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Biological reference materials for extracellular vesicle studies

S. Valkonen [a,b,*], E. van der Pol [c,d], A. Böing [c], Y. Yuana [c], M. Yliperttula [e,f], R. Nieuwland [c], S. Laitinen [b], P.R.M. Siljander [a]

[a] EV Group, Biochemistry and Biotechnology, Faculty of Biological and Environmental Sciences, Division of Pharmaceutical Biosciences, Faculty of Pharmacy, University of Helsinki, Finland
[b] Finnish Red Cross Blood Service, Helsinki, Finland
[c] Department of Clinical Chemistry, Academic Medical Center, University of Amsterdam, The Netherlands
[d] Department of Biomedical Engineering and Physics, Academic Medical Center, University of Amsterdam, The Netherlands
[e] Division of Pharmaceutical Biosciences, Faculty of Pharmacy, University of Helsinki, Finland
[f] Department of Pharmaceutical and Pharmacological Sciences, University of Padova, Italy

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ABSTRACT

Extracellular vesicles (EVs) mediate normal physiological homeostasis and pathological processes by facilitating intercellular communication. Research of EVs in basic science and clinical settings requires both methodological standardization and development of reference materials (RM). Here, we show insights and results of biological RM development for EV studies. We used a three-step approach to find and develop a biological RM. First, a literature search was done to find candidates for biological RMs. Second, a questionnaire was sent to EV researchers querying the preferences for RM and their use. Third, a biological RM was selected, developed, characterized, and evaluated.

The responses to the survey demonstrated a clear and recognized need for RM optimized for the calibration of EV measurements. Based on the literature, naturally occurring and produced biological RM, such as virus particles and liposomes, were proposed as RM. However, none of these candidate RMs have properties completely matching those of EVs, such as size and refractive index distribution. Therefore, we evaluated the use of nanoerythrosomes (NanoE), vesicles produced from erythrocytes, as a potential biological RM. The strength of NanoE is their resemblance to EVs. Compared to the erythrocyte-derived EVs (eryEVs), NanoE have similar morphology, a similar refractive index (1.37), larger diameter (70% of the NanoE are over 200 nm), and increased positive staining for CD235a and lipids (Di-8-ANEPPS) (58% and 67% in NanoE vs. 21% and 45% in eryEVs, respectively).

Altogether, our results highlight the general need to develop and validate new RM with similar physical and biochemical properties as EVs to standardize EV measurements between instruments and laboratories.

1. Introduction

Extracellular vesicles (EVs) are lipid bilayer surrounded particles that contain proteins, lipids, metabolites, and nucleic acids (Yanez-Mo et al., 2015). EVs are produced by most cells, including bacteria and plant cells, making cross-kingdom communication possible (Samuel et al., 2015). EVs have active physiological and pathophysiological roles and they are functional components of intercellular communication, thereby offering possibilities in the development of therapy and diagnostics, or collectively, theranostics (Fais et al., 2016). EVs are often classified into exosomes and microvesicles based on size and the route of formation, but increasing data have revealed this to be an oversimplification, since the isolated populations are heterogeneous and have overlapping properties including size, density, and molecular markers (van der Pol et al., 2016).

The molecular content and concentrations of EVs in human body fluids have raised increasing interest for their use as biomarkers (Fais et al., 2016). A biomarker based on EVs has not yet been realized, partly due to the lack of standardization. Standardization is difficult because the calibration of instruments, the interpretation and validation of results, and the comparison of measurements require a reference material (RM) with physical properties equal to EVs. One of the most analyzed property of an EV sample is the concentration. However, the measured EV concentration depends on the physical properties of EVs, such as the size distribution and refractive index (RI), complicating the analysis, as explained below.

EVs smaller than 300 nm constitute the majority of EV population (Aatonen et al., 2014; Arraud et al., 2014; Dragovic et al., 2011, 2013; Gercel-Taylor et al., 2012; Varga et al., 2014; Yoshioka et al., 2013). Typical size distributions of EVs start at ~30 nm, show a peak at a

* Corresponding author at: Room A327b, Viikinkaari 4, 00014, University of Helsinki, Helsinki, Finland.
E-mail address: sami.valkonen@helsinki.fi (S. Valkonen).

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diameter <100 nm, and follow a decreasing power-law function or exponential function for diameters >100 nm (Fraikin et al., 2011; van der Pol et al., 2016). With the exception of transmission electron microscopy (TEM), none of the current analytical methods are able to detect the entire population of EVs (van der Pol et al., 2016). The inability to detect the smallest EVs leads to both differences and underestimation of the determined concentration. Consequently, the reported number of EVs in normal human plasma ranges from 10^4 to 10^{12} mL^{-1} (van der Pol et al., 2014a). This 8 orders of magnitude difference in EV concentrations emphasizes the need for standardization.

In flow cytometry, which is one of the most commonly used methods in EV studies (Lacroix et al., 2010), particle detection is often based on light scattering. Because the RI of silica (1.45) and polystyrene beads (1.61) is higher than the mean RI of naturally occurring EVs (~1.39), applying a gate on the scatter signals of silica or polystyrene beads will result in erroneous estimations of EV size and concentration (van der Pol et al., 2012, 2014b). For example, a lower size gate set with 200 nm polystyrene beads, which scatter the same amount of light as EVs of ~500 nm (Chandler et al., 2011), leads to the exclusion of EVs between 200 and 500 nm (van der Pol et al., 2014b). Since the concentration of EVs decreases with increasing diameter, a polystyrene size gate generally leads to an underestimation of the actual EV concentration.

With nanoparticle tracking analysis (NTA) the Stokes–Einstein equation is used to derive the hydrodynamic diameter of EVs from their Brownian motion (Dragovic et al., 2011). Although in NTA, the RI of EVs does not affect the measured diameter, the EV size distribution and RI do affect the measured concentration (Filipe et al., 2010), because the measured concentration depends on the brightness of the scattering particle.

Altogether, these examples emphasize the urgent need to develop RM with a similar RI and size distribution, but preferably also with a morphology (for TEM) and zeta potential (for tunable resistive pulse sensing, TRPS) similar to the studied EVs. Ultimately, also other RM properties would match those of EVs, including surface molecules or internal cargo. This is challenging because the development of an optimal RM for EV studies and the analytical methods for their detection are dependent on each other. Further, the different analytical techniques depend on different properties of EVs (Table 1). In this study, we took a three-step approach to develop RM for EV studies: a literature search was performed to find candidates for biological RM, and then EV researchers were asked for the preferences for RM and their use. Finally, we took a step forward and developed an erythrocyte-derived EV-RM, nanoerythrosomes (NanoE), and evaluated its usability.

2. Materials and Methods

2.1. Literature Search

The task of discovering a potential biological RM for EV studies was initiated through discussions with various professionals of "Metrological characterization of micro-vesicles from body fluids" (METVES; www.metves.eu) program. Based on the discussions, the initial categories of RM were determined and a literature search was conducted in Google and PubMed using terms such as "erythrocyte ghost", "RBC carrier", "outer membrane vesicles", "nanobacteria", "viral particle", "coccoid bacteria", "liposome", "cell organelle", "stability", production", and "preparation" to elaborate the properties of potential RM. Initial inclusion criteria for potential RM were submicron size and organic composition. To further investigate the benefits of the selected RM, experts from the EV field were consulted regarding the properties of potential RM from the literature search. Candidates were excluded if the particles contained infection risk, did not express sufficient physical and biochemical resemblance to EVs, or were poorly storable. The literature search and expert consultation was conducted from 10/2014 to 11/2014.

Table 1
Dependency of the different detection techniques on EV properties and EV sample properties.

	AFM	DLS	FCM	NTA	SAXS	TEM	TRPS
Adhesion	+	−	−	−	−	+	−
Buoyancy	−	−	−	−	−	−	±
Charge	−	±	−	±	−	−	+
Concentration	+	+	+	+	++	+	+
Membrane proteins	±	−	±	±	−	±	−
Monodispersity	−	++	−	±	++	−	−
Refractive index	−	+	++	+	−	−	−
Size	−	+	+	+	++	−	+
Spherical shape	−	++	++	+	+	−	++
Stiffness	+	−	−	−	−	+	−

Abbreviations: AFM: atomic force microscopy; DLS: dynamic light scattering without charge option; FCM: flow cytometry; NTA: nanoparticle tracking analysis without charge option; SAXS: small-angle X-ray scattering; TEM: transmission electron microscopy; TRPS: tunable resistive pulse sensing.

2.2. Survey of RM and Their Use in EV Studies

A questionnaire (Appendix 1) was designed to collect the following information: methods in use for the characterization and quantification of EVs, current use of RM, desired and minimal physical and biochemical requirements of RM, and opinions of other potential RM. The questionnaire was sent to 14 stakeholders from the METVES program and 32 collaborators from the Laboratory of Experimental Clinical Chemistry (Academic Medical Center, Amsterdam, Netherlands) working with EVs. Replies were collected from 11/2014 to 12/2014.

2.3. Preparation of RM from Erythrocyte Concentrates

Standard leukocyte-reduced erythrocyte concentrates were used to produce NanoE. Outdated concentrates were obtained from Sanquin (Amsterdam, The Netherlands) and the Finnish Red Cross Blood Service (Helsinki, Finland). Concentrates were handled anonymously, and only concentrates that could not be administered clinically were used as accepted by the Finnish Supervisory Authority for Welfare and Health (Valvira, Finland).

To isolate erythrocyte-derived EVs (eryEVs), 25 mL of the concentrate was diluted with 25 mL of 0.22 μm filtered calcium- and magnesium-free 1× phosphate-buffered saline (PBS [Sigma-Aldrich, St. Louis, MO, USA]) and centrifuged for 20 min at 1560×*g*, room temperature (RT) without brake (Centrifuge 5810 R, Eppendorf, Hamburg, Germany) (Varga et al., 2014). Supernatant was transferred to new tubes and centrifuged 3 times under the same settings. The obtained supernatant was centrifuged for 1 h in 100,000×*g* at 4 °C (Optima™ MAX-XP Ultracentrifuge with rotor TLA-55, k-factor 66, Beckman Coulter, Brea, CA, USA), after which the pellet was washed with similar ultracentrifugation. Finally, the pellet was suspended with PBS to the initial volume and aliquoted to 100-μL aliquots for storage at −70 °C (Fig. 1A).

NanoE production was initiated by separating the erythrocytes from the concentrate: 25 mL of concentrate was diluted with 25 mL cold (+4 °C) PBS and centrifuged at 300×*g* for 10 min at 4 °C without a brake (Centrifuge 5810 R). The pellet was suspended to an equal volume of cold PBS, centrifuged 1560×*g* for 20 min at 4 °C without a brake (Centrifuge 5810 R). The washing was repeated 2 more times. Next, three different disruption methods were evaluated to produce NanoE:

Freeze-thawing: 500 μL aliquots of erythrocytes were treated with 3 consecutive freeze–thaw cycles of 5 min in liquid nitrogen and 5 min in 37 °C water bath.

N_2 bomb treatment: 5 mL of erythrocytes were diluted with 10 mL of PBS in 50 mL Falcon tube to facilitate nitrogen access to the cells. The tubes were placed in N_2 bomb (Parr Cell Disruption Bomb, Moline, IL, USA). A pressure of 75 Psi was created using nitrogen and after