Novel Tools for the Study of Cell Type-Specific Exosomes and Microvesicles

Günter Müller*

Ludwig-Maximilians-University Munich, Biocenter, Department Biology I, Genetics, 82152 Martinsried near Munich, Germany

Abstract

Many cell types release exosomes and microvesicles (EMVs) with a size range from 0.05-5 μm, harbouring receptors, bioactive and signaling proteins, molecular mediators and nucleic acids for cell-to-cell communication. Microvesicles bud directly from the plasma membrane, in contrast to exosomes which are derived from multivesicular bodies within the cell. Increased levels of EMVs have been observed in plasma, urine and other body fluids in patients suffering from a wide range of common complex diseases, including vascular, metabolic, lung, autoimmune and neurodegenerative diseases, chronic inflammation and cancer. EMVs may affect target cells directly by surface-bound ligands, transferred surface receptors and membrane-associated enzymes, such as glycosylphosphatidylinositol-anchored proteins, or delivered cytoplasmic or membrane-associated constituents, such as cytosolic proteins, microRNA/ RNAs, bioactive lipids and even mitochondria. The use of EMVs as diagnostic markers for the prediction, diagnosis, therapy monitoring and prognosis of complex diseases is becoming increasingly attractive. Novel technologies for analysis of the size, density and molecular composition of EMVs are currently emerging together with methods for their improved isolation and purification out of heterogenous vesicle populations. In addition, the recent revolution in mass-spectrometry, (micro-) flow cytometry, atomic force microscopy, nanoparticle tracking and biosensing will considerably facilitate the quantitative and qualitative analysis of all the constituents assembled in EMVs. Technologies will be preferred that provide signatures specific for EMV subsets rather than a single or a few parameter(s) averaged for the total EMV population. Those EMV signatures have to be correlated to specific disease states along cross-sectional and longitudinal clinical studies. Moreover, it has to be tested which signatures and molecular components, i.e. EMV subspecies, are most informative to obtain actionable disease information. Ultimately, the reliable, rapid and low-cost analysis of EMVs will support systems biology-based approaches for the diagnosis and therapy of complex diseases and supplements the analytical power of conventional biomarkers.

Keywords: Biomarkers; Biosensing; Exosomes; Microparticles; Microvesicles; Nanoparticle tracking

Abbreviations: EIS: Electrochemical Impedance Spectroscopy; ELISA: Enzyme-Linked Immunosorbent Assays; EMV: Exosomes and Microvesicles; FCM: Flow Cytometry; ISFET: Ion-sensitive Field Effect Transistor; miRNAs: microRNAs; MS: Mass Spectrometry; NPs: Nanoparticles; NTA: Nanoparticle Tracking

Introduction

The increased release of exosomes and microvesicles (EMVs) and their accumulation in body fluids including plasma, liquor, urine, saliva, mucus and interstitial fluids (e.g. ascites, pleural fluids) is often directly related to cell activation [1-4] and the pathogenesis of a variety of diseases [5-9]. While the exact mechanisms inducing EMV release still remain obscure [1,10,11], the releasing efficacy is often modulated by (receptor-dependent) extracellular signals, such as ligands or viral infection [12,13-16]. Apparently, the underlying vesicular trafficking, exocytotic and plasma membrane shedding processes [17] operate in infection [12,13-16]. Apparently, the underlying vesicular trafficking, exocytotic and plasma membrane shedding processes [17] operate in infection [12,13-16].

(i) High-throughput/high-content analytical platforms have to be implemented and validated (standard operation procedures) that allow the quantitation and discrimination of EMV composition and heterogeneity as related to their physicochemical properties (size, density, refractive index) on the basis of both single-parameter and signature analysis of their proteomic (by MS [mass spectrometry], single/multiplex enzyme-linked immunosorbent assays [ELISAs]), lipidomic (by MS), metabolomic (by MS) and nucleic acid (DNA, mRNA, miRNA [microRNA]) by reverse transcription-polymerase chain reaction (RT-PCR) composition. (ii) The pre-analytical procedures, including the calibration and standardization of flow cytometry (FCM) platforms, appropriate for EMV analytics have to be improved and simplified. (iii) Multi-parameter combinations of fluorescently labeled monoclonal antibodies and nucleic acid staining and fixation/permeation pre-analytical protocols for simultaneous multi-parameter FCM analysis of cell type-specific EMVs have to be implemented and validated. (iv) Quantitative MS-based proteomics as reference method and as bridging technology for the determination of protein composition and content in EMVs released from different cell types under normal and disease conditions has to be used to facilitate...
the final selection of antibody targets for single and multiplex ELISA platforms or multi-parameter FCM. (v) Quantitative MS-based lipid species analytics for EMVs from different cell types has to be introduced and validated on the basis of the high-throughput/content lipid species profiler (>800 lipid species) already developed (see www.lipidomicnet.org) with special focus on regulatory and high-affinity lipid ligands (e.g. fatty acids, lysophospholipids, sphingolipids) for G protein-coupled receptors, annexins, nuclear hormone receptors (e.g. PPARγ, LXR, RXR) and other transmembrane receptors and transporters (e.g. CD36, integrins, selectins, glycosylphosphatidylinositol-anchored proteins). (vi) Novel highly membrane-permeable dequencher dyes to increase the specificity, sensitivity and accuracy of fluorescence staining of nucleic acids (DNA, mRNA, miRNA) in EMVs with excitation/emission-profiles suitable for multi-parameter FCM have to be implemented and validated. (vii) The pre-analytics (extraction, inhibitor elimination, etc.) and analytics (primer design, assay format) of array-based and quantitative RT-PCR Taqman high-throughput measurement of the miRNA contents in EMVs have to be improved and validated. (viii) Multi-omics-, nanoparticle tracking analysis (NTA)-, and nanoparticle (NP) biosensing-based software tools and graphic data presentation for single-parameter and signature-fueled analytics of EMVs have to be implemented for routine (clinical) use. (ix) The levels and composition of EMVs released by specific cell types in vitro have to be determined for the use of specific EMV preparations appropriate as standard materials to be added for the normalization and validation of the assay for EMV analytics (sensitivity and linearity determination). (x) For validation as novel biomarkers or surrogate markers reflecting the systems biology-based risk assessment, diagnosis, therapy monitoring and prognosis, the disease specificity, sensitivity and predictive value of cell type-specific EMVs defined by single markers or complex signatures have to be established in patient cohorts with complex common diseases. (xi) A public format for multi-parameter omics-, FCM-, signature-driven as well as cell type- and disease-specific data bases has to be developed as “EMV platform” to provide connectivity and algorithms to synergize published and newly generated knowledge about proteome-, lipidome-, metabolome- and nucleic acid-based EMV targets. (xii) Finally, the knowledge gathered by the “EMV platform” has to be transferred to preclinical and clinical research, practical medicine and national and international health care authorities as well as to companies engaged in diagnostics and drug discovery and development.

Established Tools

For the majority of biomarker projects correlations between the modulation of candidate plasma EMV biomarkers and disease-specific stages by measuring target cell-specific EMVs including their biological functionality and activation status have to be elucidated in order to stage by measuring target cell-specific EMVs including their biological modulation of candidate plasma EMV biomarkers and disease-specific heterogeneity of the stress response (during development of the disease the-art ultrasensitive high content imaging techniques.

the understanding why a small subpopulation of cells apparently determines the outcome of cancer development and/or treatment. Standardized technologies will be required to profile heterogeneous cell populations and to characterize EMVs derived from individual cells by combining microscopic, flow cytometric, light scattering, particle tracking and biosensing technologies [110].

Enrichment and Purification of EMVs

A number of workflows already have been and still have to be developed for the validation of the enrichment of EMVs from a cell population. They encompass several read-outs, such as size and density, for the discrimination of exosomes, microvesicles and apoptotic bodies themselves as well as of contaminating entities, lipid (droplets), lipoproteins, protein aggregates, protein-phospholipid micelles, cell and membrane fragments, but also broken and intact cells (Table 1). In general, EMV enrichment and purification relies on three principle workflows. (i) Adsorption to nano-magnetic beads through ionic interactions between the negatively charged phospholipid surface of EMVs and positively charged beads [111,112] or EDTA-coupled beads complexed to Ca²⁺ [113-115], (ii) Differential centrifugation using sequentially increasing centrifugal forces for the removal of cells, cellular debris, larger particles and organelles from conditioned cell culture supernatants or body fluids and final collection of the microvesicles (10,000-20,000xg, 10-30 min) or exosomes (> 100,000xg > 120 min, subsequent to deprivation of the microvesicle fraction by the 10,000-20,000xg spin) during the last centrifugation step [116,117], (iii) Size exclusion chromatography involving a low-speed centrifugation step for the removal of cells, cellular debris, larger particles and organelles, followed by pre-concentration with two filtration steps (0.2 μm pore size, 100 kDa MWCO) and subsequent size exclusion chromatography of the concentrates and final collection of the separated exosome and apoptosis and cell lysis [104-108] in cell cultures derived from the tumor patients, and (ii) To characterize cell populations responding to stress at the level of individual cells by proteomic and lipidomic methods. The identification of specific alterations in membrane domain, in particular lipid raft, structures [109] leading to selectively induced expression of heat shock proteins in a heterogeneous cell population may facilitate the understanding why a small subpopulation of cells apparently determines the outcome of cancer development and/or treatment. Standardized technologies will be required to profile heterogeneous cell populations and to characterize EMVs derived from individual cells by combining microscopic, flow cytometric, light scattering, particle tracking and biosensing technologies [110].

Table 1: Some technologies recently introduced for the preparation and characterization of EMVs.

<table>
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<tr>
<th>Technology</th>
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<tbody>
<tr>
<td>Cell culture supernatants</td>
<td>[117]</td>
</tr>
<tr>
<td>Blood sampling /anti-coagulation / needle size</td>
<td>[161,182]</td>
</tr>
<tr>
<td>Freezing and thawing cycles</td>
<td>[183]</td>
</tr>
<tr>
<td>Proteomics</td>
<td>[184-186]</td>
</tr>
<tr>
<td>Immunofluorescence depletion</td>
<td>[112,127,128]</td>
</tr>
<tr>
<td>Centrifugation</td>
<td>[187]</td>
</tr>
<tr>
<td>Scanning electron microscopy</td>
<td>[188]</td>
</tr>
<tr>
<td>Atomic force microscopy</td>
<td>[123,124,189]</td>
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<tr>
<td>Micro/Filtration</td>
<td>[122,190-192]</td>
</tr>
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<td>FCM</td>
<td>[121,125,126,148, 149,151,193-196]</td>
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<td>Isotype controls for FCM</td>
<td>[197]</td>
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<tr>
<td>Fluorescent FCM</td>
<td>[198]</td>
</tr>
<tr>
<td>Calibrated beads</td>
<td>[195]</td>
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<tr>
<td>Scatter angle</td>
<td>[199,200]</td>
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<tr>
<td>Electrochemical impedance spectroscopy (EIS)</td>
<td>[201]</td>
</tr>
<tr>
<td>NTA</td>
<td>[111,119,147]</td>
</tr>
<tr>
<td>NP-based biosensing</td>
<td>[163,164]</td>
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<tr>
<td>Microfluidic isolation</td>
<td>[202]</td>
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<td>Standardization</td>
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microvesicle populations from the individual fractions by high-speed centrifugation (> 100,000xg, 120 min) [118].

Importantly, systematic investigation of the impact of the storage conditions of preparations of EMVs on their size and integrity demonstrated [119] that the vesicle diameter significantly declines with the duration of the storage period (e.g. within two days) and with increasing temperature during the storage (from 4° to 37°C). It is of practical interest that storage at -20°C, repeated freezing-thawing cycles (up to 10) and ultracentrifugation per se does not appear to affect the size and composition of EMVs to any significant degree [119].

Detection, Differentiation and Quantification of Cell-Derived EMVs

In vitro models to generate EMVs from the main cell lineages implicated in the pathogenesis of the disease of interest should encompass the following steps: (i) Cell culture protocols and standard operation procedures have to be implemented and optimized for the routine generation of EMVs from relevant and appropriate human cell culture models. (ii) Cell type-specific EMV markers have to be validated using FCM, immune blotting (using commercially available and/or newly generated mono-/polyclonal antibodies), lipid/protein/DNA arrays and nucleic acid probes, as well as NTA and biosensing on EMVs generated in vitro from the corresponding cell type. (iii) Candidate markers for relevant cell-derived EMVs have to be selected for the demonstration of their presence in the circulation and validated as candidate biomarkers in future clinical studies.

Filtration

A convenient, cheap and rapid procedure for the differentiation of EMVs, which does not depend on sophisticated and labour-intensive technology, represents filtration (Table 1). The samples are passed through filters of appropriate pore size (0.1 to 0.2 µm) and made of materials which do not support unspecific adhesion. In general, the filtration methods for the isolation of EMVs have the distinct advantage compared to procedures based on differential centrifugation of being capable of processing of larger sample volumes, in particular, of cell culture supernatants. The subpopulations of the fractionated EMVs can be further analyzed and separated according to density or function which considerably increases the resolving power compared to filtration alone [120,121]. For instance, a filtration method has been developed that enables the quantification of phosphatidylserine-positive EMVs from human plasma with diameter > 200 nm and relies on staining with annexin-V [122]. The levels of EMVs in the venous plasma of newborns and if stained with annexin-V compared to the fed and non-smoking state, increments in the levels of released EMVs which were amenable to impact on the absolute number of EMVs. In contrast, fasting and smoking monocytes. The majority of the variables investigated, including low temperature (-20°C), proband age and gender, did not have detectable impact on the absolute number of EMVs. In contrast, fasting and smoking led to significant (about 2.7-fold and 1.9-fold, respectively) increments in the levels of released EMVs which were amenable to staining with annexin-V compared to the fed and non-smoking state, respectively [122].

Centrifugation

EMVs contained in biological sample fluids can be collected by differential centrifugation, often including initially a single or two sequential “cleaning” step(s) at low speed and for short time (e.g. 300xg, 5 min) for the removal of intact and broken cells, cell debris, huge membrane fragments and subcellular organelles (e.g. mitochondria) followed by the “collection” step for microvesicles at medium speed and for intermediary time (e.g. 12,000xg, 20 min) and terminated by the “collection” step for exosomes at high speed (in an ultracentrifuge, e.g. 150,000xg, 120 min) for prolonged time [113-115]. The fractionated microvesicle and exosome pellets can be further purified by several washing cycles using appropriate centrifugation conditions adapted from the “collection” steps and modified for optimal differentiation between the separated EMV subspecies. The identity of the pelleted EMV subspecies can then be determined by immune blotting for known EMV protein components. However, this procedure does not provide information about the number and size of the separated EMV subspecies.

Enzyme-Linked Immunosorbent Assays

ELISAs typically fail to quantitatively capture all the EMVs present in biological sample fluids, detect soluble antigens, such as cytoplasmic enzymes and nuclear proteins, which may represent the soluble contents of EMVs and are prone to loss during the EMV preparation procedure, and can not differentiate between microvesicles and exosomes [114]. The latter two disadvantages could be overcome by the use of detecting antibodies raised against surface, in particular, transmembrane protein components that are specific for microvesicles or exosomes.

Electron Microscopy

In general, electron microscopy manages to reveal the presence of EMVs in the pellets obtained by ultracentrifugation of the biological sample fluids, such as plasma, urine or cell culture medium (Table 1). However, in general this method is not quantitative and necessitates extensive efforts for appropriate sample preparation. Nevertheless, transmission as well as scanning electron microscopy has been used in many studies published during the last two decades and are therefore commonly regarded as the golden standard for the size determination and classification of EMVs [123]. However, closer analysis of the multitude of electron microscopic micrographs and pictures available in the public domain reveals the inherent difficulties and pitfalls in the interpretation of the results obtained by different research groups. In particular, the considerable variability in the extent of heterogeneity as delineated from the size and morphology of the EMVs reported to be released from the same cell type in response to the same stimulus (e.g. calcium ionophores) is surprising and disappointing. It may be explained by (subtle) differences in the methods used for preparing the EMV samples and/or taking the micrographs as well as in the biased selection of the photographic fields to be analyzed and published. In any case, this study-to-study variation significantly impairs the quantitative evaluation and classification of the portions of EMV subspecies released from specific cell types upon challenge with specific stimuli. An additional disadvantage represents the pronounced time requirements for a complete cycle of the electron microscopic procedure, which do not match the demands for routine and clinical EMV analytics.

Atomic Force Microscopy

Atom force microscopy has recently been introduced for the analysis of the size (distribution), number and morphology of EMVs [123], in general (Table 1), and of CD41+ vesicles in plasma, in particular [124]. The data presented so far are of exquisite quality with regard to both the precision of the size measurement and the clarity and illumination of structural details that have never been detected up
to now. Certainly, these exciting results have to be corroborated using distinct cell types and stimuli with emphasis on analysis of the degree of variation. Unfortunately, at present the equipment requires high expenditure regarding the technological complexity, knowledge and experience in instrument handling, labor intensity and costs, which will limit the broad availability of atomic force microscopy.

Conventional FCM

The degree of light scattering recognized by commercially available (analogue or first generation digital instruments of) flow cytometers (e.g. Becton Dickinson Facs Calibur™ Flow cytometer) is adequate for certain routine clinical applications, such as cell sorting and counting of particles with diameters above 500 nm, but not sufficient to support the detection of smaller particles below 300 nm [125,126]. For future routine analysis of microvesicles (rather than exosomes) by FCM (Table 1), it will be essential to compare plasma samples with identical numbers of EMVs on two or more of instruments of the same type for standardization and calibration of EMV analytics per se as well as evaluation of the reliability of the chosen instrument series. Further improvements in resolution may result from adsorption of the EMVs to latex beads as well as the use of CD surface markers, such as CD61, CD8 or FasL, to identify, characterize and categorize EMVs released from different cell types [117,127,128].

A solution for the apparent failure of FCM to differentiate between vesicle sizes in the range of 100 to 500 nm, i.e. for the differentiation between microvesicles and exosomes, may come from the use of fluorescent signals instead of visible light, which are generated by excitation with a laser beam and become highly amplified by sensitive photomultipliers. The monitoring of scattered fluorescence results in a significantly elevated signal-to-noise ratio compared to scattered visible light. In consequence, the counting efficacy of vesicles in the range of 100 to 500 nm becomes significantly increased upon fluorescence measurement which leads to a considerable improvement of the reliability of detection and assignment of microvesicles. In contrast, at present typical exosomes fail to be detected by FCM with satisfying precision and reproducibility, even if operating with the fluorescence principle.

Dynamic Light Scattering

For more than five decades light scattering has been commonly applied for the determination of the molecular weights of large protein complexes and the sizes of small particles. This range of resolution which has been further broadened on the basis of recent technological progress seemed to be well suited for the analysis and differentiation of the individual EMV subspecies (Table 1). In fact, an interesting comparison of the performance in size determination was performed between two commercially available instruments, Zetasizer Nano S (Malvern Instruments, Ltd., Malvern UK) and N5 Submicron Particle Size Analyzer (Beckman-Coulter, Brea, CA), that are both based on dynamic light scattering but different technical realization, with EMVs from fresh-frozen human plasma samples [121]. In fact, the detection principle seemed to be compatible with the resolution requirements for the discrimination between exosomes and microvesicles and to offer the additional advantages of simple practical application and routine use. Meanwhile, the Delsa Nano series of analyzers has been introduced into the market, which will replace the Beckman instrument NP5 and relies on Brownian motion of the EMVs, in the presence or absence of an applied electrical field, that causes Doppler effects with the light scattered from the vesicles. This instrument provides a valuable combination of information about zeta potentials, electrophoretic mobility and size distribution. In theory, the huge detection range from 0.6 nm to 7 µm should guarantee the reliable classification and characterization of EMVs, which remains to be demonstrated in future studies.

Multi Parameter FCM

Numerous FCM studies using body fluids have shown a correlation between the number of EMVs and certain human diseases (Table 1). However, due to the optical performance of the currently used standard FCM platforms (scattering, frequency), microvesicles (~1 µM diameter) are accessible for measurement by conventional FCM, while the smaller exosomes (50-100 nm) remain below the detection limit. Moreover, other protein complexes, especially circulating immune complexes, protein aggregates, membrane fragments and subcellular organelles, overlap in biophysical properties (size, scattering, density and sedimentation) with EMVs. However, with the most recent FCM technology (NAVIOS Beckman-Coulter, FACSVersa Becton Dickinson, Apogee A50) the instrumental requirements to reliably quantify EMVs have greatly been improved. Moreover, important progress has been made towards standardization of sample preparation, immunostaining and vesicle size protocols. Currently, there is little agreement on the actual concentration of EMVs in blood in healthy subjects ranging from 200/µl to over 1000/µl. These discrepancies are mostly due to variation in sample processing and in the calibrator beads used in order to distinguish EMVs from cell debris, organelles or protein aggregates. A recently published protocol using 0.5 to 0.9 µm-polystyrene microspheres (meganix) allowed to define a forward scatter EMV-size gate for standardization of EMV-measurements in body fluids. In combination with latex beads the Apogee A50-Micro Flow Cytometer now manages to resolve EMVs down to about 100 nm by light scattering and to discriminate exosome and microvesicle subpopulations. With the most recent instrumentation introduced into the market (Beckman-Coulter NAVIOS, Becton Dickinson FACSVersa) the sensitivity, speed (20-bit processors) and optical resolution (scattering performance) now enable a more standardized and quantitative EMV analysis in pre-centrifuged patient blood samples.

Electrochemical Impedance Spectroscopy (EIS)

A novel label-free method for EMV detection and quantification represents EIS which reaches detection limits as low as several EMVs per µl (Table 1). Impedance-based detection and quantification of EMVs is based on fundamental interfacial electrochemistry at the sensing electrode, which is driven by electrochemical activities of EMVs that can be separated by applied electrochemical potential. Compared to light scattering in FCM, the integration of the EIS method into an automated, more reproducible and accurate EMV counting platform is conceivable. However, the detection limit of ≥ 300 nm may limit its use and introduce a bias toward the detection of larger vesicles, i.e. of microvesicles vs. exosomes [121].

Coulter Principle

The so-called Coulter technology monitors the considerable changes in the electrical impedance through a narrow channel with a defined aperture that is caused by the passage of single cells or particles and enables the determination of their number per volume unit as well as of their absolute size and size distribution with the help of appropriate signal and data processing software. The diameter of the aperture is critical for the size range of particles and cells that
are amenable to Coulter analysis. For instance, the Coulter counter, “Multisizer 4” (Beckman-Coulter, Brea, CA) was designed for the general determination of particle size with a lower cut-off of 400 to 600 nm presumably for technical reasons which limits the lower diameter to be analyzed at present. Consequently, this instrument will not enable the reliable counting of exosomes.

**Future Tools**

**Nucleic acid analytics**

A multitude of methods have been reported for the identification of nucleic acids associated with EMVs, which are based on routine next-generation sequencing, RT-PCR, Taqman analysis and chip/microarray technologies [129-138] and will not be covered in this review. Instead, the potential use of fluorescent probes and chromophores directed against EMV-associated nucleic acids is proposed.

For this, standard fluorescent nucleic acid probes could be used that detect the complementary sequence information by enhanced emission intensity or quenching [139]. However, such changes potentially result from side effects leading to artifacts in the fluorescence read-out. Undesired quenching of fluorescence emission represents a considerable problem in cell biology. Hence, dual (or even multiple) labels, that change their emission maximum, operate as superior probes for the detection and imaging of nucleic acids, as it has been shown with wavelength-shifting molecular beacons, for instance [140-142] (Figure 1). However, the disadvantage of commercially available systems is that often the signal-to-noise ratio is rather low. It has been proposed based on published results (see below) that the RNA base surrogate pairs of cyanine dyes promote a resonance dipole-dipole interaction mechanism within a ground-state complex [143,144]. This assembly follows a static quenching mechanism yielding a better signal-to-noise ratio. It is important to point out that the ground state of dye-dye interactions is determined by the RNA duplex framework holding the dyes close together and is not due to an inherent affinity of the dyes for each other since this provides the basis to discriminate optically between miRNA strands [144].

From most recent results with combinations of cyanine dyes it became clear that the type of chromophore attachment to the oligonucleotides is critical for efficient changes in emission color. In commercially available probes, for instance molecular beacons, fluorescent dyes are attached typically via flexible and long alkyl chain linkers [142]. Energy transfer can occur only inefficiently by collisional quenching. In contrast, in ongoing approaches the DNA architecture around the fluorescent chromophores incorporated as DNA base substitutions forces the two dyes in close proximity and thereby enhances the energy transfer efficiency by static quenching [139]. During the detection of the corresponding miRNAs the architectural force becomes released by opening the hairpin conformation step by step, and the resulting separation of the dyes gives the characteristic fluorescence color change, for instance from red to green [141]. In comparison with conventional probes this approach is characterized by two major advantages: (i) The fluorescence read-out allows a clear and distinct discrimination simply by the emission color (about 140 nm-shift). (ii) On basis of the well separated emission bands, the high contrast between the open and closed forms increases the signal-to-noise ratio.

These two properties hopefully characterize the corresponding newly developed probes as powerful tools for the nucleic acid analytics of EMVs. Moreover, the application of synthetic chemistry will allow the tuning of the applied chromophores. In consequence, the design, synthesis, biophysical characterization and application of chromophore-labelled oligonucleotides as specific and bright fluorescent probes for the detection and imaging of EMV-derived RNAs promise the distinct advantage of the avoidance of sophisticated and cost-intense technologies and thus justify future research efforts for the confirmation of this hypothesis.

However, each of the above procedures necessitates the disruption of the isolated EMVs in order to gain access to their luminal contents. While the extraction and purification of mRNAs and miRNAs out of EMVs for their subsequent analysis has turned out to be feasible even for routine purposes, the use of NP-based biosensing (see below) in theory may offer the opportunity to identify the nucleic acid signatures of EMVs without the need for their cracking and nucleic acid preparation due to the intrinsic capability of NPs to cross biological membranes. This hypothesis and the associated advantage of a considerably simplified mRNA/miRNA analytics merit rigorous testing by future experimentation.

**Nanoparticle tracking analysis (NTA)**

Many of the problems and disadvantages associated with the technologies mentioned above could be overcome by the recently introduced method of NTA. It enables the direct and real-time visualization as well as quantitative evaluation of nanoparticles (NPs) in fluidic samples [145] and has already been established in other areas, such as the size determination of engineered NPs, such as inks, pigments, carbon nanotubes, protein aggregates and viral particles. NTA relies on the light-scattering characteristics of laser light on vesicles that undergo Brownian motion when in solution. Thus, the underlying principle is a correlation between the Brownian motion that is monitored by light scattering using a light microscope connected to a video system, and the vesicle size. The NTA software uses the video data for tracking the Brownian motion of individual EMVs and therefore calculates their size and total concentration. For this, a video recording of the tracked EMVs is analyzed and a mean squared displacement is calculated for each vesicle [146].
Conventional NTA

Conventional NTA allows the determination of the hydrodynamic radius and thereby of the size distribution of the EMVs. For this, the diffusion coefficient and sphere-equivalent hydrodynamic radius have to be calculated on the basis of the Stokes-Einstein equation. First experimental experience is now available for the NanoSight LM10 and NS500 instruments (NanoSight Ltd., Amesbury, UK) that introduce a exactly focused laser beam via a glass prism into the sample fluid harbouring the EMVs in suspension at diluted state (Figure 2). Following refraction of the beam at a low angle when it hits the sample, a thin beam of laser light is generated at the interface of the glass prism and the sample fluid which illuminates the EMVs through the thickness of the sample chamber (about 500 µm). EMVs which reside within a beam as it crosses the sample layer are visualized with a conventional optical microscope connected to a video camera. These instruments are typically installed in parallel to the beam axis and collect all the light which becomes scattered by all EMVs present in the field of view at the time point of the photography. The beam depth is about 20 µm at the point of entry and thereby matches with the distance of the imaging optics guaranteeing optimal focus (Figure 2). Typically, the recording data for the video are 60 seconds total duration and 30 frames per second. The NTA software (Nanosight Ltd.) for monitoring the movement of the individual EMVs has been designed to first identify and classify each vesicle and then to follow each selected vesicle by frame-to-frame comparison of its tracked Brownian motion. The relative velocity of the individual EMVs calculated from the frame-to-frame comparisons and measurements is correlated to the vesicle size on the basis of the two-dimensional Stokes-Einstein equation.

In a series of studies the potential of NTA for the determination of size distribution and concentration of EMVs and their classification into exosome and microvesicle subtypes was demonstrated with biological fluidic samples. The identification and tracking of a particular EMV contained in the sample can be optimized by adjustment of a multitude of parameters affecting the video capture, such as shutter speed and camera gain, as well as the data analysis, such as background subtraction, filter setting, minimal track length and frame-to-frame search area. Importantly, the refractive index of the individual EMV critically determines the range of its size which is compatible for its detection by NTA. In general, high refractive indices, such as for colloidal gold, allow the tracking of very small particles, whereas low indices, such as for cellular vesicles including EMVs, require larger diameters of the particles for their reliable visualization. The reason for this is the strong dependence of the lower detection limit on the signal-to-noise ratio of the image. The latter is critically affected by the amount of the scattered light that for EMVs at the size limit of detection will follow the Rayleigh scattering equation. In consequence, the lower limit of detection of EMVs with their low refractive indexes by the NTA system is in the range of 50 nm. The upper limit of detection is at about 1 µm since for larger particles their Brownian motion becomes too low and thereby would limit the accurate tracking. Taken together, this theoretical range of detection makes NTA perfectly suited for the discrimination of EMV subspecies according to their size distribution.

In fact, the experimental evidence so far available [111,119,147] has clearly demonstrated that the size and concentration of EMVs can be measured using NTA accompanied by a number of decisive advantages compared to the methods discussed above: (i) In comparison to electron microscopy and atomic force microscopy, a complete operation cycle of NTA requires less time compatible with a higher throughput of sample processing and analysis and, in addition, does not contribute to shrinkage artifacts as caused by the fixation procedure, since it is performed in suspension. (ii) In comparison to conventional FCM, the demonstrated lower size limit for detection of EMVs by NTA is considerably lower (50 vs. 300 nm) and thereby NTA manages to detect vesicles as small as about 50 nm with significantly higher sensitivity. It remains to be shown whether the improved resolution power of the latest generation of digital flow cytometers with the reported discrimination of 100-nm and 300-nm latex/polystyrene beads from one another in a mixture [148,149] holds true for the minimal sizes of EMVs for their detection and separation by FCM. This seems to be questionable given the lower refractive indexes of EMVs and associated underestimation of their size compared to latex/polystyrene beads [148-150].

It is thus of considerable advantage that the detection principle of NTA relies on the Brownian motion of the EMVs rather than on their refractive index [145]. Interestingly, according to FCM the major portion (>90%) of placental vesicles, which are commonly assumed to represent a mixture of exosomes, microvesicles and apoptotic bodies over a broad size range [151], had a diameter of less than 1 µm with a peak distribution shifted leftward to the 300-nm cut-off. According to NTA, the major portion of the EMVs actually had diameters below 300 nm, albeit larger vesicles of 500 to 600 nm were identified in that preparation, too, however to a minor degree only [147]. This apparent underestimation of the number of larger compared to smaller vesicles, i.e. microvesicles vs. exosomes, in complex EMVs samples may be due to the requirement for their dilution in case of a high ratio of exosomes vs. microvesicles in those mixtures. NTA is operating most accurately with EMV concentrations of 2–20×10³/ml. Samples harbouring higher numbers of EMVs have to be diluted before measurement and the relative EMV concentration calculated according to the dilution factor. Thus, albeit vesicles with a size up to 1 µm can be tracked by NTA, a low concentration of them caused by dilution will inevitably lead to a more than proportional decrease in signal strength over background due to the limited Brownian motion of large vesicles. To overcome the resulting possibility of underestimation of vesicles from 0.5 to 1 µm, such as microvesicles in particular, alone or in complex mixtures, the determination of their numbers should be performed by FCM rather than NTA. (iii) In comparison to dynamic light scattering, NTA is capable to differentiate the EMV subspecies in complex sample...

![Figure 2: Principal configuration of the instrumentation (NanoSight) for NTA of EMVs (adapted with modifications from [147]; see text for details).](image-url)
mixtures taking into consideration the limitations as discussed above. Very small vesicles are accessible for detection by dynamic light scattering if present in the sample alone. However, in combination with other vesicles of different size the very small ones resist resolution by this method [146] since the light emitted from all the vesicles together becomes collected and measured simultaneously by the single detection element of the instrument. The calculated average value for the scattering of a given sample tends to overestimate the absolute size and size distribution of the vesicles.

At variance, NTA measures simultaneously the scattering intensity and velocity of the movement of individual vesicles and thereof calculates their size individually. This operation mode is compatible with maximal resolution in size even out of heterogeneous EMV sample mixtures. In addition, the direct monitoring of individual EMVs supports the determination of the vesicle concentration through extrapolation of the number of vesicles detected at any given time point in any microscopic field to the concentration of the vesicles per volume unit with regard to the defined and known scattering volume. Certainly, the possibility of calculating the concentration of individual EMVs, which remains unique for NTA so far, represents a huge advantage for the analysis of the EMV subspecies and their composition of biological fluids, since the physiological relevance of EMVs of different size (and composition) in health and disease apparently critically depends on alterations in their concentration and shifts in their size distribution.

In addition, the possibility to quantitatively evaluate the number of EMVs in a given sample by NTA using the NanoSight instrument allows for the first time in combination with protein determination to know how many EMVs are being added to an experimental set-up for studying their biological effects. So far, the input of EMVs into a test system has typically been reported as amount of EMV protein, which often ranges from a few to several hundred micrograms [127,152], rather than as a precise number. NTA is now likely to support a more reliable standardization for biological test systems. Furthermore, the physical state of a given EMV preparation prior to its addition to a test system has often remained uncharacterized so far. Now NTA analysis of EMVs prior to the assay may reveal whether (even minor) portions of the vesicles contained in the sample to be assayed are in the monodispersed or aggregated state, which both could considerably affect the biological activity of the EMVs in the test system. Finally, the knowledge of the average size and size distribution of EMVs as determined by NTA will help to judge about the (biophysical) quality of a given EMV population as well as the reproducibility of its preparation.

A major disadvantage of NTA represents the considerable height and variability of the background signal. To keep that within acceptable limits in many experiments serum-free medium seemed to be required since serum supplements (e.g. fetal calf serum) often harbour vesicles and particulate materials which have to be removed by ultracentrifugation prior to their addition to the culture medium. Moreover, serum proteins may tend to aggregate and thereby elicit false-positive signals during NTA analysis. Unfortunately, many cell systems do not tolerate extended periods of time under serum-free conditions without changes in physiological behaviour or loss of vitality, which both could affect the NTA results. As a consequence, the analytics of EMVs released from those cultured cells can not be assessed in serum-positive medium since it will be very difficult or even impossible to discriminate between cell-derived and serum-derived EMVs on the basis of conventional NTA. However, it is conceivable that the measurement of EMV signatures rather than single parameters in course of NP-based biosensing may enable the “substraction” of serum-derived EMVs from the total EMV population prepared from the culture medium of the releasing cells and thereby result in the identification of the cell-derived EMVs (see below).

**Fluorescent NTA**

The unmodified version of NTA did not include the possibility to characterize the EMVs at the molecular level, in particular with regard to the presence of marker proteins and the cellular origin. Such phenotyping of EMVs is of crucial importance for our future understanding of their biological roles, since the easily accessible sources for EMVs, such as plasma, urine, saliva and mucus, consist of complex mixtures of EMVs released from many different cell types. To tackle this challenge, Dragovic et al. recently modified and further developed the conventional NTA technology with the combination of fluorescent labelling [147]. Thus fluorescent NTA has been designed for the simultaneous detection of both microvesicles and exosomes that had been labelled with stable fluorophores (quantum dots) conjugated to antibodies or other biological probes for excitement with a 405-nm violet laser and measurement of fluorescence emission using a matched 430-nm long-pass fluorescence filter and a sensitive fluorescence camera. Under the fluorescence mode only the fluorescently labelled EMVs are tracked, individually and in real-time for the subsequent calculation of the size and concentration of the labelled EMVs. The light scatter mode can operate in parallel for evaluation of the total number of EMVs and/or other light-scattering particles. For instance, three videos of either 30 or 60 seconds may be recorded for each EMV sample with shutter speeds of 30, 6 and 1 milliseconds for the 100-, 200- and 400-nm control beads and with shutter speeds of 30 and 15 milliseconds for the EMVs samples. NTA 1.1 and 2.1 software (Nanosight) may be used for data analysis.

Dragovic et al. showed for the first time that the combination of NTA with the use of fluorescent antibodies directed toward EMV protein components or fluorescent quantum dot-labelled cell tracker peptides and fluorescence measurement allows the phenotyping and classification of EMVs with regard to their cellular origin, even from total pelleted vesicles out of complex biological fluids, such as human plasma [147]. For this, EMVs released from human placenta in course of perifusion in vitro or EMVs recovered from human plasma were incubated with a fluorescent mouse monoclonal antibody specific for the placental EMV marker protein, NDOG2 [153] and linked to quantum dots. Alternatively, a fluorescent cell tracker peptide conjugated to quantum dots was used for the incubation. The concentrations of NDOG2-positive placental or cell tracker-positive plasma EMVs were found to be slightly and drastically, respectively, lower compared to those of the corresponding total EMVs as revealed by light scattering. These differences are presumably due to contaminations of the perfusate with blood cells and/or EMVs derived thereof, which are recognized by light scattering rather than the anti-NDOG2 antibody, and of the plasma with lipidic structures, such as very-low-density lipoproteins, chylomicrons and lipid droplets, which are detected by light scattering rather than the cell tracker dye, respectively. Upon separation of those lipidic structures with a size similar to, but a buoyant density significantly below that of EMVs [154,155] from the EMVs in course of ultracentrifugation, the numbers of (cell-derived) fluorescence and (total) light scattering counts were determined for the pelleted EMVs and found to be very similar [147].
These data suggested for the first time the principal feasibility of NTA-based single or multiple component analysis of EMVs.

In an analogous set of experiments Soo et al. [111] compared the numbers of EMVs released from various human T-cell lines that can be detected by NTA before and after immunodepletion of the EMVs using anti-human CD45 antibodies coupled to magnetic beads [122]. Only about 50–60% of the total EMVs detected by NTA were accessible for immunodepletion [111], raising the possibility that the EMV population studied was heterogeneous with vesicles of comparable size either expressing or lacking CD45. An alternative explanation is that within an EMV subspecies the variation in size of the exosomes or microvesicles affects the expression of a given canonical marker protein. Thus a (too) small size of exosomes or microvesicles may prevent the inclusion of a certain canonical marker commonly accepted for either exosomes or microvesicles, respectively, in each individual member of that particular EMV subspecies. In consequence, a series of proven markers should be tested in order to define a complete EMV-specific pattern for the protein equipment of EMV subspecies. Altogether, these results are very encouraging for the potential routine analysis of the cellular origin and for further phenotyping of EMVs from complex biological fluids using fluorescent NTA. These objectives may be realized in course of more detailed labelling studies with plasma EMVs and (combinations of) fluorescently labelled antibodies and peptides directed against specific markers for erythrocytes, leukocytes, platelets, macrophages and endothelial cells as well as for a selected panel of tissue cells.

**Conventional NP-based biosensing**

The generation of signatures reflecting in completion or in part, at least, the combination of different components, such as proteins, phospholipids and nucleic acids, that constitutes the specific types of EMVs and EMV subspecies, rather than the identification of single or a few components as is true for the methods described so far represents the objective of biosensing. In principle, biosensors could perfectly fulfill this job of pattern recognition based on the following assumptions and theoretical considerations (Figure 3), but clearly the successful operation of biosensing for the analytics of EMV signatures remains to be demonstrated in the future and will encompass the following steps. The initial step for achieving this goal represents the design of NPs consisting of a gold core with covalently attached tentacle-like aliphatic, positively charged and aromatic substituents at their surface. They have to be produced with multiple structural variations for differential interaction with EMVs through their proteinaceous and phospholipidic surface components and possibly luminal protein as well as miRNA/mRNA constituents after passage of uncharged or amphiphilic NP variants across the EMV membranes. With regard to the biosensing of luminal EMV components, NPs of certain size and structure have been amply documented to efficiently cross biological membranes (and reach the cytoplasmic compartment of many mammalian target cells) through yet incompletely understood molecular mechanisms [156-159]. A multitude of hydrogen, electrostatic, hydrophobic and van der Waals bonds will contribute to the high avidity-interactions of the EMV protein and nucleic acid constituents with the substituted NPs. Analogous NPs which interact with selected serum proteins, viruses, bacteria or cancer cells in selective fashion have already been successfully developed and used as so-called "chemical noses" in very sensitive and specific detection assays [160-162]. For the subsequent step a readily assayable reporter enzyme, such as glucose oxidase, has to be selected and engineered via recombinant means which interacts with those EMV-specific NPs on the basis of high avidity rather than high affinity and selectivity.

It is thought that upon incubation the reporter enzyme, such as glucose oxidase, and the sample EMVs will compete with one another for binding to the NPs depending on the relative avidity and cooperativity [163]. Two possibilities can be envisaged: (i) NPs interact with the glucose oxidase rather than with the EMVs and thereby cause its inhibition, presumably through steric hindrance of substrate (i.e. glucose) binding (Figure 3) or (ii) NPs interact with certain subspecies of the sample EMVs rather than with the glucose oxidase thereby leaving it in the active state. Theoretically, this can be
determined through measurement of the produced protons, preferably by an ion-sensitive field effect transistor (ISFET) assembled onto a biosensor chip and providing small currents which after amplification are transformed by appropriate data software into signatures as hypothetically shown in Figure 4 (upper panel) [163,164]. This technology allows very precise quantitative evaluation of the activity state of glucose oxidase or of alternative proton-generating reporter enzymes. ISFET is optimal for the precise and reliable recognition of subtle differences in avidity of the NP-EMV interaction for different NP and EMV species and thereby contributes to the differentiation of distinct EMV types in a body fluid sample. An additional advantage of the biosensor technology is the possibility for measurements in the high-throughput format upon installing of a microarray of ISFETs onto microfluidic cards and lab-on-the-chips. These configurations should greatly facilitate the analysis of huge numbers of NP-EMV interactions with NP structural variants that is required for the delineation of signatures specific for each EMV subspecies (Figure 4).

In general, the unequivocal identification of EMV subspecies out of total sample EMVs can not be achieved with the use of a single or a few NP structural variants. This will lead to almost identical or similar signatures provoked by overlapping recognition patterns of the various NPs as well as by similar molecular composition and structural features of the various EMV subspecies in the sample. Rather EMV analytics by NP-based biosensing necessitates the reliable assignment of a multitude of structurally distinct NPs to the EMV subspecies. In fact, the intelligent and systematic design of NPs with structurally distinct surface substituents considerably differing in hydrophobicity, hydrogen donors/acceptors, charges and size by combinatorial chemistry and their inclusion in the biosensing array will drastically improve its resolving power. A hypothetical example for typical complex combinations of signatures specific for EMVs, that have been released from different cells and tissues into plasma of normal probands, and generated along six separate cycles of biosensor analysis (Figure 5). In addition to receiving the EMV-specific signatures after multiple cycles of measurement with differently substituted NPs, (Figure 4; lower panel) as is the case for conventional EMV analytics for EMV analytics will ultimately reveal the validity of this paradigm and thereby the usefulness of EMVs as “systems biology-based” biomarkers.

The number of EMV-associated components, which can be analyzed simultaneously, depends on the number of different fluorophores available for conjugation to the differently substituted NPs. For consecutive analysis, it will be sufficient to label the different NPs with the same fluorophore. The interaction of NPs with EMVs is monitored by fluorescence scattering, which replaces the measurement of reporter enzyme activity by ISFET as performed for NP-based biosensing (Figure 5). In addition to receiving the EMV-specific signatures after multiple cycles of measurement with differently substituted NPs, (Figure 4; lower panel) as is the case for conventional NP-based biosensing, NTA-based biosensing could allow concomitant monitoring of the movement (velocity) of individual EMVs and thereby determination of their size and size distribution as well as physical state. Again, future intense experimentation will be required for the

**NTA-based biosensing**

Currently we are trying to integrate the NTA and NP-based biosensing technologies into a single assay format in order to combine the unique advantages of the former, i.e. rapid and reliable determination of the size and number of individual EMVs in a body fluid sample, and latter, i.e. unequivocal annotation of identity and cellular origin of the EMVs released from a multitude of different tissues/cells into a body fluid. For this, the NPs, which have been designed for differential interaction with the sample EMVs, have to be coupled to distinct fluorophores. Only those NPs will be selected for the fluorescent labelling that are known to recognize surface markers specific for a subset of EMVs, such as those released from a specific cell type or in response to a specific stimulus. Those cell type-specific EMV candidate markers have already been elucidated for rodent and human adipocytes, such as Gce1, CD73 [165-171], MFG-E8 [172,173], murine and human macrophages and T cells, such as IL-16, Caspase-1 [174], TCR/CD3 [20], CD45, Alix, Tsg101 [111] as well as human dendritic tumor cells, such as ERBB2 [175] and hopefully their number will increase in course of the ongoing characterization of EMVs released from other cell types using NTA and biosensing. However, it is the current paradigm that cell type-specific EMVs are characterized by specific signatures constituted for by quantitative rather than qualitative differences in the expression level of many (but not all) EMV components and do not differ in the presence or absence of a single typical “marker” component. The ongoing efforts for the introduction of NTA- and NP-based biosensing for EMV analytics will ultimately reveal the validity of this paradigm and thereby the usefulness of EMVs as “systems biology-based” biomarkers.

![Figure 4: Determination of signatures specific for EMVs released into the blood from different types of blood and tissue cells by NP-based biosensing (upper panel) and NTA (lower panel) with measurement of current (upper panel) and fluorescence (lower panel) as the read-outs and using the same six types of NPs indicated by the distinct colors. The signatures obtained with the two read-outs are very similar with regard to the relative strength of the interactions between the individual NP types and EMV subspecies, but differ in quantitative fashion. Thus biosensing with both read-outs will reliably provide signatures that unequivocally identify the subspecies and cellular origin of EMVs in the blood (see text for details).](image)
Exerted by a multitude of distinct EMV-specific markers by parallel different fluorophores (Figure 5), determination of the signature of EMV-specific markers by parallel analysis using NPs labelled with the same fluorophore, determination of the identity and amount of EMV-specific markers by consecutive analysis using NPs (Figure 3), determination of the identity and amount of absence of NPs (Figure 2), determination of the signature/identity the size distribution, total number and physical state of EMVs in the following operation modes are conceivable: determination of the impact of NPs on EMV movement/velocity, the hydrodynamic radius of the NPs has to be adapted to the size of exosomes or microvesicles, respectively. From a simplifying point of view, NTA (in its fluorescent version) and NP-based biosensing differ only in the use of fluorophore-conjugated antibodies vs. NP, respectively. But this makes the critical difference. The creation of EMV-specific signatures follows an “evolutionary” process according to the needs at the time point of assay implementation. Their informative value, i.e. resolving power, can be gradually increased with the number and quality (with regard to avidity and selectivity) of the NPs included in the assay. The synthesis of NP surface substituents starting from previously adequate structures follows the rules of combinatorial chemistry and can be automated thereby resulting in a huge variety of structural variants in short time. This optimization process is conveniently followed, for instance by NP-based biosensing, and terminated upon fulfillment of the present criteria for the signature. Those NPs may form the substrate for further optimization in the case of future challenges. Thus there is no need for re-inventing the wheel in order to meet higher demands for EMV interaction/recognition. In contrast, this case would require the generation and identification of novel antibodies with improved affinity and specificity by chance in time-consuming and cost-intensive processes.

In the ultimate and ideal experimental setting of NTA-based biosensing, the following operation modes are conceivable: (i) Determination of the size distribution, total number and physical state of EMVs in the absence of NPs (Figure 2), (ii) Determination of the signature/identity and number of specific EMVs by consecutive analysis using unlabelled NPs (Figure 3), (iii) Determination of the identity and amount of EMV-specific markers by consecutive analysis using NPs labelled with the same fluorophore, (iv) Determination of the identity and amount of EMV-specific markers by parallel analysis using NPs labelled with different fluorophores (Figure 5), (v) Determination of the signature exerted by a multitude of distinct EMV-specific markers by parallel analysis using NPs labelled with different fluorophores and quenching/shifting of the emitted and scattered fluorescence (Figure 4; lower panel). For gaining a complete picture about the nature of the EMVs in a body fluid sample it may be useful to integrate two or more of these operation modes into automated sequential (or if possible parallel) cycles. Future experimentation will reveal which of these hypothetical operation modes can actually be realized on the basis of the currently available instrumentation and whether the NTA-based biosensing will considerably improve our tool box for a more thorough understanding of the systems biology of EMVs [176-190].

Conclusions

EMVs released from many cell types into body fluids in response to specific (environmental) stimuli during the pathogenesis of complex diseases form the intermediary scale between (macro) molecules and cells with regard to size, volume and complexity in their molecular composition and thus are difficult to analyze. Importantly, the size and composition of EMVs seem to rely on their biogenesis and to determine their biological roles. The development of novel technologies, in particular those that are based on the recognition of signatures encompassing the protein, lipid and nucleic acid components, as well as physical characteristics, such as size, curvature and surface charge, rather than on the measurement of single or a few parameters, only, will significantly facilitate the unambiguous assignment of EMV subtypes, their cellular origin and their relevance for the prediction, diagnosis and therapeutic monitoring of common complex diseases and contribute to a better understanding of their pathogenesis from a systems biological point of view [190-203].

References


Figure 5: Proposed configuration of the instrumentation for NTA-based biosensing for the determination of EMV-specific signatures (see text for details).


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