TechNote TN-10

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Title: Extracellular vesicles (EVs) as a novel source for biomarker development – analysis pitfalls and important considerations

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Scope

Extracellular vesicles (EVs) are membrane-enclosed particles that can be released by almost any cell. They were first described in 1967 in a paper reporting the release of membrane particles, termed 'platelet dust', from activated platelets [1]. It is now widely accepted that EVs play an important role in intercellular communication. The fact that healthy and diseased cells release vesicles with distinct RNA cargo to the extracellular environment has only been recently realized and led to the investigation of EVs as potential resource of biomarkers for various applications from pregnancy [2] to oncology [3].

The aim of this technical note is to address the promises, major challenges and potential application areas of EVs.

Background: Origin and classification of EVs

Many different names have been used to refer to these vesicles released including ectosomes, microparticles, and shedding microvesicles, just to name a few. In order to bring harmonization to the field, researchers are now encouraged to use the term "extracellular vesicles (EVs)" as a generic term for all secreted vesicles [4]. Although confusion on the nomenclature of EVs has spread throughout the literature, EVs can be broadly classified into exosomes, microvesicles (MVs) and apoptotic bodies according to their cellular origin as shown in figure 1 and table 1.

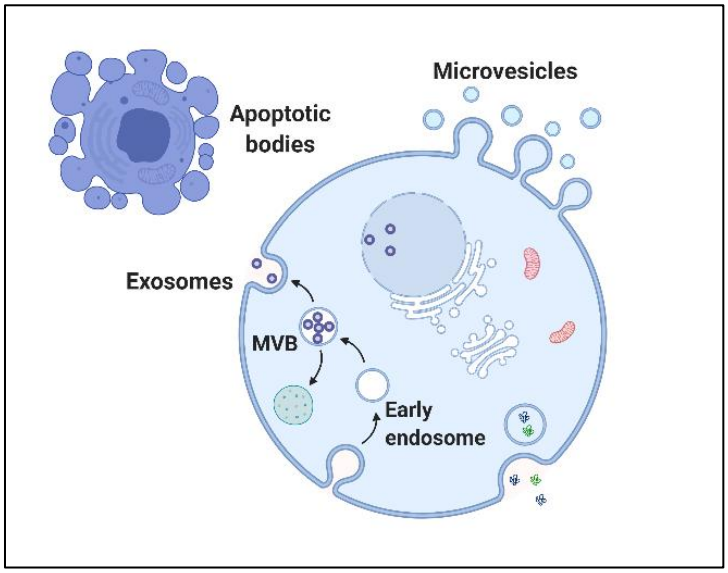

Figure 1: Schematic of the formation and release of extracellular vesicles (Source: www.biorender.com)

Table 1: Classification of EVs

	Exosomes	Microvesicles	Apoptotic Bodies
Origin	Endocytic pathway	Plasma membrane	Plasma membrane
Size	40-120 nm	50-1,000 nm	500-2,000 nm
Function	Intercellular communication	Intercellular communication	Facilitate phagocytosis
Contents	Proteins and nucleic acids (mRNA, miRNA and other non-coding RNAs)	Proteins and nucleic acids (mRNA, miRNA and other non-coding RNAs)	Nuclear fractions, cell organelles

During their formation, EVs incorporate various bioactive molecules from their cell of origin, including membrane receptors, soluble proteins, nucleic acids (mRNAs and microRNAs) and lipids, which can be transferred to target cells [5].

Extracellular vesicles as novel source for biomarker development

The U. S. Food and Drug Administration defines the term biomarker as "a defined characteristic that is measured as an indicator of normal biological processes, pathogenic processes, or responses to an exposure or intervention, including therapeutic interventions" [6]. Therefore, in order to apply EVs as biomarkers there is a need to standardize methodology and develop technologies that make the isolation and detection of EVs reproducible and scalable to the high throughput of a hospital laboratory. In the following, we will discuss the key aspects of EV analysis.

1. Why to use EVs as a novel source of biomarkers?

Because EVs are released in-vivo from many different cell types, biofluids (so-called "liquid biopsies") can be used as a basis for the isolation of EVs. This offers a number of advantages over other diagnostic methods:

a) Specific cargo: Overall EV levels are often elevated in disease – a finding that has been proposed as a simple disease measurement tool in itself, but also caused skepticism over disease specific value. Thus, a 'general stress signal' view was adopted by many [7,8]. However, repeated evidence has emerged of EVs being loaded with specific molecular components (RNAs, proteins, and lipids) that reflect the status of the parental cell, and that are enriched in a disease-specific manner [8–10].

b) Stability: The lipid bilayer of EVs contributes further to diagnostic utility by protecting biomacromolecules from RNases, proteinases, and other enzymatic activity present in biofluids. For example, in a multiplex study on ovarian cancer

patients which identified eight miRNAs for discrimination of ovarian cancer from benign ovarian disease, miRNA levels were not altered by pre-analytical variables such as collection and storage time [11]. Thus, EVs are very stable allowing storage for an extended period of time, in contrast to many biomarker assays that require processing of fresh biofluids [12]. Thus, analysis of biomarkers within the EV fraction of biofluids promises a potential solution against poor analyte stability and deviation from sample handling standard operating procedures (SOP), factors well known to confound the outcomes of clinical trials [8,13].

c) Sensitivity: Notwithstanding therapeutic relevance and sampling robustness, EV-based analysis offers a substantial statistical advantage in reducing biological matrix complexity and thereby overall assay noise. This enables a more specific and sensitive detection of low abundant biomacromolecules [8,9] or analytes with varying levels between sub-compartments of a complex biological matrix. Several studies reported increased sensitivity for EV-based biomarkers compared to whole serum and urine biomarkers [14,15]. For instance, miRNAs found in EVs isolated from sera of patients with colorectal cancers showed higher sensitivity (90%) compared to serum biomarkers CEA and CA19-9 (30.7 and 16% respectively) [14].

2. EV – isolation and measurement - getting closer to a gold standard

In theory, EVs can be purified solely based on their physicochemical properties, because they are larger in size than the protein fraction yet smaller than whole cells, denser than the lipid fraction and with a rather narrow density range. In addition to these physical properties, EVs possess a palette of surface markers specific for the parent cell type. Thus, there are many possible approaches for EV isolation (Table 2).

Table 2: Methods for EV isolation (adapted from Konoshenko et al. 2018[16])

Method	Time	Advantages	Disadvantages
Ultracentri-fugation	140 - 160 min	Low cost, isolation can be performed from large input volumes, absence of additional chemicals.	Expensive equipment, complexity, non-exosomal impurities, low reproducibility, low RNA yield, damage of exosomes; efficiency is affected by the type of rotor, force g, sample viscosity; low throughput.
Density gradient	250 min to 2 days	Pure preparations; no contamination with viral particles when using iodixanol as density	Complexity, loss of sample, ultracentrifugation; fails to separate large vesicles with similar sedimentation rates; contamination with viral

		gradient medium; absence of additional chemicals.	particles when using sucrose as density gradient medium; low throughput.
Size-exclusion chromato-graphy (SEC)	1 ml/min + column washing	Reproducibility and purity; preserves vesicle integrity; use of the buffers with a high ionic strength enhances elimination of nonspecific impurities; high sensitivity, no losses, scalability, large amount of exosomal proteins; prevents EV aggregation; insensitive to high viscosity of samples; no additional chemicals.	Limitations on sample volume and number of separated peaks (necessary difference of the components in molecular weight, ≥10%); specialized equipment; complexity; co-isolation of large protein aggregates and lipoproteins; low throughput; cost.
Precipitation with polymers	65 min	Cost and simplicity of procedure; preservation of EV integrity; no need of additional equipment; pH close to physiological range; high ion concentrations.	Contamination with and retention of the polymer and soluble proteins.
Two-phase isolation - incubation in PEG-dextran mixture	75–195 min	Cost; simple procedure; no EV deformation; purity; efficiency; no protein contamination in the EV fraction; preserving the integrity of EV membranes	Repeated replacement of PEG phase and presence of polymer

a) Ultracentrifugation

The most widely used method remains centrifugation in its variants density gradient, differential and ultracentrifugation. The small size of EVs is selected for in a number of sequential centrifugation steps, starting at low speed (300 g) to remove any intact cells or cell debris, and continuing at higher speeds to obtain fractions enriched in microvesicles (20,000 g) and exosomes (100,000 g). The sedimentation behavior of EVs can be modified by using density gradients, allowing separation from proteins and other components. Although processing times can be quite long, a clear advantage of centrifugation is the possibility to process larger volumes, such as collected cell culture supernatants. The use of centrifugation is losing popularity since the report of (lipo)protein and/or RNA-protein complex contaminations and loss of EV integrity after pelleting [17,18].

b) Size Exclusion Chromatography (SEC)

SEC makes it possible to separate the molecules differing in their hydrodynamic radius and is widely used for separation of biopolymers (proteins, polysaccharides, proteoglycans, etc.). As has been shown, this method can also be used for efficient separation of EVs from protein complexes and lipoproteins using diverse samples such as human blood plasma, urine or cell-conditioned medium [19–22]. However, as samples are often diluted during SEC, this might lead to reduced sensitivity which can be counteracted by combining SEC with concentration of samples using ultracentrifugation [23] or ultrafiltration [22,24].

Taken together, there is no gold standard for EV isolation. The optimal method of EV purification depends, as often, on the characteristics of the starting material and the demands of the downstream applications.

From our own experience: At TAmiRNA, we are mainly using SEC in combination with ultrafiltration of SEC-fractions to reduce working volumes and increase EV concentrations. We have observed high purity of EV fractions (little to no protein contamination) and high-quality small RNA-seq and RT-qPCR results.

3. Qualitative and quantitative characterization of EVs

The high research interest in EVs combined with its challenges resulted in the development and implementation of a large variety of approaches and technologies to quantify and characterize EVs. However, thus far, no single technology has been shown to capture the full spectrum of EV properties (size distribution and number of EVs of all sizes) in complex biological or clinical samples.

The most popular approaches in recent literature are: immunoblotting (a) of specific proteins to confirm EV origin, transmission electron microscopy (TEM) (b) to confirm EV structure and nanoparticle tracking analysis (NTA) (c) and flow cytometry (d) to quantify the number of EVs in a sample volume and their size distribution

a. According to the most recent “Minimal Information for the Study of EVs” (MISEV2018) guidelines[25] the presence of EVs should be demonstrated by the analysis of at least one transmembrane protein associated to the plasma membrane (e.g., CD9, CD63, CD81) and one cytosolic protein in EVs (e.g., TSG101, ALIX). Moreover, for EVs isolated from biofluids (e.g., urine, plasma), the Internationl Society for Extracellular Vesicles (ISEV) recommends additional quantification of common protein contaminants often co-isolated with EVs (e.g., apolipoproteins, albumin, uromodulin) to assess the purity of EVs [26].

b. The most direct method to determine the size and morphology of individual EVs is electron microscopy (EM). Importantly, to assess the heterogeneity in size of EVs within a sample, the MISEV initiative suggests analysing a sufficient number of overview images containing multiple EVs accompanied by close-up images of single EVs [26].

c. Several commercial NTA operating platforms have been developed. The method is based on recording a time-lapse of particles undergoing Brownian motion by imaging them using either scattered light (Sc-NTA) or emitted fluorescence (Fl-NTA) [27]. By analysing a large number of individual trajectories, it is possible to make an estimate of the particle concentration and size distribution even in polydisperse samples.

d. Fluorescence-triggered flow cytometry (FT-FC) can be used for reproducible quantification of extracellular vesicles using a fluorescent lipid dye [28]. In addition, this method can also be used for simultaneously demonstrating the presence of EV markers (such as CD9, CD63, CD81) by combining the lipid dye with fluorescently labelled antibody staining, or DNA/RNA cargo by combining with SYBR® Gold staining.

From our own experience: At TAmiRNA, we are routinely using NTA and FT-FC analyses to characterize the concentration and size of enriched EV-fractions before applying defined amounts of EVs to downstream analyses such as RT-qPCR, NGS or protein analysis. Our NTA/FT-FC EV characterization can be combined with fluorescence-based analysis of nucleic acid cargo (SYBR® Gold) or detection of specific surface epitopes.

4. Quantitative (mi)RNA analysis

In principal there are two established techniques for the quantification of RNA molecules in total RNA extractions from extracellular vesicles.

a. Next-generation sequencing (NGS) analysis is a so-called genome-wide and “untargeted” (no pre-selection of RNA species required) methodology. Small RNA-seq analysis generates information for several types of small RNAs such as tRNA, rRNA, piRNAs, microRNAs and microRNA isoforms. NGS analysis is a cost-efficient platform for high-throughput analysis of thousands of RNA molecules.
b. Real-time quantitative PCR (RT-qPCR) is the most sensitive and reproducible method to quantify RNA transcript levels, and is extensively used in biomarker discovery, validation and clinical diagnostics. RT-qPCR is ideal for profiling smaller panels of (pre-selected, e.g. based on NGS data) microRNAs in many samples and yields highest data quality.

Proper data normalization is a key obstacle encountered in biomarker discovery and validation based on EV-enclosed miRNA. Many algorithms and strategies for RT-qPCR based data normalization are used, while there is still lack of consensus on issues such as stably expressed reference genes and the differential expression bias produced by outliers, often complicating the comparability of data sets produced by different laboratories. Figure 2 summarizes all steps that are necessary for in-depth characterization of EVs in biomarker research.

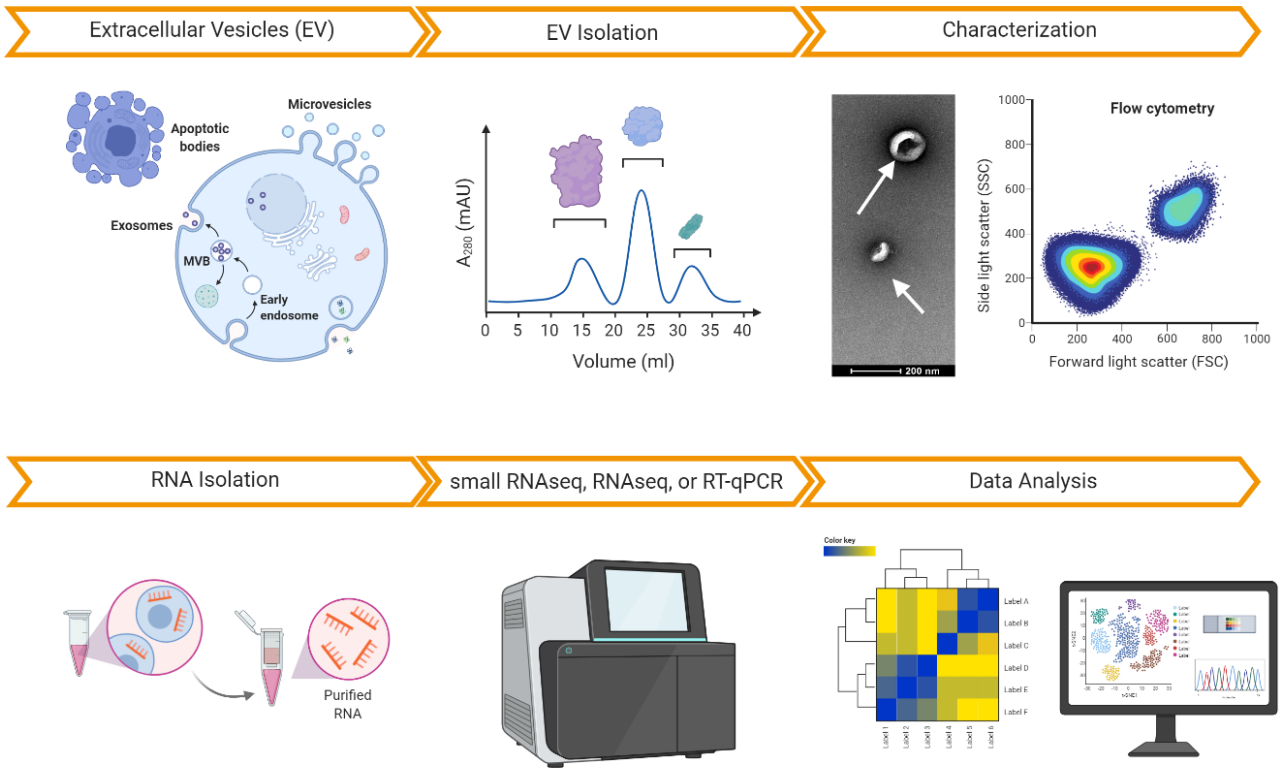

Figure 2: Summary of all important steps from EV isolation to data analysis, which are offered by TAmiRNA as a one-stop-shop service (source: www.biorender.com)

From our own experience: NGS analysis has become our platform-of-choice for conducting genome-wide analysis of non-coding and coding RNAs in biological matrices. We have successfully applied NGS to analyse the RNA cargo of EVs purified from cell culture media, plasma, urine, and synovial fluid. Compared to other screening technologies such as microarrays, low-density qPCR arrays, or other technologies (e.g. Nanostring), NGS shows high cost-efficiency and provides the deepest insights into the transcriptomes of biological samples. However, NGS assays need to be carefully optimized according to the sample type as well as upstream laboratory methods (e.g. RNA extraction and EV isolation).

Extracellular miRNAs as potential candidates for therapy

The use of miRNA-containing complexes for therapeutic applications harbors a number of potential. Given the relatively easy access to these complexes, particularly circulating EVs, one could envision collecting EVs from healthy donors and injecting them in patients to treat disease (Figure 3).

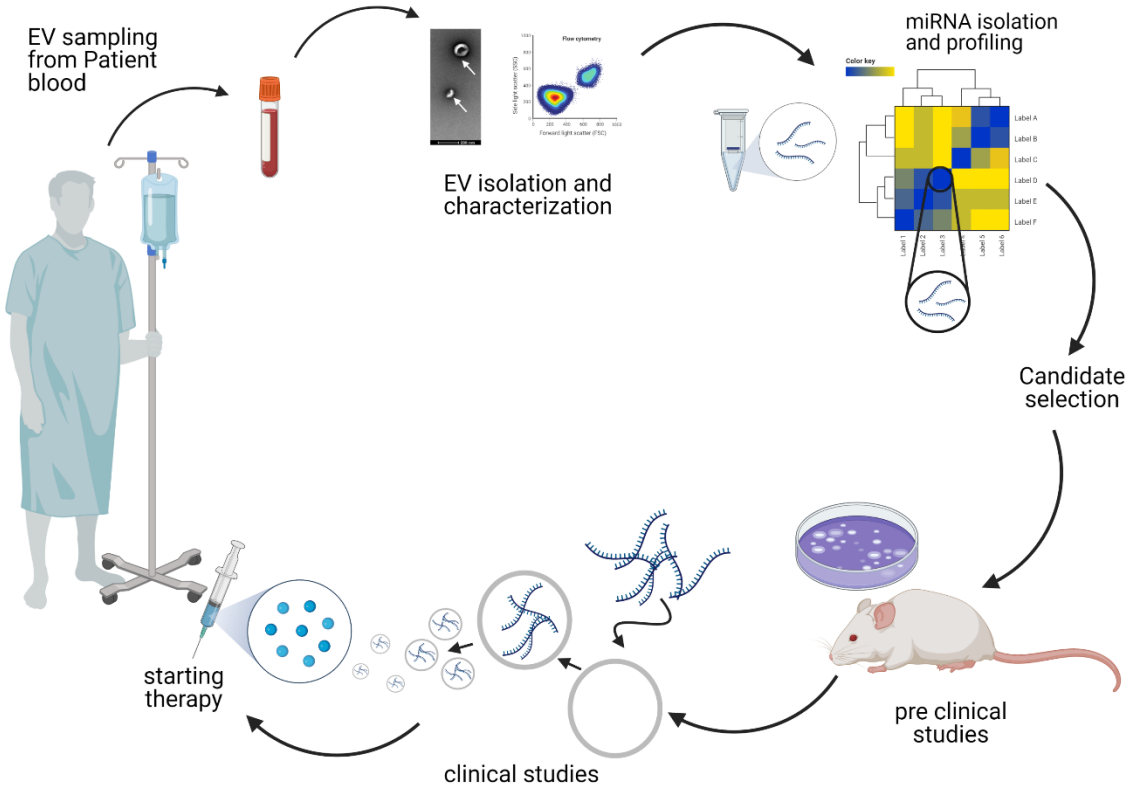

Figure 3: From EVs to therapies (source: www.biorender.com)

Currently, the diagnostic potential of circulating exosomes and their microRNA cargo has been recognized and is being explored in a variety of diseases. In particular, miRNA and extracellular vesicles are discussed as putative early prognostic indicators in a wide variety of different acute and chronic liver diseases. Barutta et al. reported an increase in extracellular miR-145 in patients with early diabetic nephropathy and microalbuminuria, as well as in animal models of diabetes [29]. Cell culture experiments demonstrated that high glucose levels induced mesangial cell expression of miR-145, probably through increased levels of transforming growth factor β1 (TGFβ1). Moreover, extracellular miRNA has also been examined as a source of non-mutated RNA to repair damaged or diseased kidneys. Exosomes from healthy Sprague–Dawley rats could transfer wild-type Pkhd1 RNA to polycystic kidney cells in vitro and in vivo, and thereby restrict cyst formation and improve renal structure and function [30]. In humans, a phase I trial investigating the effect of microvesicles derived from cell-free cord blood on β cell mass in type 1 diabetes mellitus is ongoing (NCT02138331) (US National Library of Medicine. ClinicalTrials.gov. 2014. https://clinicaltrials.gov/ct2/show/NCT02138331).

Considered together, these findings indicate that exosomes from urine and other biofluids may provide a source of therapeutic agents and targets for the treatment of renal diseases.

Vision for the future

In the future, extracellular miRNA opens a new era of superior biomarkers. As current techniques evolve, we anticipate that extracellular miRNAs will become a routine approach in the development of personalized patient profiles, therefore allowing targeted therapeutic interventions. To this end, more details about the interaction between EV formation and function are necessary in achieve this knowledge for individualized patient management.

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