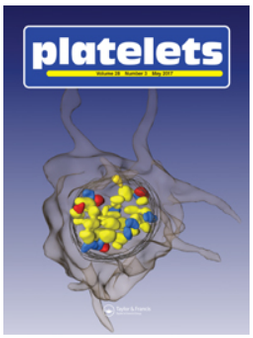

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SPECIAL REVIEW: PLATELET MICROVESICLES

Bulk immunoassays for analysis of extracellular vesicles

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ABSTRACT

There is increasing clinical interest in extracellular vesicles (EV) for diagnostic and treatment purposes. This review provides an overview of bulk immunoassays to analyse EV. Western blot and enzyme-linked immunosorbent assay are still the two predominant bulk immunoassays. Recently, new assays have become available that can detect exposure to EV concentrations that are up to 10,000-fold lower. This is advantageous for applications that detect rare EV. Other important parameters are the detectable concentration range, the required sample volume, whether simultaneous presence of different antigens on a single EV can be detected, size selectivity of each assay and practical considerations. In this review, we will explain the working principles of the traditional and novel assays together with their performance parameters. The most sensitive assays are micro-nuclear magnetic resonance, surface plasmon resonance, and time-resolved fluorescent immunoassay.

KEYWORDS

analysis, bulk, detection, extracellular vesicles, exosomes, immunochemistry

History

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Introduction

There is increasing clinical interest in extracellular vesicles (EV), such as "microparticles" and "exosomes", for diagnostic and treatment purposes. To study EV, bulk assays are widely used because they are affordable, widely available, and applicable to EV research. We define bulk assay as any assay that performs a biochemical analysis on an ensemble of EV. In practice, bulk assays determine one or more biochemical properties from thousands to trillions of EV simultaneously. Because bulk assays analyze an ensemble of EV, bulk assays are generally more sensitive and faster than single EV techniques. A typical disadvantage of bulk assays is that information on the EV concentration, biochemical heterogeneity, and polydispersity is lost [1, 2]. We will limit the scope of this review by exclusion of proteomics, lipidomics, nucleotide sequencing assays, and polymerase chain reaction [3-8], enabling us to focus on bulk immunoassays (BIA). An example of a mechanism employed in BIA is the use of a fluorescent antibody conjugate, where the amount of fluorescence is proportional to the number of antigens. Besides fluorescence, other reporters include enzymes, proximity sensors, radioactive isotopes, and metal nanoparticles. In this review, we will discuss the use of BIA for the detection of EV from any origin, including platelet-derived EV.

For this review, a literature study was performed in Scopus (Elsevier, Amsterdam, The Netherlands; May 2016). Included articles (1) contain at least one BIA in the abstract, (2) apply the BIA to study EV, and (3) were published since January 2010. The search resulted in nine different BIA, which include Western blot (WB) and enzyme-linked immunosorbent assay (ELISA), as well as seven newly developed BIA.

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Classification and assay description

We will classify the nine BIA by whether they (A) are performed after gel electrophoresis, (B) are performed in suspension, or (C) are immunosorbent assays. See Figure 1 for an overview of the principles of operation of all described assays.

A. Gel electrophoresis-based assays

A.1. Western blot (WB)

WB provides information about the presence and molecular weight of an antigen of interest by gel electrophoresis and subsequent labeling of the target antigen [9].

A commonly applied WB procedure starts with EV lysis. Subsequently, all proteins are separated by gel electrophoresis based on differences in their molecular-weight and charge [10-12]. Thereafter, the antigens are transferred or "blotted" by electrophoresis to a carrier membrane, such as nitrocellulose or polyvinylidene fluoride. Subsequently, the sample is incubated with an antibody against the antigen of interest, followed by a second antibody targeting the first antibody. The second antibody is conjugated to horseradish peroxidase or alkaline phosphatase, which catalyzes a colorimetric reaction in a substrate. Alternatively, either enzyme can be utilized to perform conversion of a substrate into chemiluminescence or fluorescence. Readout of the blot is done through digitization of the blot, or directly by eye.

B Assays in suspension

B.1. Micro-nuclear magnetic resonance (μNMR)

μNMR measures the degree of sample magnetization in response to an applied magnetic field.

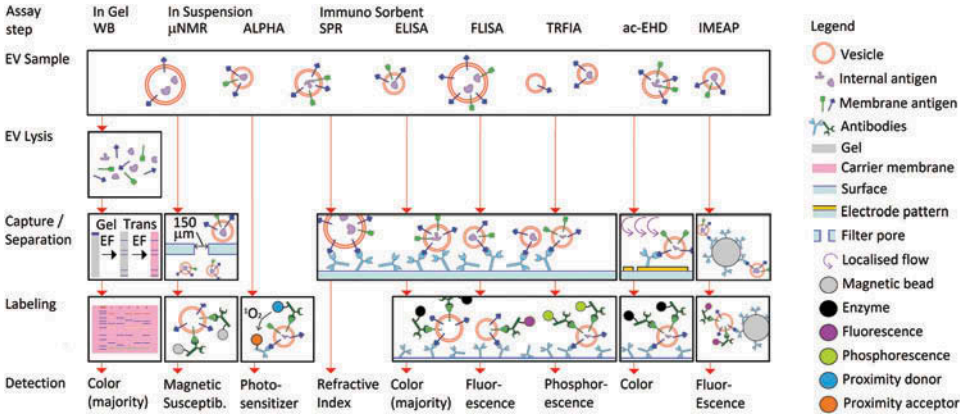

Figure 1. Schematic overview of bulk immunoassays for EV characterization. Western Blot (WB) performs EV lysis, gel electrophoresis (EF) of all protein content, and transfer EF to a membrane for subsequent immuno-labeling. Micro-Nuclear Magnetic Resonance (µNMR) selects EV sizes by filtration, and labels EV with immunomagnetic particles for subsequent detection of the magnetic susceptibility of the sample. Amplified Luminescent Proximity Homogeneous Assay (ALPHA) detects transfer of singlet oxygen from the donor to the acceptor if they are in close proximity, i.e. bound to an EV. Surface Plasmon Resonance (SPR) captures EV on an immuno-functionalized surface, and detects the resulting change in refractive index. The other immunosorbent assays are all variations of the sandwich Enzyme Linked Immuno Sorbent Assay (ELISA), where capture of EV on a functionalized surface is complemented by detection of a different antigen, or epitope. The reporter molecules can be enzymes (ELISA), fluorescent molecules (FLISA, Fluorescent Immuno Sorbent Assay), or phosphorescent molecules (TRFIA, Time-Resolved Fluorescent ImmunoAssay). To overcome the mass transport limitations near a surface, electrohydrodynamic-induced nanoshearing (ac-EHD) generates additional flow near the surface, while the Integrated Microfluidic Exosome Analysis Platform (IMEAP) mobilizes the surface itself by means of immunomagnetic beads.

In the µNMR protocol, EV are labeled with 7 nm magnetic beads in a two-step labeling protocol inside a microfluidic chip. A 150 nm filter at the entrance port limits the maximum EV size, and a 50 nm pore size filter is used to retain the sample inside the chamber during washing of unbound labels. Subsequently labeled EV are detected with an embedded magnetic coil. Biological samples have negligible magnetic susceptibility, allowing detection of immunomagnetic nanoparticles bound to membrane antigens on EV [13]. This results in a ~1,000-fold more sensitivity improvement over ELISA [13]. Selection of different filters allows changes in the size range.

B.2. Amplified luminescent proximity homogeneous assay (ALPHA)

A proximity assay measures the emitted signal as a function of the proximity of two components of the detection system by means of some energy transfer process.

In the ALPHA system two different types of beads are used. A donor bead releases singlet oxygen ($^1O_2$) upon excitation, and an acceptor bead emits light upon uptake of $^1O_2$. Due to the rapid decay time of $^1O_2$ to the stable triplet oxygen ($^3O_2$), these beads need to be within 200 nm of each other. Therefore, a signal is only found if both donor and acceptor are held near to each other, for example on two antigens of an EV of interest [14]. The emitted light intensity of these "bead-EV-bead" complexes correlates linearly with the number of EV. The assay can be performed in a 96 well plate format and requires a plate reader dedicated to the proximity luminescent assay.

C. Immunosorbent assays

C.1. Surface plasmon resonance (SPR)

SPR detects the refractive index change due to immune mediated adhesion of EV at a detection surface.

In the basic protocol, the sensor surface is coated with antibody, followed by washing the sensor and blocking any exposed sensor surface by incubation with a protein like bovine serum albumin. Then, the sample is loaded into the sensor, and left to incubate. EV are captured by antibodies at the surface. This capture results in a refractive index change which can be monitored in time. The time data allow the study of kinetics of interaction between EV and the surface. Because the refractive index contrast between buffer and EV is sufficiently large, no reporter is needed for readout [15-18], which is why SPR is sometimes referred to as a label-free technique. The change in refractive index provides information on the amount of captured material [19]. Different commercial systems exist, and each detection system requires dedicated sensor chips. The sensor design allows a single, or up to 96 parallel antigen measurements.

C.2. Enzyme linked immunosorbent assay (ELISA)

The sandwich ELISA performs immune mediated EV capture at a surface and reads out the captured quantity through a secondary labeling procedure. All the immunosorbent assays discussed in the rest of this section are variations of the sandwich ELISA assay.

A sandwich ELISA begins with the coating of a capture antibody to the well(s) of a microplate. EV samples, control samples and, if available, a reference standard containing protein of interest, are all pipetted into different wells. After incubation and washing, a second antibody targeting the captured EV is added. After again incubation and washing, a third antibody targeting the second antibody is added. This third antibody is linked to an enzyme, for example horse radish peroxidase. The amount of enzyme can be measured analogous to WB. Although colorimetric quantification is encountered most frequently, chemiluminescence may be more sensitive [20].

The ELISA method is a benchmark for quantitation of antigens in solution. This quantitation relies on calibration with a standard containing a known concentration of protein of interest, for example a recombinant antigen. However, for intact EV this calibration is of limited value because the diffusion of EV toward the capture surface is 10-100-fold slower than the protein solution used for calibration.

C.3 Fluorescent immunosorbent assay (FLISA)

FLISA is an ELISA-based assay where the captured quantity is determined through a fluorescent antibody conjugate.

A recent example applied to EV is the "EV array" [21, 22]. The EV array utilizes slides printed with an array of antibodies to capture EV and then determines the amount of captured EV by

labeling with a cocktail of biotinylated CD9, CD63 and CD81 and the use of streptavidin-Cy5 as a reporter molecule. Advantages of the use of a fluorescent reporter molecule instead of an enzyme may be an increase in sensitivity and a less time sensitive protocol. However, the auto fluorescence of the plastic of standard 96 well plates limits the detection of low density antigens. To mitigate this auto fluorescence, the EV array is performed on glass microscopy slides with an "ArrayIt" system to temporarily generate 96 wells.

C.4. Time-resolved fluorescent immunoassay (TRFIA)

TRFIA is an ELISA-based assay where the captured quantity is determined through a phosphorescent antibody conjugate

This exploits the difference in time delay in emission of phosphorescent molecules relative to auto fluorescence of the sample and sample holder. For example, the amount of bound antibody labeled with europium is quantified using a dedicated time delay plate reader [23]. The time delay measurement results in an ~10-fold improvement in sensitivity over ELISA and FLISA due to elimination of the background signals of the sample and the 96-well plate.

C.5. Alternating current electrohydrodynamics-induced nanoshearing (ac-EHD)

ac-EHD is an ELISA-based assay in a microfluidic chip, in which capture of EV is enhanced 3-fold by a local flow induced by an electric field [24].

The electric field induced local flow also causes an increase of shear at the capture surface, which enhances the release of weakly bound EV, thus reducing non-specific signal. Enzyme-based detection has been demonstrated [24], but there is no fundamental limitation to the application of fluorescence or phosphorescence.

C. 6. Immunomagnetic beads in a microchip ELISA (IMEAP)

IMEAP is an ELISA-based assay, where the capture surface has been mobilized onto immunomagnetic beads.

In the microchip, EV-bead complexes are captured through the application of a magnetic field for all washing steps and for sample enrichment. This has been demonstrated in the "Integrated microfluidic exosome analysis platform" (IMEAP) [20]. In IMEAP, chemiluminescent detection is performed by means of a sandwich assay similar to ELISA. If desired, the captured EV can be lysed in a follow up analysis, and the released proteins are recaptured, but now with magnetic beads conjugated to the antibody of interest prior to detection.

The main advantage is that the capture surface is made mobile, thus potentially increasing the contact between capture surface and sample, and thus reducing the limitations imposed in ELISA by the relatively slow diffusion of EV. However, it is unknown what the gain is, and we encountered only one immunomagnetic bead capture assay.

Comparison of BIA performance

To determine the usefulness of BIA in a clinical setting, we have compared the described methods with regard to their limit of detection, the concentration range that can be handled, the required sample volume, and the ability to perform multiple experiments in parallel. The results of this comparison are shown in Table 1 and discussed below.

Limit of detection

For BIA, we define the limit of detection as the minimal required EV concentration for the signal to reliably exceed the noise. Although the limit of detection is a critical parameter there is no uniform method to define nor determine the limit of detection.

In the literature, the limit of detection is expressed in μg antigen, μg antigen/mL, total number of EV, number of EV/mL, or compared to another assay, such as ELISA. The variation in definitions is most extreme in the case of SPR, where authors have determined the detection limit as number of EV/mL [16], μg EV/mL [17], or number of EV/cm^2 [18]. While each of the units for limit of detection are sensible for some of the discussed assays, we selected number of EV/mL because it is definable for all assays. The alternative μg antigen/mL is not definable for all assays, and μg antigen or EV alone can be misleading for methods that do not see the whole sample volume.

To determine the limit of detection of a method, NTA is frequently used to determine the concentration of EV in a reference sample. NTA does not detect the concentration of EV, but the concentration of all particles inside the detection size range (typically 80-500 nm [25]). Furthermore, a wide array of reference samples is used, including EV derived from different cell cultures [13, 14, 16, 17, 20, 21, 23, 26] and/or plasma [21, 27] were used. All these factors lead to considerable uncertainty in the limit of detection. Therefore, we report the order of magnitude of the limit of detection. The actual

Table 1. Comparison of bulk immunoassays for extracellular vesicles (EV).

	In gel	In Suspension		Immunosorbent					
Method	WB	μNMR	ALPHA	SPR	ELISA	FLISA	TRFIA	ac-EHD	IMEAP
Limit of detection (EV/mL)	10^{12} ⁰	10^{7} †	10^{10} ‡	10^{7} *	10^{10} *	10^{10} †	10^{9} ⁰	10^{9} ‡	10^{8} ‡
Dynamic range◇	10^{2}	10^{2}	10^{3}	10^{6}	10^{3}	-	10^{5}	-	10^{4}
Sample volume (μL)	10-1000	1-100	5	20-150	100	1-10	100 ⁰	500	30
Targets on same EV (n)	1	1	2	1	2	2	2	2	2
Parallel experiments	10	3	96	96	96	96	96	96	1
Analysis duration (h)	3	5	3	2	2	24	8	4	2
Articles (n)	49	1	1	5	18	2	1	1	1
Data references	[27]	[13]	[14]	[16, 17]	[16, 20]	[21]	[23]	[24]	[26]

Source: * Directly provided, † calculated from minimal number of EV /sample volume, ‡ relative to ELISA with sensitivity of 10^{10}/mL, ⁰ Inquiry with author, ◇ maximum/minimum detected signal

WB = western blot, μNMR = micro-nuclear magnetic resonance, ALPHA = Amplified Luminescent Proximity Homogeneous Assay, SPR = surface plasmon resonance, ELISA = enzyme-linked immunosorbent assay, FLISA = Fluorescent immunosorbent assay, TRFIA = time-resolved fluorescence immunoassay, ac-EHD = alternating current electrohydrodynamic-induced nanoshearing, IMEAP = integrated microfluidic exosome analysis platform

limit may still prove to be 1-2 orders of magnitude different if all BIA were to be compared using a uniform definition for limit of detection, and with a single culture cell line and a single antibody clone. The latter two factors are important to ensure that the EV antigen exposure level and the affinity of the antibody do not influence the comparison. Comparison of new BIA assays would greatly be facilitated by standardization of the method to determine the limit of detection.

The reported or calculated detection limits range between 10^7 and 10^{12} EV/mL. WB and ELISA are both relatively insensitive, and require - on average - 10^4-fold higher concentrations of EV than SPR and μNMR to allow detection [13, 16].

Dynamic range

The highest detectable EV concentration is the concentration at which the signal is nearest to saturation. At the saturation level, an increase in EV concentration no longer results in an increase in signal. The lowest detectable concentration of EV is the limit of detection, earlier defined as the EV concentration at which the signal is just discernible from noise. The dynamic range is the ratio between the highest and lowest detectable concentration. This parameter is important for quantitative measurements, because with a low dynamic range samples need to be analysed at multiple dilutions, adding time and cost to the assay. Because the dynamic range was not stated in any publication, we derived a lower boundary for dynamic range from the maximum measured signal relative to the limit of detection. The order of magnitude is given, with an uncertainty less than one order of magnitude.

WB has the lowest dynamic range, approximately 100:1. Partially because of this low dynamic range, WB is typically not used quantitatively. TRFIA and SPR have the highest dynamic ranges of 10^5 and 10^6, respectively.

Sample volume

Assays with high minimum EV concentration may need to concentrate their samples prior to measurement. Therefore, we define the sample volume as the total (initial) volume required for analysis per measurement. This sample volume varies between 1 and 500 μL for the different BIA and therefore is no limitation for conditioned cell media and commonly used body fluids in EV research, such as blood plasma or urine. However, a sample volume of 500 μL may pose a problem for murine experiments or measurements on neonate blood samples.

Number of targets

This parameter describes the capability to detect a combination of one, two or more antigens on the *same* EV. Typical single antigen assays are WB, SPR, and μNMR. These assays may be capable of measuring multiple targets in parallel experiments, but this will not prove that EV in the sample expose all targets simultaneously. The sandwich ELISA and the fluorescent and phosphorescent analogues thereof, as well as ALPHA may detect two antigens on the same EV by targeting a different antigen with the primary and secondary antibody. No BIA was found that could measure more than two targets on the same EV.

Parallel experiments

Parallel experiments allow analysis of multiple samples and/or targets, and therefore are advantageous for analysis of biorepositories. For parallel samples, the 96 well plate compatible ELISA and fluorescent/phosphorescent analogues together with the proximity assay Alpha have the best possibilities. For parallel targets, the imaging version of SPR can measure up to 96 different antibodies on a single sample.

Analysis duration

The overall analysis duration is estimated from the described methods in literature and represents an estimate of the time required to prepare and perform an experiment. It includes the time required for sample preparation, surface or bead modifications, sample incubation and the actual detection. Performing a set of parallel experiments does not change this duration for any BIA if the increase in time due to pipetting actions is neglected.

Most BIA require two to four hours to prepare and perform an experiment, but FLISA and TRFIA require 24 and 8 hours, respectively, due to longer sample incubation steps [21, 23]. Longer incubation allows more contact between sample and capture surface, and thus longer incubation may benefit the limit of detection for ELISA, SPR, and ac-EHD. However, the sample integrity may be compromised if the time to result becomes too long.

EV size

All discussed BIA have some EV size dependence in their detected signal. For example, in all surface-based immunosorbent assays the transport of EV to the surface is limited by diffusion [17]. Diffusion scales with the inverse of the diameter, so a 50 nm EV will have a 20-fold higher diffusion constant as a 1,000 nm EV. This means that while surface-based immunosorbent assays may detect EV of all sizes, they will detect a lower proportion of larger EV. On the other hand, it is easier to lyse a large EV [28], which means that assays that require lysis of EV may measure proportionally more large EV. For ALPHA, which uses two antigen targets, a larger EV presents a higher chance that a singlet oxygen is absorbed by an acceptor bead. μNMR limits the size of EV in the sample to 50-150 nm by means of filtration [13]. Note that this latter size range is inferred from the filter pore diameters, and has not been verified. In fact, for none of the assays described in this review size selectivity has been thoroughly studied, although a theoretical basis has been described for SPR [17]. Moreover, in the case of ELISA and its analogues, calibration is needed prior to quantification, in part to correct for the sample volume that comes in contact with the capture surface. The typical reference is a concentration series of recombinant antigen in solution, but this does not account for the 10-100-fold difference in diffusion constant between EV and antigens in suspension.

Differentiation between intravesicular and extravesicular domains

If the assay requires lysis of EV, it is not possible to differentiate between whether the target antigen was found on the outer surface of the EV, or intravesicular. The only assay that requires lysis is WB. For detection of intravesicular antigens, all other assays allow but do not require lysis.

Popularity

Western blot is widely used [9-12, 29-73] to confirm the presence of EV, through their proteins, but rarely the concentration of EV. ELISA is the most widely used immunosorbent assay [9, 56-76], and detects the concentration of target antigens in a sample. Most other assays discussed in this review were discussed by fewer than three laboratories per assay. SPR has been applied at least five laboratories.

Conclusion

BIA have become an important tool in EV studies. WB and ELISA are established in the field and at least seven more BIA are currently being tested and may become useful for detection and/or biomolecular characterization of EV. Because BIA are mainly used to detect or quantify the presence of specific markers on EV, we expect that the most sensitive assays, μNMR, TRFIA, and SPR, will empower progression of the EV field.

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