**Review**

**Novel methods for microparticles’ detection: From expectations to implementation**

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**ABSTRACT**

Microparticles (MPs) are considered important diagnostic biological markers in many diseases, with promising predictive value. There are several methods that are currently used for the detection of the numbers and the characterization of structure of MPs. MP detection methods are sufficiently distinguished in their cost and time consumption. The review depicts the perspectives of using coupling methods for MP measurement and structure assay. Indeed, there is a large body of evidence regarding that the combination of atomic force microscopy or the coupling of nanoparticle tracking analysis (NTA) with microbeads, plasmon resonance method and fluorescence quantum dots, which could exhibit high accuracy to detect both concentration and structure of MPs when compared with traditional flow cytometry and fluorescent microscopy. Whether several combined methods would be useful for advanced MP detection, it is not fully clear, although it is extremely promising.

**Keywords:** microparticles; detection; analytical limitations; biomarker; probability

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**Introduction**

Microparticles (MPs) are specific small membrane vesicles with diameters ranging from 50 to 1,000 nm[1]. They are produced and actively secreted by several cells due to activation and/or apoptotic stimuli[2]. Transferring active molecules, proteins, peptides, DNAs, RNAs/micro-RNAs, and hormones, circulating free-cell MPs play a pivotal role in various biological processes including immune reaction, cell-to-cell cooperation, endogamous reparation, inflammation, proliferation, and growth[3,4]. MPs possess a wide spectrum of biological effects on intercellular communication by transferring different molecules, which are able to modulate other cells, affecting intercellular communication, differentiation of cells, growth of tissue, reparation, vasculogenesis, inflammation, apoptosis, infection, and malignancy. Additionally, MPs are not only cargo for biological active substances. There is strong association between immune patterns of MPs originating from different cells (endothelial cells, mononucelars, dendritic cells, and platelets) and the natural evolution of various diseases including cardiovascular (CV) diseases, diabetes mellitus, abdominal obesity, cancer, sepsis, eclampsia, autoimmune states, infections, and thrombosis[5–10]. Moreover, the concentration of circulating MPs has been hypothesized to be responsible for the prediction of CV risk, thromboembolic events, autoimmune crisis, bleeding, as well as risk of all-cause mortality and CV death[11–14]. In this context, the measurement of MPs in circulation is considered a fairly promising tool for improving personal risk stratification. On the way there are several technical limitations regarding purification of samples, determination of MPs, and calculation of their concentration. The review depicts the perspectives of using coupling methods for the measurement of MP concentration and the assay of their structure.
Determination, nomenclature and origin of microparticles

MPs belong to heterogeneous family extracellular vesicles (EVs) that originate from plasma membranes having diameter 1,000 nm and less (Table 1). The family of EVs include exosomes (30–100 nm in diameter), MPs (50–1,000 nm in diameter), membrane particles (<50 nm in diameter) and apoptotic vesicles (1–5 µm in diameter) [16]. Exosomes are characterized by the presence of proteins, which are involved in membrane transport (cholesterol, sphingomyelin, and ceramide) and fusion (Rab, GTPases, annexins, and flotillin). The identification of exosomes is based on their cup-shaped morphology after negative staining and presentation of CD63 [16]. Microvesicles, also known as MPs, are actively released from the cell membrane during activation and apoptosis [16]. Because all types of cells may produce at least two types of EV with a wide range of diameters, MPs and exosomes may overlap when diameters are measured in biofluid. There seems to be an interest on whether consequent methods should be used to quantitatively distinguish both populations of EVs. On this circumstance, the determination of morphology and immune phenotype may be useful to detect exosomes and MPs separately. Membrane MPs are shed directly from the plasma membrane and are very variable in their diameters; they also show a slightly lower density when compared with exosomes and do not optionally expressed CD63 [16]. In contrast, CD133 is discussed as an essential immune phenotype marker best fitted for this population. Apoptotic vesicles originate from the plasma membrane of apoptotic cells and are detected in the circulation as large bodies with diameter 1,000 nm and more. They contain chromatin components (DNAs, microRNAs, and histones), whereas there is evidence that endoplasmic reticulum-derived apoptotic vesicles do not contain genetic material but expose immature glycoepitopes. Additionally, proteomics of the main populations of EVs, especially the signatures of microRNAs, may be unique for several types of apoptotic vesicles.

Current methods for microparticles’ determination

Nowadays, there are several methods that are currently used for the detection of concentration (flow cytometry technique, optical microscopy, nanoparticle tracking analysis (NTA), and dynamic light scattering) and characterization of structure (electronic and atomic force microscopy, fluorescent microscopy, and surface plasmon resonance (SPR) technique) of MPs [17,18].

Flow cytometry technique

Flow cytometry technique with polystyrene beads is the gold standard to determine MP sizes, which has now been standardized by the collaborative workshop of the Scientific Standardization Committee (SSC) of the International Society of Thrombosis and Hemostasis [18]. 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 [19]. The liposome-based size calibration with fluorescently labeled liposomes could improve MP size assessment with flow cytometry because there is a single-event signal at sufficiently high concentrations, irrespective of the applied gating [20]. Unfortunately, the determination of MPs sized 50 nm and less remains to be serious limitation for this method [21]. Indeed, small-sized MPs have a low refractive index and are heterogeneous in their size and composition, which require advanced methods for detection [22]. Therefore, some organelles and macromolecules (RNAs, DNAs, etc.) that are released 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 the specificity and sensitivity of flow cytometry-based enumerations of MPs. Overall, the main limitations of flow cytometry methods of MPs’ identification are the several requirements for biofluid fractionation, particularly the use of exosome-enriched fractions, high risk of sample contamination, and increased biological variability that negatively affects precision of measurement [22,23].

Electron microscopy

Electron microscopy (EM) is a widely used technique which allows having strong evidence regarding the structure of MPs including their morphology, size and presence of immune markers (immune complexes). The main limiting factor of EM is a need to have an enriched MP sample, which requires it to be fixed with some agents (e.g., 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 measuring MPs with EM using frozen samples. This approach appears to be promising in the investigation of MP structure in detail because there are no effects of dehydration and fixation by the chemicals of the samples.
**Atomic force microscopy**

Atomic force microscopy (AFM) is used to investigate the structure of MPs due to its higher resolution when compared with EM\(^{[24]}\). This fact is especially important for the so-called small-sized (<50 nm) vesicles. Frequently, AFM is performed prior to other methods of quantitative MP determination\(^{[25]}\).

**Nanoparticle tracking analysis**

Nanoparticle tracking analysis (NTA) is based on an optical method which allows the fetching of particle tracing for an independent measure of both parameters (concentration and size distribution of MPs) with very low limit (<50 nm)\(^{[26]}\). On this instance, 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 the quality of preparation of biofluid with enriched MP in order to avoid contamination with lipoprotein particles, microbial/ viral bodies, and protein complexes. Note that even after careful purification, some particle may be found in the final mixture prepared for MP measurement, and hence the estimated concentrations of MPs with the use of this technique may be not quite accurate. Finally, all these findings require the 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 the assessment of MPs’ size distribution because the biofluid-enriched MPs should be relatively monodispersed to avoid some problems with the enumeration of vesicles by specific software\(^{[27]}\).\(^{[27]}\)

**Resistive pulse sensing**

The resistive pulse sensing (RPS) allows for detecting the absolute size of MPs in average of 50 nm to 1,000 nm, depending on the pores’ diameter of the non-conductive membrane\(^{[28]}\). The resistive pulse detector counts MPs when they pass in the flow through an appropriate pore in the membrane under electrical power. The method is fairly accurate in utilizing the MPs in suspension, which is on the camera with probe volume. The calculation of MPs requires beads of known concentration and utilized using the calibration with liposomes with known diameter. The main limitation of the method is the heterogeneity of suspension fetched by the flow under electrical power through pores with known diameter. Using membrane with pores 500 nm, it has been postulated that MPs ranging 50–500 nm with other small-sized molecules (fibrinogen and other low weight molecules, apoptotic bodies, small cells, etc.) could be detected and enumerated as MPs. Consequently, the method requires high accuracy in the purification and preparation of samples prior to measurement. Therefore, a calibration is needed before each investigation.

**Raman spectroscopy**

Raman spectroscopy used monochromatic laser-based scattering of inelastic features of living cells that allows for the detection of 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\(^{[29]}\). Moreover, a quantitative assay is possible, too, as a result in the measurement of amplitude of signal obtained by Raman microspectroscope. On the other hand, this method is relatively expensive and requires a large experience especially with the affected data interpretation.

**Small-angle X-ray scattering**

The small-angle X-ray scattering (SAXS) is a useful method that is based on the scattering of elastic features by X-ray photons at low angles\(^{[30]}\). 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 the determination of membrane-related proteins and the organization of lipid layers of membranes\(^{[31]}\). 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 a well-established method that, in cooperation with fiber optic technology, may determine MPs after their absorption to beads\(^{[32]}\). Because both gold and silver are plasmon-active metals, they are used as main components for graphene-coated bead, which helps in preventing oxidation and shows better absorption to biomolecules. This graphene-based surface is a key tool for performing SPR and features to ensure complex processes of metal deposition, which are needed for the absorption of the investigating substrate to the surface. SPR allows the detection of morphology of MPs, as well as the
calculation of their numbers in the solution by laser-based scattering. The main advantage of the method is its low cost and high reproducibility, which are met rarely amongst similar methods. Moreover, conventional prism-based SPR platforms currently in use have cost-effectiveness and miniaturization [33].

**Western blotting**

Western blot is a useful tool for detecting some proteins which express on the surfaces of MPs in a carefully purified biofluid. Although Western blotting is not able to provide quantitative information regarding MPs in mixture, this method is useful for determining several sub-populations of MPs that distinguished each other with immune phenotypes. The cost and difficulty of this method are some leading limitations of using one in MP detection.

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

**Couple methods for MP identification**

A combination of optical or non-optical enumeration as well as functional methods may be required for a complete profiling of circulating MPs [34]. There is a large body of evidence regarding that the combination of SPR or RPS method with atomic force microscopy, or coupling NTA with Raman microscopy, microbeads, and fluorescence quantum dots, which exhibited much more accurate ability to detect both the concentration and structure of MPs when compared with traditional flow cytometry and fluorescent microscopy [35–37].

To fetch axillary information regarding the concentration, size distribution, and accurate chemical compositions of MPs in biofluid, Raman microspectroscopy with RPS could be useful. The additional attractive feature of the methods’ combination is avoiding fluorescence-labeling, with appropriate antibodies versus specific antigens, which contributes in reducing analytical time. It has been postulated that consequently performing SAXS, AFM, and X-ray diffraction techniques may be a useful tool for the identification of structural, mechanical, and electrical properties of MPs [38]. Interestingly, SPR imaging could be more useful in determining MPs with low expression of antigens, but NTA analysis contributes axillary data to SPR that could improve information regarding both structure and morphology of MPs. A signal of SPR is weaker than in NTA, which creates a possibility to perform both methods consequently to increase sensitivity and specificity in the enumeration of MPs [39].

The advanced bright-field light optical polarization microscopy could be an alternate free-labeled optical method for quantified measurement of sizes and size-related characteristics of MPs. Increased resolution of a new method of optical microscopy is based on the interaction of polarized light with the thin film of monolayer of the whole blood, with further mathematical analysis of the image through mutual superposition of each next layer over previous one. All these relate to the sufficiently increased capture ability and dynamic diapason extension by way of using the same optical magnification. This is an essential advantage of advanced bright-field light optical polarization microscopy in comparison with conventional highly-sensitive optical-coherent microscopy. Therefore, technically, we have confirmed that the advanced bright-field light optical polarization microscopy exhibits great potential to accurately obtain all clinically relevant properties of single MPs at a high speed, although reproducibility requires more investigations in future [40].

Moreover, all these new methods could be used as screening methods for MP detection. They should not only have much more reproducibility, specificity and sensitivity, but also they should be

<table>
<thead>
<tr>
<th>Population of vesicles</th>
<th>Diameter (nm)</th>
<th>Origin</th>
<th>Main contained components</th>
<th>Best characterized cellular sources</th>
<th>Markers</th>
</tr>
</thead>
<tbody>
<tr>
<td>Exosomes</td>
<td>50–100</td>
<td>Plasma membranes, endosomal membranes</td>
<td>Regulatory proteins (<em>i.e.</em>, heat-shock proteins, tetraspanin), lipids, active molecules, nucleic acids (mRNA, miRNA), cytokines, growth factors, hormones, procoagulant phosphatidylserine, likely complement</td>
<td>Most cell types</td>
<td>There is no unique lipids and proteins for exosomes</td>
</tr>
<tr>
<td>Microvesicles</td>
<td>20–1,000</td>
<td>Plasma membranes</td>
<td></td>
<td>Most cell types</td>
<td>Insufficiently known</td>
</tr>
<tr>
<td>Membrane particles</td>
<td>50–80</td>
<td>Plasma membranes</td>
<td></td>
<td>Epithelial cells</td>
<td>CD133</td>
</tr>
<tr>
<td>Apoptotic vesicles</td>
<td>1,000–5,000</td>
<td>Plasma membranes</td>
<td>Pro-apoptotic molecules, oncogenic receptors</td>
<td>All cell types</td>
<td>Annexin V binding, DNA content</td>
</tr>
</tbody>
</table>
fairly inexpensive and assessable\textsuperscript{[41,42]}. Therefore, combined methods might assay some components of MPs, including RNAs, lipids, proteins, and active molecule profiling\textsuperscript{[43]}. Probably, similar approach would attenuate pre- and intra-analytical errors and improve the entire precision of the methods\textsuperscript{[44]}. Thus, coupling some methods based on different principles might allow detecting the numbers and structure of MPs. All these may be useful for providing the necessary information to clear the biological role of MPs as diagnostic and predictive biomarkers.

**Conclusion**

In conclusion, analytical methods currently used as the only techniques for detecting MPs exhibit serious limitation to the interpretation of received results. Each of these biosensor diagnostic platforms has its own advantages and disadvantages in detecting MPs and in identifying their size distribution and composite chemicals. The combination of MP detection methods allows for sufficiently increasing their accuracy, specificity, sensitivity, and probability.

**Conflicts of interest**

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Nano-zymography using laser-scanning confocal


