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Silica microspheres are superior to polystyrene for microvesicle analysis by flow cytometry ☆

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ABSTRACT

Background: Cell-derived microvesicles (MVs) in biological fluids are studied for their potential role in pathological conditions. Flow cytometry is used to characterize MVs. Polystyrene microspheres are often used in flow cytometry to distinguish MV from cells by setting a 1-μm MV gate in a side-scatter (SSC) vs. forward-scatter (FSC) dot plot. Polystyrene microspheres, however, exhibit higher FSC and SSC than MVs of equal size. Consequently, some platelets are included within the MV gate, which incorrectly increases the reported percentage of platelet-derived MVs. Silica microspheres exhibit FSC that is closer to that of cellular vesicles and, therefore, should permit more accurate discrimination of MV from platelets.
Objective: Compare silica with polystyrene microspheres to calibrate flow cytometers for definition of MV population and estimation of MV sizes.
Methods: Silica and polystyrene microspheres of various sizes were used in flow cytometry assays to define MV populations and determine platelet and MV sizes in human plasma samples. Sizes determined by flow cytometry were compared to sizes determined by resistive pulse sensing (RPS) method.
Results/Conclusion: Use of 1.0-μm polystyrene microspheres to define the upper MV gate produced a median platelet contamination of 16.53% (8.24, 20.98) of the MV population; whereas, use of 1.0-μm silica microspheres excluded platelet events completely. Calibration with silica microspheres resulted in significantly better estimation of MV diameter than calibration with polystyrene microspheres. We conclude that silica microspheres are superior to polystyrene microspheres as standards to define MV populations without platelet contamination and to determine MV sizes by flow cytometry for a given cytometer.

Introduction

Cell-derived microvesicles (MVs) are submicron vesicles released from activated, apoptotic, or injured cells [1,2]. Microvesicles derived from platelets, leukocytes, and endothelial cells have been reported in a variety of biological fluids that include plasma, cerebral spinal fluid, and alveolar lavage fluid and have been associated with several pathological conditions that include thrombosis, diabetes, myocardial infarction, traumatic brain injury, pulmonary hypertension, brain hemorrhage, and acute respiratory distress syndrome [3–7]. Microvesicles have also been described as mediators of intercellular signaling and transport [8,9].

To gain insight into the various functions of MVs, it is important to accurately characterize their cellular origin, phenotype, concentration, and size. Size distributions of MV populations have recently emerged as a parameter of interest [10–12]. Microvesicle size and its relation to composition, functional activity, and clinical significance has been reviewed by Jy et al. [13]. In an earlier study, Jy et al. stated that platelet-derived procoagulant activity in thrombotic patients is attributed to larger-size MVs that are > 1.0 micron in size [14]. A study by Dean et al. indicated that different sizes of platelet-derived microvesicles (PMVs) differed significantly in their contents of plasma membrane receptors and adhesion molecules, chemokines, growth factors, and protease inhibitors. In that study, the authors reported four different size ranges of PMVs (separated by gel filtration chromatography), of which the smaller two PMV size ranges inhibited collagen/adenosine-diphosphate-mediated platelet thrombus formation [15]. Therefore, accurate determination of the sizes of MV populations in biological fluids is important in elucidation of MV functions.

Microvesicles in biological fluids can be quantified by several techniques that include resistive pulse sensing (RPS), nanoparticle tracking analysis (NTA), dynamic light scattering (DLS), and flow cytometry (FCM) [16]. Flow cytometry is the most prevalent method of MV detection and characterization since in addition to concentration, information on the origin, phenotype, and size of MVs can be acquired on thousands of MVs in each sample within a short time. In flow cytometry, MV are identified as events that are ≤1 μm in size as defined by side scatter

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(SSC) and forward scatter (FSC) characteristics of 1-μm polystyrene microspheres used to set the upper limit of the MV SSC vs. FSC gate [17,18]. The lower limit of the MV gate is defined by the smallest polystyrene microsphere FSC that is discernable from noise, which is 400- to 500-nm polystyrene microspheres for many cytometers, while some new generation cytometers equipped with a photomultiplier tube FSC detector (FSC-PMT) and/or wide angle forward scatter detection or with high sensitivity SSC detection system are capable of discerning as low as 160-200-nm polystyrene microspheres from noise [19].

Polystyrene microspheres, however, have a higher refractive index (η_{PS}) than cellular material (η_{cells}) (1.59 vs.1.39, respectively), which causes polystyrene microspheres to scatter more light than cellular vesicles of the same size [19,20]. Consequently, FSC from polystyrene microspheres underestimates MV diameter. Reports have shown that vesicles are 2-3 times larger than polystyrene microspheres with the same FSC depending on the instrument type, optical configuration, and settings (16, 19). Thus, establishing the upper MV gate using 1-μm polystyrene microspheres actually includes vesicles that are within the platelet size range.

Silica microspheres, however, have a lower refractive index ($\eta_{Si} = 1.45$) that is closer to the refractive index of cellular vesicles ($\eta_{cells} = 1.36\text{-}1.42$). Therefore, the optical properties of silica microspheres are more similar to the optical properties of vesicles than those of polystyrene microspheres [21]. Consequently, FSC of silica microspheres is closer to FSC of cells and cellular particles. We, therefore, hypothesized that use of FSC from silica microspheres will yield closer estimates of the actual sizes of MV and of the 1-μm upper limit of the MV gate than use of FSC from polystyrene microspheres.

In this study, we demonstrated that calibration of a flow cytometer using silica microspheres of various sizes enables establishment of MV SSC vs FSC gates that exclude platelets; whereas, MV gates established using polystyrene microspheres include platelet-sized events.

Materials and Methods

Reagents and Supplies

Anti-human CD41a labeled with APC-H7 (clone HIP8), citrated Vacutainer® tubes, and 19-gauge needles were obtained from BD Biosciences (San Diego, CA, USA). Hanks' balanced salt solution (HBSS) was obtained from Life Technologies (New York, USA). Polystyrene microspheres were obtained from Bangs Laboratories (Fishers, IN, USA), and silica microspheres were obtained from Polysciences (Warrington, PA, USA). The microsphere standards are traceable to National Institute of Standards and Technology (NIST) Standard Reference Materials.

Plasma Isolation

In accordance with a protocol approved by the institutional review board, a 19-gauge needle was used to collect blood from seven healthy non-fasting volunteers into Vacutainer® tubes (BD Biosciences, San Jose, CA, USA) that contained sodium citrate. Platelet-rich plasma (PRP) was prepared by centrifugation of the blood tube at 200 ×g for 10 min. Platelet-poor plasma (PPP) was prepared by centrifugation of the blood tube at 3,000 ×g for 10 min followed by a second centrifugation of the upper two thirds of the plasma fraction at 3,000 ×g for 10 min in 12 mm x 75 mm polypropylene tubes. The upper two thirds of the plasma were collected carefully using a pipet without disturbing the pellet and transferred to 12 mm x 75 mm tubes for the second centrifugation.

Calibration of Flow Cytometer for Size Determination

Flow cytometric measurements were carried out on a Canto II (BD Biosciences) flow cytometer equipped with a 405-nm laser, a high power (200 mW) 488-nm laser, and a 640-nm laser. Forward and SSC were measured off of the 488-nm laser and the SSC threshold was set to 200. To minimize background noise from small dust particles in the sheath fluid, the two standard in-line 0.2-μm filters on the Canto II were replaced with 0.1-μm filters. To reduce background particles in sample suspensions, HBSS used to resuspend polystyrene and silica microspheres and to dilute PRP and PPP was passed through a Stericup Express Plus 0.1-μm filtration system (Millipore Corp., Billerica, MA) prior to use.

Polystyrene microspheres (390 nm, 505 nm, 794 nm, and 990 nm) and silica microspheres (500 nm, 700 nm, 1000 nm, and 2000 nm) were used to calibrate the Canto II flow cytometer for size determination. Calibrations were performed daily. One drop (~50 μl) of each size of polystyrene microsphere stock from their bottles was diluted with 10 ml of filtered HBSS in separate tubes and mixed thoroughly by vortexing. This was repeated for silica microspheres of different sizes in a separate set of tubes. Specific volumes (5, 25, 50, and 75 μl) of diluted polystyrene microspheres or diluted silica microspheres were added to tubes containing 2 ml of filtered HBSS. A mix of all four sizes of microspheres in 2 ml of filtered HBSS was also prepared from diluted stock suspensions. Forward scatter and SSC data were acquired on individual microsphere suspensions, and a tight gate was drawn around each microsphere population in an SSC vs. FSC plot. After the microsphere gates were defined, data was acquired on the polystyrene and silica microsphere mixtures. Mean FSC value for each microsphere population within that mixture was obtained and logarithmically transformed. Size calibration curves were generated by linear regression analyses of log mean FSC vs. microsphere size data. Following acquisition of FSC and SSC data on both, polystyrene and silica microspheres, FSC and SSC data was acquired on PRP and PPP samples. The cytometer configuration and settings remained the same for microsphere and plasma samples.

Platelet and Platelet MV Identification and Size Determination

To identify platelets, 5 μl of PRP or PPP was mixed with 5 μl of anti-CD41a antibody conjugated with APC-H7 (titrated on platelets) plus 90 μl of 0.1-μm filtered HBSS, and incubated at 4 °C for 30 min in dark followed by 10-fold dilution with 0.1-μm filtered HBSS prior to analysis. Samples were then analyzed on a BD Canto II flow cytometer using a 640-nm laser to measure anti-CD41a-APC-H7 fluorescence and a 488 nm laser to measure FSC and SSC. Twenty thousand platelets were counted for each PRP sample and 100,000 MVs were counted for each PPP sample. Platelets were identified by anti-CD41a-APC-H7 fluorescence vs. FSC density plots (2% probability). Mean platelet size in PRP was calculated from the mean FSC within the platelet gates using each calibration curve. Microvesicle size in PPP between 600 nm and 800 nm was calculated from each calibration curve and from platelet-derived vesicle:microsphere size equivalency ratios (SERs) as described in "Results."

Platelet and MV sizes were also measured by RPS with a qNano particle size analyzer. Resistive pulse sensing measures transient changes in current flow through pores of a membrane as particles pass through, and the magnitude of each transient change is proportional to particle size according to the Coulter principle [16]. The mean size of platelets in each PRP sample determined by flow cytometry using polystyrene and silica calibration curves was compared with mean sizes determined by RPS. Similarly, the mean size of MV in PPP between 600 nm and 800 nm was determined by each method and compared.

Statistical Analyses

Repeated-measures analysis of variance (ANOVA) with Tukey adjustment post hoc was performed to compare platelet sizes measured by flow cytometry (polystyrene vs. silica microspheres) and by RPS.