THE APPLICATION OF NOVEL COUPLING METHOD FOR DETECTION OF MICRO VESICLES: THE ROLE OF THE ADVANCED BRIGHT-FIELD LIGHT OPTICAL POLARIZATION MICROSCOPY

Introduction

Micro vesicles (MVs) are determined as small membrane particles the diameter of which ranged from 50 to 1000 nm [1]. They are secreted by various cells and play a pivotal role in cell-to-cell communications, cargo of active molecules, immune reaction, inflammation, proliferation, growth and malignancy [2]. Additionally, MVs may coordinate biological activity of some cell components incorporated in the endogenous repair systems. Therefore, number and / or immune patterns of MPs predicted a risk of manifestation of different diseases including cardiovascular (CV) and metabolic diseases, autoimmune diseases, malignancy, infections and eclampsia [3]. In this context, measure of MVs in circulation is discussed fairly promising to personify risk of CV disease and premature death rate.

The aim of the study was to investigate the role of wide range of the methods of MV determination based on various analytical principles and the best fitted to the routine laboratory practice.

Determination of micro vesicles

MVs belong to heterogeneous family extra vesicles (EVs) that originate from plasma membranes having diameter 1000 nm and less (Table 1). In family of EVs are included the exosomes (30–100 nm in diameter), MVs (50–1000 nm in diameter), ectosomes (100–350 nm in diameter), small-size micro particles (<50 nm in diameter) known as membrane particles and apoptotic bodies (1–5 μm in diameter). MVs and ectosomes have originated by direct budding from the plasma membrane, otherwise the exosomes are formed by inward budding of the endosomal membrane and then they are released on the exocytosis of multiple vesicular bodies known as late endosomes. However, the exosomes have been predominantly labeled in the case of immune cells and tumor cells. Unlike the exosomes, the ectosomes are ubiquitous MVs assembled at and released from the plasma membrane [3].

The modern methods of MV determination

There are several methods regarding purification of MV samples, determination of MVs, and identification of origin and measure their concentration [4]. Nowadays, there are several methods that currently used for the detection of number (flow cytometry technique, optical microscopy, nanoparticle tracking analysis [NTA], dynamic light scattering) and characterization of structure and features (electronic and atomic force microscopy, fluorescent microscopy, surface plasmon resonance [SPR] technique) of MVs.

Table 1 – Classification and key features of extracellular vesicles

Population of vesicles	Diameter, nm	Origin	Main contained components	Best characterized cellular sources	Markers
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, procoagulant phosphatidylserine, likely complement	All cell types	Annexin V binding, tissue factor and cell-specific markers
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 and 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 and tumors	Annexin V binding, DNA content
Apoptotic bodies	0.5–3.0 μm	plasma membranes	Pro-apoptotic molecules, oncogenic receptors	Cell lines	

Abbreviations: EVs, extracellular vesicles; MPs, microparticles; MV, microvesicles; RBC, red blood cells.

Flow cytometry technique

Flow cytometry technique with polystyrene beads is gold standard to determine the MP sizes that has now standardized by the Scientific Standardization Committee collaborative workshop of the International Society of Thrombosis and Hemostasis [5]. However, this method of size assessment based on SSC has a low resolution of MPs that is roughly estimated to be between 60 and 200 nm, dependent on the vesicle size. The liposome-based size calibration with fluorescently labeled liposomes could be improved MP size assessment with flow cytometry, because there is a single event signal at sufficiently high concentrations irrespective of the applied gating. Unfortunately, determination of MP size 50 nm and less remains to be serious limitation for this method. Indeed, small-size MPs have a low refractive index and are heterogeneous in their size and composition that requires advanced methods for detection. Therefore, some organelles and macromolecules (i.e., DNAs) that release from necrotic cells may bind to MPs and lead to «big» aggregates with altered optical and density and impaired immune features. All these may negatively affect specificity and sensitivity of flow cytometry-based enumerations of MPs. Overall, the main limitations of flow cytometry methods of MPs' identification are several requirements for biofluid fractionation in particularly use of exosome enriched fractions, high risk of sample contamination and increased biological variability that negatively effects on precision of measurement.

Electron microscopy

Electron microscopy (EM) is widely used technique, which allows having strong evidence regarding structure of MPs including their morphology, size and the presence of immune markers (immune complexes). The main limiting factor of EM is a need to have an enriched MP sample, which requires to be fixed with some agents, i.e. paraformaldehyde. Rarely, suspension with MPs could be much simpler object for EM, while some MPs concentrated in suspension are not able to adhere to the grid prior to EM. There is a method of measure of MPs with EM using frozen samples. This approach appears to be promising in investigation of MP structure in detail, because there are no effects of dehydration and fixation by chemicals of samples [4].

Atomic force microscopy

Atomic force microscopy (AFM) is used to investigate the structure of MPs due to high resolution when compared with EM [6]. This fact is especially important for so called small-size (< 50 nm) vesicles. Frequently, AFM is performed prior to other methods of quantitative MP determination [4].

Nanoparticle Tracking Analysis

Nanoparticle tracking analysis (NTA) is based on optical method, which allows fetching of particle tracing for independent measure of both concentration and size distribution of MPs with very low limit (< 50 nm) [4]. On this occasion it is so difficult to distinguish MPs from other particles and vesicles with similar size distribution, which could express similar Brownian motion. Thus, NTA analysis is extremely sensitive to quality of preparation of biofluid with enriched MP avoiding contamination with lipoprotein particles, microbial/ viral bodies, and protein complexes. Noted that even after careful purification some particle may be found in final mixture prepared for MP measurement and the estimated concentrations of MPs with a use of this technique may be not pretty accurate [7]. Finally, all these finding require improvement of the NTA technique using some fluorescence technologies or non-optical enumeration of MPs.

Dynamic light scattering

Dynamic light scattering (DLS) is recommended for assessment of the MP size distribution, while the biofluid enriched of MPs should be relatively monodispersed to avoid some problem with enumeration of vesicles by specific software [8].

Resistive Pulse Sensing

The resistive pulse sensing (RPS) allows detecting the absolute size of MPs in average from 50 nm to 1000 nm in depending on pores' diameter of non-conductive membrane [9]. The resistive pulse detector counts MPs when they pass in flow through an appropriate pore in the membrane under electrical power. The method is pretty accurate utilize the MPs in suspension, which is in the camera with probe volume. The calculation of MPs requires beads of known concentration and preforms using calibration with liposomes with known diameter. The main limitation of the method is heterogeneity of suspension fetching by flow under electrical power through pores with known diameter. Using membrane with pores 500 nm, it has been postulating that MPs ranged 50–500 nm with other small-size molecules (fibrinogen and other low weight molecules, apoptotic bodies, small cells,) could be detected and enumerated as MPs [4]. Consequently, the method requires high accuracy in purification and preparation of samples prior to measure. Therefore, calibration needs before each investigation.

Raman spectroscopy

Raman spectroscopy is used the monochromatic laser-based scattering of inelastic features of living cells that allows detecting their structure and chemical compositions. The main advantage of the method is avoiding labeled marker use,

because the wavelength spectrum is highly specific for each molecule [10]. Moreover, a quantitative assay is possible too as a result in measure of amplitude of signal obtained by Raman microspectroscope. On the other hand, this method is relatively expensive and requires a large experience especially affected data interpretation.

Small-angle X-ray scattering

The small-angle X-ray scattering (SAXS) is the useful method that is based on scattering of the elastic features by X-ray photons at low angles [11]. In contrast to X-Ray crystallography SAXS is able to perform in closer-to-native molecular conditions, but in respectively low resolution manner. However, SAXS could present an ultrastructure model for compositions incorporated into MPs and provide more information about molecular conformation that may have an important value for determination of membrane-related proteins and organization of lipid layers of membranes [4]. Overall, the role of SAXS in the identification of MPs is not clear and requires more investigations.

Surface plasmon resonance technique

Surface plasmon resonance (SPR) technique is well-established methods that in cooperation with to fiber optic technology may determine MPs after their absorption to beads [12]. Because gold and silver are plasmon active metals, they are used as a component a graphene-coated bead, which helps in preventing oxidation and shows better adsorption to biomolecules. This graphene-based surface is a key tool for performing SPR and features of one ensure complex processes of metal deposition, which are needed to absorption of the investigating substrate to the surface. SPR allows detecting morphology of MPs, as well as calculation of them in the solution by laser-based scattering. The main advantage of the method is pretty low cost and high reproducibility that meets rarely amongst similar methods. Moreover, currently conventional prism-based SPR platforms are simply in used, have cost-effectiveness and miniaturization [4].

Western blotting

Western blot is a useful tool for detecting some proteins, which express on the surfaces of MPs in carefully purified biofluid. Although Western blotting is not able to provide quantitative information regarding MPs in mixture, this method could be useful for determining several sub-populations of MPs distinguished each other with immune phenotypes. The cost / difficulty of these methods is one of leading limitation to use one in MPs' detecting [4].

Overall, the current MP detection methods have been remained pretty costly and time consuming. Additionally, majority of them requires be standardizing and approving. In this context, combined methods might to quantify and qualify MP detection.

The advanced bright-field light optical polarization microscopy

To increase sensitivity and specificity in the enumeration of MVs we have been used the advanced bright-field light optical polarization microscopy [13]. Recently we have reported practical advantages of this method as an alternative free-labeled optical method for quantified measured of sizes and size-related characteristics of MVs [14]. We focused the research on a development of photodiode grid and PDA matrix, both of which are able to reply immediately, but not consequently as it was performed in the traditional polarized spectrophotometry. Additionally, original soft helps to recognize replies from surfaces under interfering of various length waves and produced by two source of polarized light, i.e. ultraviolet (λ = from 240 nm to 410 nm) and visual spectrum VIS (= from 590 to 950 nm). We obligatory excluded from grid photo electronic multiplier for range of length waves about 240–680 nm. The diapason of scanning was one second and less that leads to increased performance and reproducibility of results even applied for single MV in the sample. The original types of prisms and diffraction grids mediate a narrow strip of the light about 5 nm and even less and thereby they produce higher quality and clarity of the light that go around a sample. The original images are recognized MV in wide range of diameter in the mater cells that allow determining origin of the MVs on real time in higher resolution and automatic manner. The examples of the low-contrasted images received by light optical polarization microscopy and mathematically modelled images received through advanced bright-field light optical polarization microscopy are reported Figures A, B, C and D. The low-contrasted objects in red blood cells (RBCs) are visualized by application of monochromatic light with =370+30 nm (Fig A). At the figure B we can see MVs with diameter less 1 mcm secreting by RBCs. Because the cell free RBC-MVs and cell debris could not be distinguished with the traditional optical polarization microscopy (C), we consequently applied ultraviolet emanation with high sensitive polarized capture through original soft to construct the image with improved contrasted features suitable for analysis of shaping, number and structure of RBC-EVs (Fig D). Finally, this method can lead to measure a concentration of MVs in the sample without higher cost expenditure and it does not require much equipment and staff persons.

In conclusion, the advanced bright-field light optical polarization microscopy is simple method of MVs determination with low cost, high resolution and reproducibility that requires to be investigated in future.

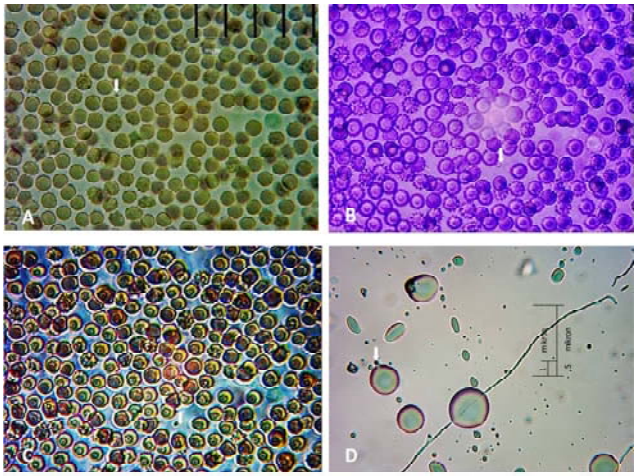

Figure. The example of the images received through traditional (A, B, C) and advanced bright-field (D) light optical polarization microscopy
Notes: Arrows are indicated MVs

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Д-р мед. наук А. Е. Березин, Р. Э. Мохнач Применение нового метода обнаружения микровезикул с использованием усовершенствованной оптической поляризационной микроскопии