. The exosomes are derivate of the endosomal membrane of predominantly immune and tumor cells, whereas the ectosomes are released from the plasma membrane of broad spectrum of the cells including antigen-presenting cells [18]. Microparticles (MPs) and microvesicles are resulting in cellular membrane vesiculation due to an impact of several triggers (i.e., shear stress, inflammation, cell activation through growth factor and hormones, direct mechanical injury, coagulation on the surface of endothelium) affecting rebuilding of cell skeleton [19,20].

Biological Function and Regulation of Extracellular Vesicles

Recently some investigations have deemed that EVs are transport form for different molecules (tissue coagulation factors, autoantigens, cytokines, mRNA, miRNA, hormones, and surface receptors), which could be paracrine regulators of target cell metabolism and function [21-23]. The opinion was maintained a large body of evidence regarding the role of biological molecules incorporated into EVs in the various processes, such as inflammation, infections, growth and differentiation of tissue, reparation, vasculogenesis, and malignancy. Within last

***Corresponding author:** Alexander E. Berezin, Internal Medicine Department, State Medical University of Zaporozhye, 26, Mayakovsky av., Zaporozhye, UA-69035, Ukraine, Tel: +380612894585; E-mail: dr_berezin@mail.ru; aeberezin@gmail.com

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Populations of vesicles	Diameter	Origin	Main contained components	Best characterized cellular sources	Markers	Detection
EV	30-1000 nm	Cell membranes	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	All cell types	Annexin V binding, tissue factor and cell-specific markers	Flow cytometry western blotting, mass spectrometry, electron microscopic technique, SPRi microscopy, NTA
MPs	100-1000 nm	Plasma membranes		Platelets, RBC and endothelial cells		
MV	50–1000 nm	Plasma membranes		Platelets, RBC and endothelial cells		
Small-size MPs	<50 nm	Plasma membranes		Endothelial cells	CD133+, CD63-	
Exosomes	30–100 nm	Endosomal membranes		Immune cells and tumors	CD63, CD61, CD63, CD81, CD9, LAMP1, TSG101	
Ectosomes	100–350 nm	Plasma membranes		Platelets, RBC, activated neutrophils, and endothelial cells	TyA, C1q	
Late endosomes	50–1000 nm	Endosomal membranes	Close-packed lumenal vesicles	Immune cells, dendritic cells and tumors	Annexin V binding, DNA content	
Apoptotic bodies	0.5-3.0 μm	Plasma membranes	Pro-apoptotic molecules, oncogenic receptors	Cell lines		Flow cytometry

Table 1: Classification and key features of extracellular vesicles.

decade it has been became to know that the EVs are not only cargo form for biological molecules, but they might produce direct impact on target cells through presented on their surface mother cell-specific receptors and active substances. Additionally, the changing in number and worsening in immune pattern of MPs originated from different cells (endothelial cells, mononuclears, dendritic cells, platelets) have been found in several settings including CV and metabolic diseases [24-26].

Some controversies in recognizing of molecular mechanisms regarding regulation of EVs' forming and secretion were recently found. There are at least two distinguished mechanisms of vesiculation, i.e., spontaneous and trigger-induced. Up to date, the mentioned above mechanisms of EV release are mandatory of physiological and pathological conditions. Whether both mechanisms are similar in cellular changing aspects is not fully clear, although shear stress on endothelium, coagulation/platelet aggregation on the surface of the endothelial cells, microbial toxins-related endothelium injury, and activated/apoptotic cells may stimulate EV forming and secretion [27-31]. However, the EVs originated from activated and apoptotic cells may distinguish in their structure, antigen and tissue factor presentation, ability to transfer of biological substance including miRNAs, and consequently they might trigger variable biological responses. Indeed, EVs produced by activated cells may involve in the reparation of the tissue, angiogenesis, and cell-to-cell cooperation, whereas EVs secreted by apoptotic cells are able to mediate direct tissue injury via promoting oxidative stress, inflammation, platelet aggregation and thrombus formation [30,31].

The Methodology of Detection of Extracellular Vesicles

The most published data regarding immune biology, structure, and proteomics of free-cells EVs have been presented conflicting results [32,33]. Basically the analytical obstacles and methodological limitations to recognize and distinguish several types of EVs are the main source of unsatisfactory knowledge about 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].

In routine laboratory practice fluorescent methods (i.e. flow cytometry) for EV detection and distinguish are predominantly used [37]. However, small sizes, low concentration and lack of consensus regarding standardization remain the main challenging to measure EVs in samples [37]. Therefore, the other methods of EV detection (i.e. nano-particle tracking analysis, Western blot analysis, dynamic light scattering, resistive pulse sensing and electron microscopy, mass spectrometry-based proteomic methods, etc.) are costly, require more time for performing [38,39], and exhibit several technical limitations regarding their sensitivity and accuracy [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 labeled 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 labeling [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]. Probably, advanced cytofluorimetric method based on BD Horizon Violet Proliferation dye could be used optionally to detect small-sizes MPs [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. Furthermore, AFM might use to determine the morphology structure of the membranes and subsurface layers more carefully than it could be characterized by scanning electron microscope (SEM) and Fourier Transform Infrared (FTIR) spectroscopy. It might have an important value, because of changes in cellular mechanical properties closely correlate with the functionality of the cells and their response to the several stimuli. Moreover, membrane-bound proteins are involved in the cell-to-cell cooperation in vivo and directly mediate passive bead rheology and mechanical ability of the cells and sub-cell structures. Thus, AFM may present information with respect to both nanostructure of the cells/cell-derived EVs and their functionality.

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]. Overall, AFM appears to be non-destructive and quantitative way to characterize the structure of atomically thin, layered materials, essential properties of EV membrane, i.e., EV shape and size, which allows to compare the features with control. Additionally, AFM is capable to use of screening for changes in mechanical phenotype of EVs.

Nano-particle Tracking Analysis

Nanoparticle tracking analysis (NTA) is a non-invasive light-scattering technique that is useful for the rapid sizing and enumeration of EVs in real time [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, which calculates the size and total concentration of the vesicles in solution. The minimum detectable EV sizes for NTA are 70-90 nm [50] and the analytical variation is generally below 10%. It is needed to take into consideration the NTA is measured NTA the hydrodynamic diameter of the particles only. Overall, the use of fluorescent-labeled antibodies against specific markers with NTA allows the determination of the "phenotype" of the cell-derived vesicles.

There are a lot of numbers of investigations regarding comparison between NTA and other methods enable measurement of EVs in blood plasma. Mork et al. [53] reported that NTA and tunable resistive pulse sensing (TRPS) enabled acceptably precise concentration and size measurement of submicron particles in fresh, fasting samples of plasma. It is suggested that NTA is able to have better sensitivity for EVs with diameter less 100 nm [54], whereas fluorescence technique exhibits better results in measurement of EV size ranged >100 nm [55].

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 [56]. Although most calibration of NTA measurement has been performed using polystyrene microspheres, silica microspheres may be better in estimation of MV diameter [57,58]. 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 [59]. In this context, EV-enriched fractions in the sample and high concentrations of particles in the size-range of exosomes are essential for NTA. To the best of our knowledge, isolation of EVs is necessary to use before NTA that is considered a crucial step in this analytical technique. However, the complete isolation of EVs from similarly sized particles with full EV recovery is currently not possible due to limitations in existing isolation techniques. Finally, NTA is defined as easy to use, fast, robust, accurate and cost effective methods to measurement of EVs.

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 les 1 nm [60]. There is a possibility to measure the full particle characteristic distribution including size, weigh, shape, and charge [61]. 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. Optionally, the results of EV measurement by DLS comprise either a simple z-average particle size or polydispersity or a very limited-resolution particle size distribution profile. Contamination of the samples with other particles, i.e., low-density lipoproteins or aggregated microparticles/cell debris, may dramatically worse the data quality [62]. Additionally, DLS cannot measure fluorescently-labeled EVs.

Resistive Pulse Sensing

The technology of resistive pulse sensing uses the qNano system and implements to determine the concentration and size of EVs based on the Coulter principle [63]. Resistive pulse sensing (RPS) is used a membrane with pores of size with a diameter less than 100 nm [64]. Thus, resistive pulse sensing may detect small-size EVs. It appears to be promising, because of widely used techniques are very sensitive to concentration and sizes of detecting particles. In this context, RPS could be allowed to measure EVs below detection limits that are suitable for fluorescent methods. However, the sensitivity and specificity of RPS in detection of EVs in samples receive from humans require more investigation and comparison to other methods.

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 [65]. 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. Methodologically, Western blot analysis consists of five steps including electrophoretic separation of the proteins; transfer to a nitrocellulose or polyvinylidene difluoride membrane; labeling using a primary antibody specific to the protein of interest; incubation with a secondary antibody directed against the primary antibody; and visualization. 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. Indeed, nuclei and cell debris should be removed by centrifugation prior Western blot analysis to avoid to false positive results. The leading advantages of the methods are determination of the presence or absence of the proteins of interest, and also detection of the level of expression of a selected protein. To directly measure the expression of the proteins in the sample the quantitative fluorescent western blotting analysis might use. The method is also validated for measurement of component of EVs, such as miRNAs, tissue factors, and several membrane antigens. However, Western blot analysis could be a part of consequently performing combined EVs' analysis based on fluorescent technique (flow cytometry, NTA) and electron microscopy.

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 microscope 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 [66].

There is cryo-electron microscopy that is applied at temperatures below −100°C to analyze form and structure of EVs [67]. This method does not require staining and fixing of sample prior to the analyzation. 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 [68]. In contrast, the high concentration of EVs in the probe is essential to obtain the size distribution [68]. Thus, electron microscopy is a useful research tool for studying EVs, but at the expense of capital running costs, extensive sample preparation, slow throughput time and sample integrity following sample preparation.

Nano Particles Surface Plasmon Resonance Based Imaging Microscopy

Nano-particles- surface plasmon resonance - based imaging microscopy (SPRi microscopy) has currently found an alternate free-labeled optical method for quantified measured of sizes and size-related characteristics of sub-micron and nano-particles [69-73]. The essential principle of the SPRi microscopy is based on recently known phenomenon so called "surface plasmon resonance", which is defined as interaction of polarized light with thin film of metal [69-73]. The essential advantages of SPR are free label real time detection, higher sensitivity and reproducibility, simple method of detection even small-sized particles and low cost [73,74]. There are some attempts to combine SPR with high-sensitive fluorescent microscopy to merge sensitivity and selectivity of final detection of EVs [72-75]. However, the routine use of SPR technology in small-sized EV biosensing requires standardization and more investigations in field of quality of measurements.

Highly Sensitive Fluorescent Microscopy

A highly sensitive fluorescent (HSF) microscopy is based on objective-type internal reflection regarding wavelength-modulation and it may sufficiently improve nano-particle scattering. Unless SPRi microscopy and other fluorescent techniques, light dose is a limiting factor for the method that is considered a serious limitation for data interpretation [76,77]. At the same time, fluorescence performance of HSF may allow to visualize wide spectrum of sub-micro and nano-particles with higher accuracy and measurement limit of 40 nm. In this setting, highly sensitive fluorescent approach to capture and detect smaller EVs appears to be promised.

Novel Methods of EV Detection and Measurement

Not all currently available analytic methods of EV detection exhibit commercial affordability in routine laboratory practice due to its sophisticated methodology and respectively higher cost. In this context, there are several techniques, i.e., surface-assisted laser desorption/ionization mass spectrometry, Raman micro-spectroscopy, micro nuclear magnetic resonance technique, small-angle X-ray scattering, that could be considered a promising methods to evaluate widely ranged size particles irrespective their concentration in the samples [78].

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 [79]. The essential principle of EV detection in SALDI-MS is similar mass spectrometry. However, unless traditionally mass spectrometry, in the SALDI-MS the organic matrix is used to prevent the interference with matrix molecules after laser pulses and thereby the combination of soft and hard ionized substrate compounds creates a large surface area for nano-particle detection with limit of 10-30 nm. Importantly, there is no necessary to isolate EVs from fluids to further use SALDI-MS technique. Moreover, size and size-related features of EVs might investigate also. Currently available direct measurement of nano-particles on real time by SALDI-MS appears to be promised for determination EVs.

Raman micro-spectroscopy

Raman micro-spectroscopy is a spectroscopic method, based on inelastic scattering of monochromatic light using directly labeled fluorescent probes or of indirect labeling with mono- and polyclonal

antibodies [80,81]. 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 [81]. 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 [82]. 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 labeled with target-specific magnetic nanoparticles [83]. 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 [84].

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 [65]. 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 [85]. 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

Although several commercial platforms offer various possibilities to perform multiple label-free detection of EVs with aim to minimize the expenditures per single sample analysis [86], the analytical limitations that are suitable for conventional assay used in these combined techniques remain to be challenged. In the future, the novel methods regarding improvement of isolation, purification and detection of EVs are required to sufficiently low the cost of the analysis and increase the affordability of the technologies for routine laboratory practice.

Conclusion

A standardization of the methods of EVs' determination, isolation and characterization are extremely required, because are yet largely lacking. Conventional flow cytometry is the most prevalent technique, whereas NTA, DLS, and resistive pulse sensing have also been used to detect EVs. The accurate measurement of EVs is challenged by size heterogeneity, low refractive index, and the lack of dynamic measurement range for most of the available technologies. Consequently, combined methods, i.e., flow cytometry combining with NTA, Western blot analysis, and electron microscopy, remain to be optionally used methods regarding EV detection, whereas conveying to measure small-size EVs on real-time require principally novel techniques based on advanced technologies, i.e., SPR or SAXS.

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