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Possibilities and limitations of current technologies for quantification of biological extracellular vesicles and synthetic mimics

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ABSTRACT

Nano-sized extracelullar vesicles (EVs) released by various cell types play important roles in a plethora of (patho)physiological processes and are increasingly recognized as biomarkers for disease. In addition, engineered EV and EV-inspired liposomes hold great potential as drug delivery systems. Major technologies developed for high-throughput analysis of individual EV include nanoparticle tracking analysis (NTA), tunable resistive pulse sensing (tRPS) and high-resolution flow cytometry (hFC). Currently, there is a need for comparative studies on the available technologies to improve standardization of vesicle analysis in diagnostic or therapeutic settings.

We investigated the possibilities, limitations and comparability of NTA, tRPS and hFC for analysis of tumor cell-derived EVs and synthetic mimics (i.e. differently sized liposomes). NTA and tRPS instrument settings were identified that significantly affected the quantification of these particles. Furthermore, we detailed the differences in absolute quantification of EVs and liposomes using the three technologies. This study increases our understanding of possibilities and pitfalls of NTA, tRPS and hFC, which will benefit standardized and large-scale clinical application of (engineered) EVs and EV-mimics in the future.

1. Introduction

Extracellular vesicles (EVs) are lipid membrane-enclosed vesicles released by cells and present in bodily fluids. EVs are heterogeneous in composition and size, ranging from approximately 50 to 1000 nm, with the vast majority <200 nm in size [1,2]. EVs originate from their donor cell as a result of outward budding of the plasma membrane. Alternatively, EVs form as a result of intracellular budding within late endosomes, from which vesicles are released upon fusion of these multivesicular bodies with the plasma membrane [3]. Regardless of their size and origin, 'EVs' is the collective term adopted to designate any type of cell-derived vesicle in the extracellular space. In recent years, multiple reports have demonstrated EVs to play an important role in (patho)physiological processes, such as immune responses [4], blood coagulation [5], tissue repair [6] and tumor growth [7,8]. Current research focuses on obtaining improved insight into the formation and function of EVs and on studying the potential of EVs for medical applications. One of these applications is to use EVs present in body fluids as biomarkers for diagnosis and monitoring of diseases [9,10]. In cancer, tumor-derived EVs can serve as biomarkers since they contain proteins and RNAs from their malignant donor cells [7,8]. Since tumor-derived EVs are released in easily accessible bodily fluids, such as blood or urine [7,11], analysis of these EVs for disease monitoring may circumvent biopsies [11], thereby reducing biopsy related morbidity and mortality. A second important application of EV in the medical field is their use as drug delivery systems. Although liposomes, which share the bilayered membrane structure with EVs, have been employed as drug delivery systems for many years, cross-pollination of knowledge in the liposome and EV research fields now holds high promise for improvement of current delivery systems. Various studies have indicated that EVs can be exploited as carriers for delivery of exogenous therapeutic cargoes, e.g. siRNAs, in vivo [12]. EV characteristics that facilitate efficient delivery of biological drugs include their capacity to traverse intact biological barriers (e.g. blood–brain barrier) and to deliver functional RNA into cells, as well as their stability in blood (reviewed in [13]).

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Current research focuses on exploiting these features to either engineer natural EV for drug delivery to specific tissues, or to design EV mimics formulated as liposomes containing relevant EV components [14].

Even though EVs are increasingly recognized as important biological and therapeutical entities, standardized methods for their analysis are still lacking [15]. Establishment of such methods is crucial for safe application of (engineered) EV in clinical practice, but EV quantification has proven technically difficult due to the small size of EVs and their heterogeneity in size and composition.

In recent years, several instruments have become available that allow detection and characterization of individual EVs. These techniques include nanoparticle tracking analysis (NTA) [16,17], tunable resistive pulse sensing (tRPS) [18] and high-resolution flow cytometry (hFC) [19]. EV detection and quantification with these single-particle analysis techniques rely on distinct principles. NTA is based on the illumination of particles in suspension with a laser beam, followed by the recording of the scattered light by a light-microscope. The Brownian motion of each particle is individually tracked to determine the mean square displacement of the individual particle. Since temperature and viscosity of the suspension are known and controlled, the Stokes–Einstein equation can be used to determine the hydrodynamic diameter of each individual particle. The total number of particles is used for particle concentration estimation [16,20]. In tRPS, a non-conductive polyurethane membrane, punctured to contain a single opening, separates two fluid cells [21]. By applying a voltage across the membrane a flow of ions is induced. Once a particle moves through the nanopore, the flow of ions is altered resulting in a brief "resistive pulse" which is recorded by the instrument [22]. The size-distribution [23] and concentration [24,25] of particles can be calculated by referring the observed pulse height and rate to pulses induced by reference particles of known volume and concentration. Flow cytometric analysis of particles involves the sequential excitation of individual, fluorescently labeled particles in a liquid stream and detection of emitted light by diodes or photomultipliers [26]. In hFC, a high-end flow cytometer is optimized for the analysis of nano-particles. This optimization consists of light scattering detection at customized angles, the usage of high power lasers and high-performance photomultiplier tubes for more sensitive light detection, and application of fluorescence-based thresholding to distinguish particles of interest from noise signals [19]. In-depth description of the technical backgrounds of the techniques is beyond the scope of this manuscript and described elsewhere for NTA [16,20,27,28], tRPS [22–24] and hFC [19,29].

For accurate EV quantification and characterization, it is important to know to what extent instrument-specific variables influence particle characterization. For NTA, studies on how instrument settings affect the analysis of heterogeneous EV populations are limited [20,28,17], and the effects of specific variables on EV quantification and size-profiling by tRPS are largely unknown. For hFC, detailed reports on optimizing the instrument configuration and settings for accurate analysis of EVs and other nano-sized particles have recently been published [19,29]. In a few studies, two or three of the above described techniques have been compared. However, these studies either focused on size-profiling of synthetic beads [30,31], or did not address effects of instrument settings on EV characterization and quantification [32,33].

Here, we report a comprehensive comparative study on NTA, tRPS and hFC for analysis of populations of heterogeneous nano-sized EVs and synthetic mimics (i.e. polystyrene beads and calcein-loaded liposomes). We identified different NTA- and tRPS-variables that significantly influenced the quantification of these particles. Furthermore, we assessed the comparability of NTA, tRPS and hFC in absolute quantification of liposomes and EVs. Based on these data, we stress the importance of technical knowledge of the instruments, awareness of analytical variables, and recognition of how instrument settings affect measurements when analyzing EV populations with unknown concentration and size heterogeneity.

2. Materials and methods

2.1. Polystyrene beads

115 and 203 nm polystyrene beads (Izon Science, Christchurch, New Zealand) were analyzed using tRPS and NTA. For hFC, fluorescent 100 and 200 nm polystyrene beads (yellow–green-fluorescent FluoSpheres, Invitrogen) were used.

2.2. Liposome preparation and characterization

Egg phosphatidylcholine (EPC), egg phosphatidylglycerol (EPG) (Lipoid GmbH, Ludwigshafen, Germany) and cholesterol (Sigma-Aldrich Chemie B.V., Zwijndrecht, The Netherlands) were dissolved in chloroform/methanol (1:1, v/v) in a round-bottom flask in a molar ratio of 2:0.06:1, respectively. A lipid film was prepared by rotary evaporation (Rotavapor R3, Büchi Labortechnik AG, Flawil, Switzerland), followed by drying under a stream of nitrogen. The lipid film was hydrated with 10 mM calcein for 105 nm liposomes or 250 μM calcein for "L146" and "L212" liposomes in HEPES buffered saline (HBS, 10 mM HEPES, 137 mM NaCl, pH 7.4). Liposomes were sized by multiple extrusion under nitrogen pressure using polycarbonate membranes (Nuclepore, Pleasanton, CA, USA) with pore sizes of 200 nm and 100 nm in a Lipex high pressure extruder (Lipex, Northern Lipids, Vancouver, Canada) or a Liposofast Extruder (Avestin, Inc, Ottawa, Canada). Non-entrapped calcein was removed with dialysis against HBS for at least 3 days using Slide-A-Lyzer dialysis cassettes with a cut off of 10 kD (Thermo Scientific, Bremen, Germany). The mean particle size of the liposomes and the polydispersity index (PDI) was determined by means of dynamic light scattering (DLS) using a Malvern ALV CGS-3 with a He–Ne laser source (Malvern Instruments, Malvern, UK). Liposome sizes (L146 and L212) were 146 nm with a PDI of 0.03 and 212 nm with a PDI of 0.07. The zeta-potential of the liposomes (ζ potential) was determined using a Malvern Zetasizer Nano-Z (Malvern Instruments, Malvern, UK). The phosphate concentrations of the liposomes were determined with a phosphate assay described by Rouser et al. [34]. For final use, L146 and L212 liposomes were diluted with HBS till a final total lipid (including cholesterol) concentration of 65 mM.

2.3. Cell culture and EV isolation

The human glioblastoma cell line U87-MG and the lymphoblastoma cell line RN were cultured in medium containing FCS depleted from bovine EVs as described previously [18,19]. After 24 h of incubation the supernatant was isolated and centrifuged at 200 ×*g* for 10 min, two times at 500 ×*g* for 10 min, followed by 10,000 ×*g* for 30 min. 100,000 ×*g* pelleted EVs were resuspended in phosphate buffered saline (PBS) containing 0.2% BSA from an ultracentrifuged stock solution [29]. EVs were fluorescently labeled with 7.5 μM PKH67 (Sigma-Aldrich), mixed with 2.5 M sucrose, overlaid with a linear sucrose gradient (2.0–0.4 M sucrose in PBS) in an SW60 tube (Beckman) and floated into the gradient by centrifugation for 16 h at 192,000 ×*g* [29]. Gradient fractions were collected, diluted in PBS and analyzed. Fraction densities were determined by refractometry.

2.4. NTA

An LM14 Nanosight instrument (Nanosight Ltd, Salisbury, UK) equipped with a CMOS camera (Hamamatsu Photonics, Hamamatsu, Japan) and a 488 nm laser was used. Data acquisition and processing were performed using NTA software 2.3 build 0025. Background extraction was applied, and automatic settings were applied for the minimum expected particle size, minimum track length and blur settings. Since samples were diluted at least 20 times in PBS, viscosity settings for water were applied and automatically corrected for the temperature used. Detection threshold and camera level settings varied as described