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Efficient quantification and characterization of bacterial outer membrane derived nano-particles with flow cytometric analysis

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ABSTRACT

There currently exists no efficient and easy method for size profiling and counting of membranous nano-scale particles, such as bacterial outer membrane vesicles (OMVs). We present here a cost-effective and fast method capable of profiling and counting small sample volumes of nano-scale membranous vesicles with standard laboratory equipment without the need for any washing steps. OMV populations of different bacterial species are compared and even subpopulations of OMVs can be identified after a simple labelling procedure. Counting is possible over three orders of magnitude without any changes to the protocol. Protein contaminations do not alter the described measurements.

Introduction

Nanometre-sized particles – especially bacterial outer membrane vesicles (OMVs) – are in the focus of biomedical research and have a multitude of different uses in the fields of bacteriology, vaccinology and immunology. OMV formulations are used for scientific experiments where dose determination is critical for repeatability and comparability of results. For the first time this would also allow for experiments based on multiplicity of infection (MOI) regarding OMVs and eukaryotic or bacterial cells. OMV production rates of bacteria are also unclear so far and might vary considerably, the same is true for vaccination doses based on purified OMVs (Kulp and Kuehn, 2010; Van de Waterbeemd et al., 2013). Thus, accurate and efficient enumeration of OMVs is an extremely desirable goal (Berleman and Auer, 2013; Camacho et al., 2013; Ellis and Kuehn, 2010; Schaar et al., 2013; Shen et al., 2012; Wai et al., 2003; Zhao et al., 2013). Quality control of these isolated particles is very challenging because the methods used today are not sufficient to provide any information about the number or size of particles, their distribution in the solution or the presence of markers on a subset of the OMV population of interest (Granoff, 2010; Jun et al., 2013; Roier et al., 2012; Schild et al., 2009). For better standardization, accurate enumeration and characterization is clearly necessary.

The commonly performed Western blot analysis, Coomassie-stained SDS gels or Bradford protein content measurements can easily give incorrect results by contaminations, such as capsular fragments, broken flagella or secreted proteins. None of these assays can distinguish between proteins, which are part of the particles or are just isolated together with OMVs. Moreover, there are no controls to adjust for such effects.

Furthermore, drawing conclusions from the protein content to the number of OMVs can be misleading, because OMVs can have different sizes and protein content depending on the bacterial strain and individual growth conditions.

Conventional microscopic quantification and type identification of such particles is impossible due to their small size (50–200 nm) below the wavelength of visible light. Therefore, imaging the sample is limited to institutions where expensive and time consuming techniques like electron microscopy, atomic force microscopy or X-ray diffraction are readily available (Aldick et al., 2009). Even

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when such techniques are available, accurate quantification is very complex and liable to large statistical errors.

Here, we present a fast, accurate and cost-effective method capable of enumerating and profiling OMVs using standard laboratory equipment and staining solutions. The assay is based on flow-cytometry and does not require previous genetic modifications to the bacteria producing the OMVs. Furthermore, no washing steps during the staining procedure and analysis are required. This prevents losses and provides more accurate enumeration of OMVs from purified stock solutions or growth supernatants.

Materials and methods

OMV isolation

Bacterial outer membrane vesicles were isolated from different bacterial species from their culture supernatants. For preparation of larger batches of OMVs used for dilution and subpopulation studies, culture supernatants were subjected to ultra-centrifugation and sterile filtering (Wai et al., 2003). In brief, bacterial cultures were inoculated in a standard LB medium and incubated in a shaker with appropriate temperature under aeration. Bacterial cells were pelleted by a first centrifugation step (5000 × *g* 20 min, 4 °C). Then, the supernatants were filtered through a 0.45 μm pore size sterile Minisart High Flow syringe filter (Sartorius Stedim) and the resulting supernatants were re-filtered through a 0.2 μm pore size filter. Subsequently, the supernatant samples were ultracentrifuged at 150,000 × *g* for 3 h at 4 °C. The pellet was resuspended in PBS buffer. The volume for resuspension was adjusted according to the volume of initial culture supernatant used for isolation. Isolation was also performed using a gradient centrifugation step to ensure higher purity. Data were compared and no significant differences could be observed.

For direct analysis of OMVs in bacterial growth supernatant, as used for size profiles in Fig. 2, growth media (Luria Broth, LB) was filtered in 0.22 μm filters (Millipore Corporation, Billerica, MA, USA) before starting the bacterial culture to remove larger debris present in the fresh media formulation. Bacteria were subsequently cultured within the media for a period of time and removed thereafter (11,000 × *g* for 30 min at 4 °C). To avoid any disturbance of the bacterial pellet, only 5 ml of the whole solution (50 ml) were carefully removed from the uppermost part of the centrifuge tube. Staining was performed within the removed part of the cleared supernatant by directly adding dye as described below.

Staining

OMV solutions were diluted in styrylic membrane dye in PBS/BSA to a final concentration of 0.5 μg/ml and were incubated for 5–10 min at room temperature. OMVs in direct culture supernatants were stained in the medium by addition of FM dye to the final concentration of also 0.5 μg/ml.

For two colour analysis, fragment antigen binding (fab) were produced from StrepMAB-Immo antibodies (IBA GmbH, Goettingen, Germany) using the Pierce Mouse IgG1 Fab Micro Preparation Kit Kit (Thermo Scientific – Pierce, Rockford, IL, USA). Secondary antibodies Goat Alexa Fluor 488-AP anti-mouse IgG/IgM Fab (Jackson ImmunoResearch Laboratories Inc., London, UK) were used for staining.

First, 200 μl of BSA (2 mg/ml) was pipetted into the FACS tube, incubated and vortex mixed for 10 s. OMV solution was added to equal volumes (200 μl) and briefly vortex mixed again. To the 400 μl of solution, FM dye was added to the final concentration of 0.5 μg/ml. After a brief vortex mixing, 5 μl of StrepMAB-Immo-Fab solution were added and mixed by pipetting up and down and very short vortex mixing. A volume of 1 μl pure Alexa Fluor 488-AP anti mouse IgG/IgM Fab (1.5 mg/ml) was added and very briefly vortex mixed. After an incubation period of 5–10 min at RT, samples were analysed in a timely manner. Signal to noise ratio was found to worsen after >10 min due to increased unspecific binding.

Flow cytometer changes

The beam path of the detection system used in the flow cytometer (BD FACS Canto II, BD Biosciences San Jose, CA, USA) was altered for the measurements to project the vast majority of fluorescent signal into one of the first photomultipliers. Red light (610 nm ± 20 nm) was directed into the second detector to minimize losses in the optic system and enhance the signal to noise ratio. The threshold for the photomultiplier was adjusted to exclude noise and debris from triggering fluorescent events at significant rates. Software changes in the settings were performed accordingly.

Flow rate standardization

The flow rate of the instrument was determined using fluorescently labelled beads at standardized concentrations (TruCount™ Becton Dickinson, Heidelberg, Germany) suspended in PBS buffer solution. High quality FACS tubes (Becton Dickinson, Heidelberg, Germany) were used for best fit in combination with a thin film of silicone grease to avoid any leaks at the connection site between tube and cytometer.

Software

Flowcytometric data was analysed with FlowJo 7.6.1 software for Windows (TreeStar Inc. Ashland Oregon, USA). Statistical analysis was performed with Sigma Stat 32 (build 3.00.0; Systat Software, San Jose, California, USA); Charts were plotted with Sigma Plot (build 10.0.0.54; Systat Software, San Jose, California, USA).

Results and discussion

Flow-cytometry is based on the detection, quantification and separation of particles based on the way light passes through them and scatters perpendicular to them (Dittrich and Göhde, 1969). This is convenient for particles larger than the wavelength of light (e.g. cells, bacteria), but impossible for particles smaller than the wavelength of light, as no significant scatter is produced. Fluorescent dyes present in individual particles however, can be detected independent of the particle's size. This fluorescence can be amplified from the sideways scatter beam path as long as the fluorescence intensity is above the detection limit and noise level of the instrument. To quantify outer membrane vesicles with size of around 100 nm by unspecific fluorescent labelling, FM membrane dye was used. The intensity was still found to be insufficient to be detected efficiently and accurately above the machine's noise level. Therefore, the beam path in the detection system was altered as described in Section 2. By reducing losses in the optical system, the signal intensity was increased leading to an optimized signal to noise ratio, enabling the detection and identification of single OMVs with minimal background. Staining of the OMVs membranous fraction was performed with styrylic membrane dye FM 1–43 (Murthy and Stevens, 1998) at a final concentration of 0.5 μg/ml. OMVs stained with a defined concentration of dye display a distinct fluorescence signal which is dependent on their surface area, as the dye reversibly inserts into the lipid bilayer molecules in a stoichiometric relation. Thereby, a strong fluorescent signal is only produced once the dye molecules have integrated into the phospholipid-bilayer. Therefore, analysis can be performed with the analyte still