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Bead-based flow-cytometry for semi-quantitative analysis of complex membrane vesicle populations released by bacteria and host cells

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ABSTRACT

During infection, the release of nano-sized membrane vesicle is a process which is common both for bacteria and host cells. Host cell-derived membrane vesicles can be involved in innate and adaptive immunity whereas bacterial membrane vesicles can contribute to bacterial pathogenicity. To study the contribution of both membrane vesicle populations during infection is highly complicated as most vesicles fall within a similar size range of 30–300 nm. Specialized techniques for purification are required and often no single technique complies on its own. Moreover, techniques for vesicle quantification are either complicated to use or do not distinguish between host cell-derived and bacterial membrane vesicle subpopulations. Here we demonstrate a bead-based platform that allows a semi-quantitatively analysis by flow-cytometry of bacterial and host-cell derived membrane vesicles. We show this method can be used to study heterogeneous and complex vesicle populations composed of bacterial and host-cell membrane vesicles. The easy accessible design of the protocol makes it also highly suitable for screening procedures to assess how intrinsic and environmental factors affect vesicle release.

1. Introduction

The shedding of nano-sized (30–300 nm)-membrane vesicles is a highly conserved process amongst bacteria, archea, and eukaryotes. Depending on their origin and the context for release, these membrane vesicles govern functions in survival, communication and defense (Deatherage et al., 2009). Thus, membrane vesicles released during infection constitute a mixture of bacterial and host-cell vesicles that can operate in host-pathogen interactions (Berleman and Auer, 2013; Schorey and Harding, 2016; Schwechheimer and Kuehn, 2015).

To study vesicle release kinetics as well as their phenotypic and functional aspects specialized techniques are required. Currently used assays for detection or quantitative analysis of individual particles include flow-cytometry- and microscopy-based methods as well as particle detection by nanoparticle tracking or tunable resistive pulse sensing (TRPS) analysis (Hoen et al., 2012; van der Pol et al., 2014; van der Vlist et al., 2012; Witwer et al., 2013). Methods for the analysis of the gross vesicle population include Western blotting and conventional flow-cytometry. Amidst, there are flow-cytometry-based techniques for the analysis of vesicle subgroups that are more sensitive than methods for gross analysis, but less sensitive than approaches for single particle detection (Inglis et al., 2015; Pospichalova et al., 2015; Wieser et al., 2014; Witwer et al., 2013). Each of these techniques has its own strengths and weaknesses and optimal vesicle analysis usually requires the use of a combination of techniques (Lacroix et al., 2010; Witwer et al., 2013). Assays that enable the quantification and comparison of membrane vesicle populations are essential but most techniques are either complicated to use, not able to distinguish between vesicle subpopulations, or both. A relatively easy to use technique for the detection of membrane vesicles is based on bead-based flow-cytometry. By using 4-μM-sized latex beads, this assay overturns the size-related problems which prevent membrane vesicle analysis by conventional flow-cytometry. As these beads are coated with antibodies directed against specific marker proteins, they can be used to capture specific membrane vesicle subpopulations. Bead-bound vesicles can subsequently be stained with fluorescently labeled detection antibodies whereupon the total complex can be analysed by flow-cytometry. The beads are gated on their forward and side scatter so that fluorescent intensity of the bead-bound vesicles can be determined. This assay has already proven its value in the semi-quantitative determination of host-cell membrane vesicle release (Clayton et al., 2001; Thery et al., 2006; Ostrowski et al., 2010).

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In this study, we present a novel application for this methodology. We modified the assay and made it appropriate for assessing bacterial vesicle release, i.c. membrane vesicles from two common respiratory pathogens *Moraxella catarrhalis* (Mrc) and *Pseudomonas aeruginosa* (Psa). This modified assay can be used to distinguish bacterial and host-cell vesicle populations in complex samples such as obtained upon infection.

2. Material and methods

2.1. Reagents and antibodies

Latex aldehyde beads (4-μM) were purchased from Invitrogen (Paisley, UK). The antibody against Psa was from Aviva (OAMA02609, Aviva Systems Biology, San Diego, CA. USA). The rabbit serum against Mrc (strain A 1.39N, isolated from children in a primary school in Nieuwegein, the Netherlands, 1989), was kindly provided by Dr. J.P. Hays (Erasmus University Medical Centre, Rotterdam, the Netherlands). Antibodies were purified using the antibody serum purification kit based on protein A (Abcam, Cambridge, MA, USA). Hereafter, antibody were quantified by the MicroBCA protein assay (Pierce, Rockford, IL, USA). Antibody conjugations were performed using a PE-labeling kit from Abcam (Cambridge, MA, USA). Antibodies (100 μg per conjugation) were PE-conjugated according the manufacturers' instructions (Abcam, Cambridge, MA, USA). Antibodies against CD63 (unconjugated, mouse-anti-human clone H5C6), CD81 (PE conjugated, mouse-anti-human clone JS-81) and isotype control (PE mouse IgG1, κ) antibodies were from BD (BD Bioscience, Franklin Lakes, NJ, USA). HRP-conjugated secondary antibodies were from R & D systems (Minneapolis, MN, USA). For Western blot analysis all working dilutions were 1:1000, except for Mrc where a dilution of 1:40 was used. Samples for Western blot analysis were diluted in sample buffer prepared with XT-sample buffer (Bio-Rad, Hercules, CA, USA), urea (8 M), protease inhibitor complete (Roche GmbH, Mannheim, Germany), and reducing agent TCEP (Bio-Rad, Hercules, CA, USA) or water, to obtain reducing or non-reducing conditions, respectively.

2.2. Bacterial culture

The bacterial strains used were Psa ATCC 27853 and a local clinical Mrc isolate (Maastricht University Medical Center, The Netherlands). All strains were cultured at 5% CO_2 and 37 °C overnight on blood plates. After culture, several colonies were isolated and resuspended until an optical density of 0.5 McFarland (1.5×10^8 colony forming units (cfu)/ml) in RPMI1640. Bacteria were then cultured in complete vesicle-depleted culture medium with 5% fetal calf serum (FCS). Vesicle-depleted medium was obtained by the overnight centrifugation of RPMI1640 containing 30% FCS, glucose (22.5%), and sodium pyruvate (100 mM) at 100.000 × *g* using a 70Ti rotor with a κ-factor of 44 in an Optima L-90K ultracentrifuge (Beckman Coulter, Fullterton, CA, USA). This medium was then combined with FCS-free RPMI1640 with glucose and sodium pyruvate to obtain vesicle-depleted complete culture medium containing 5% FCS. Bacteria (5×10^8 cfu/ml) were cultured in this vesicle-depleted medium for 4 h for flow-cytometric analysis.

2.3. Cell culture

The human monocytic THP-1 cell line (ATCC-TIB202) was maintained in RPMI1640 (Sigma, St. Louis, MO, USA) supplemented with 10% FCS (Lonza, Verviers, Belgium), glucose (22.5%), sodium pyruvate (100 mM), and β-mercaptoethanol (25 mM) and cultured at 5% CO_2 and 37 °C. For monocyte differentiation, cells were seeded at 0.5×10^6 cells per well in a 24-wells plate or at 1×10^7 cells per flask in a T75 flask and stimulated for 72 h with 200 nM phorbol 12-myristate 13-acetate (PMA; Sigma, St. Louis, MO, USA).

2.4. Macrophage infection

Prior to infection, PMA-differentiated monocytes were washed 3 times with PBS and the medium was replaced with complete vesicle-depleted culture medium with 5% FCS (prepared as described above). Hereupon, macrophages were infected for 6 h with either one of the bacteria or both at a multiplicity of infection of 10. After infection, the medium was harvested, processed and used for bead-based flow-cytometry as described below.

2.5. Isolation of bacterial and macrophage-derived membrane vesicles

For Western blott analysis of the vesicles and the cell lysates, macrophages in T75 flasks and bacteria were cultured for 6 h after which the conditioned media were used for vesicle isolation. Cells were harvested by scraping in PBS, pelleted and lysed in NP-40 lysis buffer (150 mM NaCl, 1% NP-40, and 50 mM Tris). Additionally, bacteria were cultured for 4 h at 4.5×10^8, hereafter the cells were pelleted and lysed in NP-40 and the conditioned media were used for vesicle isolation. Membrane vesicle isolation from the conditioned media was performed by ultracentrifugation. Herefore, the conditioned media were first cleared from cells and cell debris by sequential centrifugation at 300 × g and 1200 × g for 10 min. Then, the supernatants were filtered using 0.22 μM filters and transferred to ultraclear Quickseal tubes (Beckman Coulter, Fullerton, CA, USA) and centrifuged for 90 min at 100.000 × g using a 70Ti rotor, κ-factor 44 in an Optima L-90K ultracentrifuge (both Beckman Coulter, Fullterton, CA, USA). The vesicle pellet was resuspended in 150 μl PBS and kept at −80°C until further use. The protein content of the whole cell lysates (WCL) and the vesicle pellets was determined by the Micro BCA protein assay.

2.6. Western blotting

For Western blot analysis, ultracentrifugation pellets or cell lysates were diluted in sample buffer (Biorad, Hercules, CA, USA, non-reducing for α-CD81 and α-CD63), and loaded on a 12% SDS-page gel. After electrophoresis and protein transfer to a nitrocellulose membrane (Fisher Scientific, Waltham, MA, USA), the membranes were blocked overnight in Tris-buffered saline (TBS) with 5% (w/v) milk powder after which they were washed with TBS with 0.05% (v/v) Tween and incubated with primary antibodies against CD63, CD81, Mrc (1:20) or Psa in TBS with 5% (w/v) BSA for 3 h. Next, the membranes were washed and incubated with a horse radish peroxidase (HRP)-labeled secondary antibody in TBS for 2 h. Antibodies were used at 1:1000 unless otherwise indicated. On incubation, the membranes were washed and the detection was performed by using chemiluminescence substrate (Sigma, St. Louis, MO, USA). Whole cell lysates were used as a positive control.

2.7. Flow-cytometric analysis

2.7.1. Preparation of antibody-coated latex aldehyde beads

This method is based on the assay developed by Ostrowski et al. and was used accordingly for the determination of CD63/CD81$^+$ host cell-derived vesicles or adopted for the determination of bacterial vesicle release (Ostrowski et al., 2010). Overall, 3 different bead sets were prepared as follows: a total of 1×10^8 4-μM aldehyde-sulfate beads was washed in 150 μl MES buffer (all washing steps were performed at 3000 × *g* for 10 min) and coated with 35 μg antibody against CD63, Mrc, or Psa (in a total of 250 μl), overnight at 4 °C while keeping the solution under constant agitation at 6500 rpm. Antibodies supplied as serum were purified before use (as described in the reagents and antibodies section). After coating, the remaining free binding sites on the beads were blocked by washing the beads 3 times with 0.22-μM filtered PBS with 4% (w/v) bovine serum albumin (BSA). Hereafter the beads were resuspended and kept in a total of 500 μl storage buffer (PBS

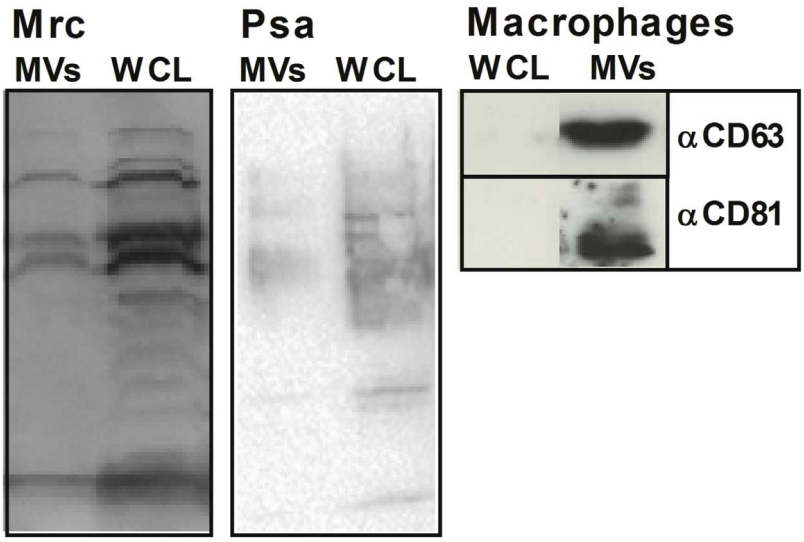

Fig. 1. Analysis of membrane vesicles and whole cells lysates of bacteria and macrophages by Western blotting. The reactivity of antibodies with bacterial membrane vesicles and host cell-derived vesicles (MVs) as well as whole bacterial and host cell lysates (WCL) was determined by Western blotting. *Moraxella catarrhalis* (Mrc)- and *Pseudomonas aeruginosa* (Psa)-derived bacterial membrane vesicles (respectively 10 and 25 µg) isolated from conditioned media from bacterial cultures by ultracentrifugation, were subjected to SDS-PAGE, transferred to nitrocellulose membranes and stained using antibodies against Mrc, Psa, CD63, and CD81. Whole bacterial and host cell lysates (50 µg) were used as a positive control.

with 0.1% (v/v) glycine and 0.1% (w/v) sodium azide) at 4 °C.

2.7.2. Bead-based flow-cytometry

After culture or infection the supernatants were processed by centrifugation. Supernatants from bacterial cultures were processed by 2 subsequent centrifugation steps at 1200 × *g* while supernatants obtained after infection were processed by 2 centrifugation steps (300 × *g* followed by 1200 × *g* both for 10 min). Then, the supernatants were filtered using 0.22-µM filters and a total of 50,000 (1 µl) of α-Mrc- and α-CD63-coated beads and 250,000 (5 µl) of α-Psa-coated beads were added to 200 µl of the processed supernatants and incubated overnight at room temperature under constant agitation at 6500 rpm. The optimal number of beads was determined in earlier experiments. Several bead-bacteria ratios (with a range between 1:20 and 1:200) were tested to reach an amount that resulted in sufficient vesicle binding without reaching a saturation, as this would prevent to determine an increase in the vesicle concentration. This way the optimal bead concentrations for each platform was established. After overnight vesicle capture, the beads were washed twice with 0.22-µM filtered PBS with 2% (w/v) BSA and incubated with an analogous secondary PE-conjugated antibody for 1 h at room temperature under constant agitation and protected from light. For the macrophage-derived vesicles, a PE-labeled isotype antibody was used as a control for non-specific Fc receptor binding and for the bacterial vesicles unconditioned medium was used as a control. Then, the beads were washed twice in 0.22µM filtered PBS with 2% (w/v) BSA, whereafter the pellets were resuspended in 300 µl PBS and analyzed by flow-cytometry on a FACSCanto™ (BD Bioscience, Franklin Lakes, NJ, USA). Analyses were performed using FACSDiva Software. Single beads were gated on their forward and side scatter. The quartile distribution within a dot plot, based on the fluorescent intensity of single beads, was then used to calculate the relative fluorescent intensity. Quartile gate 4 was set on 2% for beads incubated with unconditioned medium. The relative fluorescence intensity, expressed in arbitrary units (AU), was then calculated by multiplying the number and the fluorescence intensity of the positive beads in quartile 4.

2.8. Tunable resistive pulse sensing (TRPS)-based analysis

Prior to TRPS-based analysis, the media were concentrated to 500 µl on Ultra-15 10-kDa Amicon centrifugal filter units (Millipore, Billerica, MA, USA) by 15 min of centrifugation at 4000 × *g* at room temperature. Then, the absolute vesicle concentration in the supernatants was determined by TRPS-based analysis using the qNano Gold (Izon Science Ltd., Oxford, UK) using Izon Control Suite Software v3.2. Measurements were conducted using an NP150-pore with a stretch of 47 mm, a transmembrane voltage of 0.48 V (giving a baseline current of ± 100 nA), and a pressure of 6 mbar. Measurements were performed for 10 min and repeated when system instabilities occurred. The samples were calibrated with 114 nm polystyrene calibration beads that were diluted in culture medium at a concentration of 1×10^9 particles/ml. Concentration calculations were performed using Graph-Pad Prism 5 Software (Graph-Pad, San Diego, CA, USA) and Microsoft Office Excel (version 2010, Microsoft).

2.9. Statistical analyses

Linear regression was performed using Graph-Pad Prism 5 Software (Graph-Pad, San Diego, CA, USA). A non-parametric Mann-Whitney test was performed to determine the statistical variance. Differences were considered significant when $p \leq 0.05$.

3. Results

3.1. Reactivity of the antibodies

Western blott analysis was performed to assess whether antibodies were reactive with epitopes on bacterial membrane vesicles isolated by ultracentrifugation. All antibodies were found to be reactive with whole cell lysates as well as the membrane vesicles (Fig. 1). The reactivity of the α-CD63 and α-CD81 antibodies (CD63 and CD81 are proteins that are highly enriched on extracellular vesicles) to macrophage-derived membrane vesicles was confirmed while cell lysates remained negative. Hereafter, the antibodies were used to establish the platform for flow-cytometric analysis. The first step was to validate each platform for its reactivity and its specificity. As is shown in Fig. 2, all assays were confirmed to be positive only for the corresponding MVs.

3.2. Analysis of purified bacterial vesicles by bead-based flow-cytometry

Next, we aimed to determine if this platform qualifies for the semi-quantitative detection of bacterial membrane vesicles and to determine

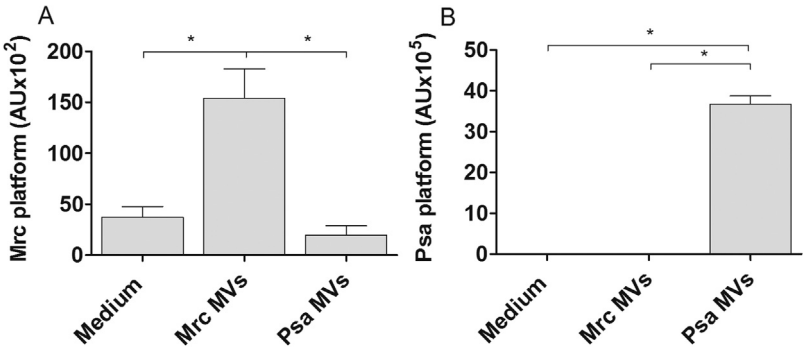

Fig. 2. Validation of the bead-based platforms by flow-cytometric analysis. Validation of the specificity of the bead-based platforms to detect Mrc- and Psa-derived membrane vesicles by flow-cytometric analysis. Beads coated with antibodies directed against either Mrc or Psa were added to control medium or media that were conditioned by Mrc and Psa for 4 h at 0.5×10^8 CFU/ml. The arbitrary units for the Mrc- (A) and Psa- (B) platforms were determined by flow-cytometry (mean ± SEM, n = 3). *$p < 0.05$.

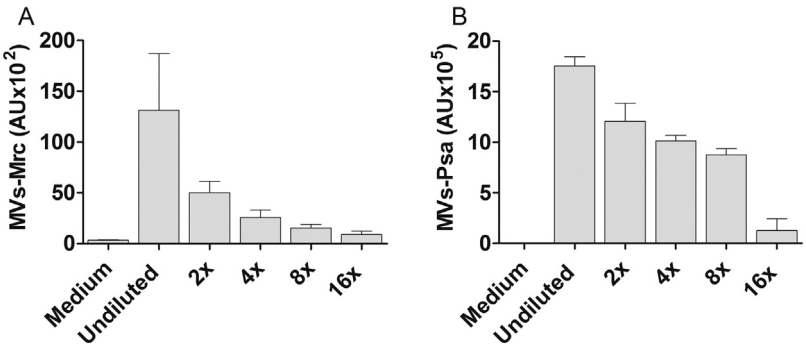

Fig. 3. Flow-cytometric analysis of bacterial vesicles. Beads were added to 2-fold dilution series with a total of 5 dilutions, using supernatants from Mrc or Psa monocultures, cultured for 4 h at 0.5×10^8 CFU/ml. After vesicle capture, the arbitrary units for Mrc-MVs (A) and Psa-MVs (B) were determined by flow-cytometry (mean ± SEM, n = 3).

the experimental variation of this assay. To test this, conditioned media from bacterial suspensions at 0.5×10^8 CFU/ml were obtained, aliquoted in 3 portions to be tested in separate experiments. Then, the assay was applied on 4 sequential 1:1 serial dilutions. Fig. 3 shows a concentration dependent decrease in the fluorescence intensity and shows there is a low inter-experimental variation. Moreover, these results show a high variation in the absolute fluorescence intensity among the vesicle-bead complexes from the different bacteria.

3.3. Correlation between the relative and absolute concentration

The correlation between the absolute and the relative concentration is shown in Fig. 4. Linear regression of the slopes revealed R^2 values close to 1 indicating that there is a strong correlation between both variables (Mrc: $R^2 = 0.954$ and Psa: $R^2 = 0.967$). These data demonstrate that bead-based flow cytometry assay can indeed be used for semi-quantitative analyses. Moreover, the high correlation indicates that the combination of bead-based flow-cytometry with TRPS-based analysis can also be used to infer the absolute concentration of vesicle subpopulations.

3.4. Bead-based flow-cytometry to determine bacterial and host-cell vesicle release upon (co-)infection

For analysis of mixed populations we used an *in-vitro* model based on THP-1 macrophages infected with Mrc, Psa, both Mrc and Psa or

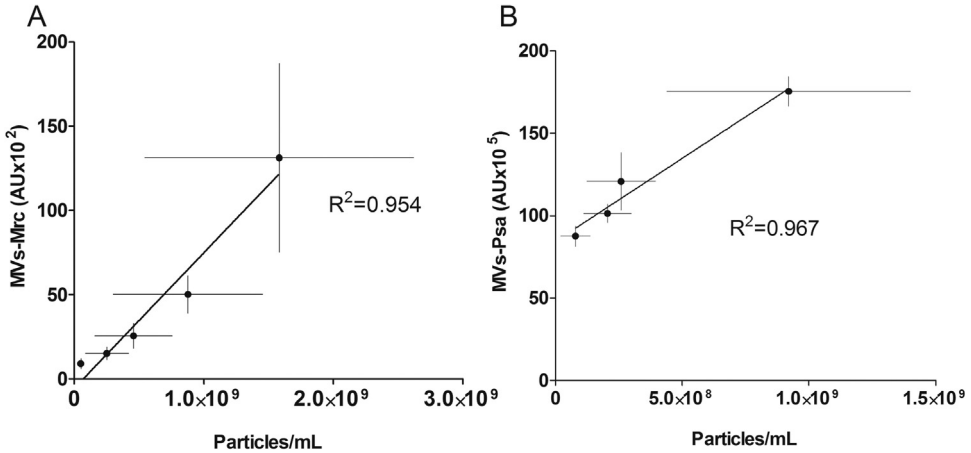

Fig. 4. Correlation between the absolute and the relative vesicle concentration. The absolute vesicle concentration was determined by TRPS-based analysis of supernatants from bacterial cultures that were concentrated (20-fold) on 10-kDa Amicon filter units. The relative membrane vesicle concentration was then plotted against the absolute concentration to determine the correlation. Linear regression of the slopes revealed R^2 correlations of 0.954 for Mrc-MVs (A) and 0.967 for Psa-MVs (B). Plots are based on the average values of 3 independent experiments.

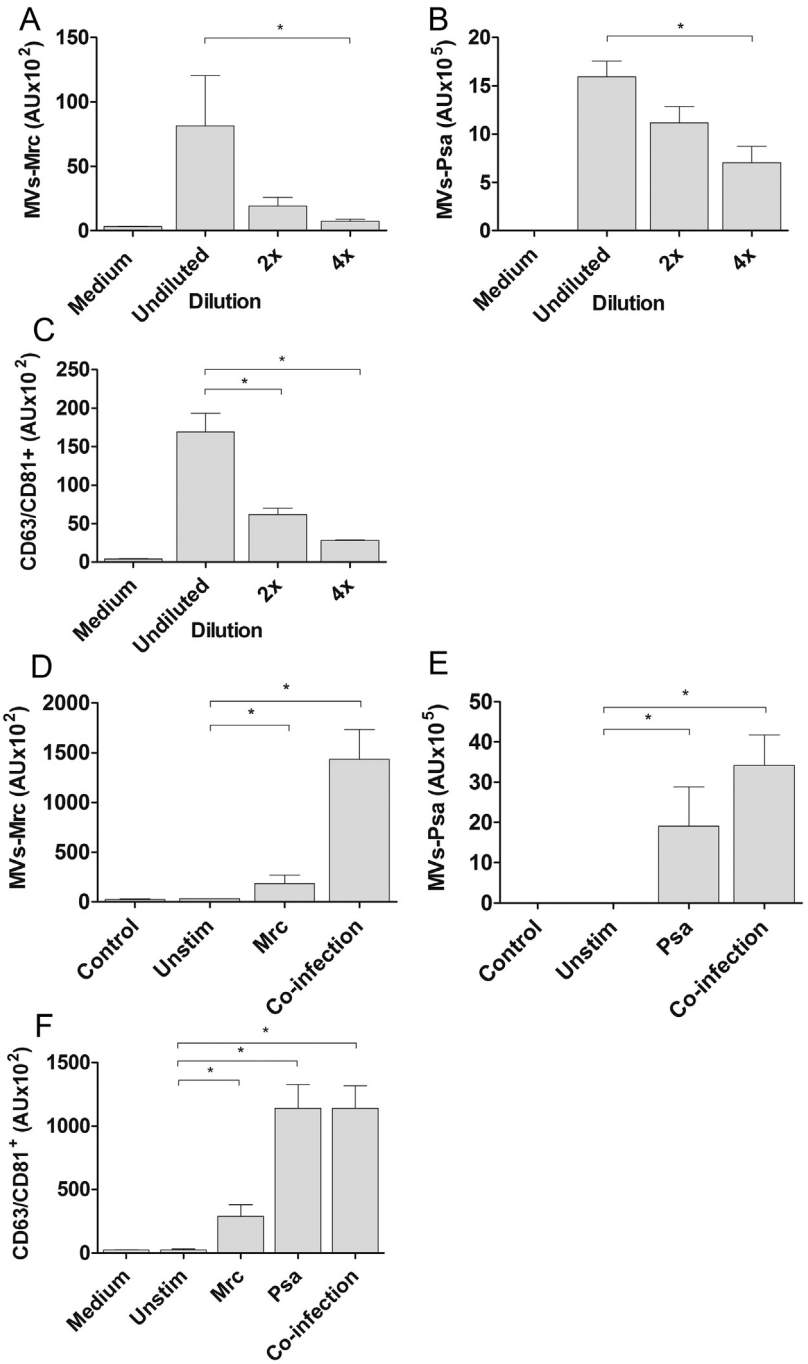

Fig. 5. Flow-cytometric analysis of bacterial and host cell vesicle release upon infection. Semi-quantitative determination of bacterial and host-cell vesicles in supernatants of macrophages infected for 6 h. Supernatants obtained after infection with Mrc (A) or Psa (B) or supernatants of uninfected macrophages (C) were used undiluted and 2- and 4-times diluted and assessed by flow-cytometry. The bacterial membrane vesicle release during infection with Mrc (D) and Psa (E) was compared to the release during co-infection. The release of host cell vesicles by macrophages after infection by Mrc, Psa, or co-infection (F). Results are mean ± SEM from 3 independent experiments. *$p < 0.05$.

were left uninfected. To demonstrate that this assay indeed allows the semi-quantitative detection method of membrane vesicles, the harvested supernatants of Mrc- or Psa-infected macrophages and uninfected macrophages were used undiluted or 2- and 4-times diluted. Fig. 5A–C show dilution-dependent decreases in the amount of bacterial vesicles during infection and host cell-derived CD63/CD81$^+$ vesicles by uninfected macrophages. Additionally, we demonstrate that infection results in a significantly increased release of CD63/CD81$^+$ host cell-derived vesicles, while co-infection significantly increased the release of Mrc-, Psa-, and CD63/CD81$^+$ membrane vesicles. (Fig. 5D–F).

3.5. Workflow and principle of bead based-flow-cytometry

The typical workflow for the improved method is depicted in Fig. 6. After infection, the supernatants are harvested and processed by centrifugation and filtration whereupon antibody-coated beads are added for overnight vesicle capture. Then, the bead-vesicle complexes are washed and stained by PE-conjugated secondary antibodies after which the fluorescence intensity can be determined by flow-cytometry. Scatter plots and fluorescence intensity histograms can subsequently be used for analysis. A scatter plot on the forward and side scatter of the bead-vesicle complexes allows to gate (gate 1) on single bead-vesicle complexes (the events in gate 2 and 3 are complexes clustered in

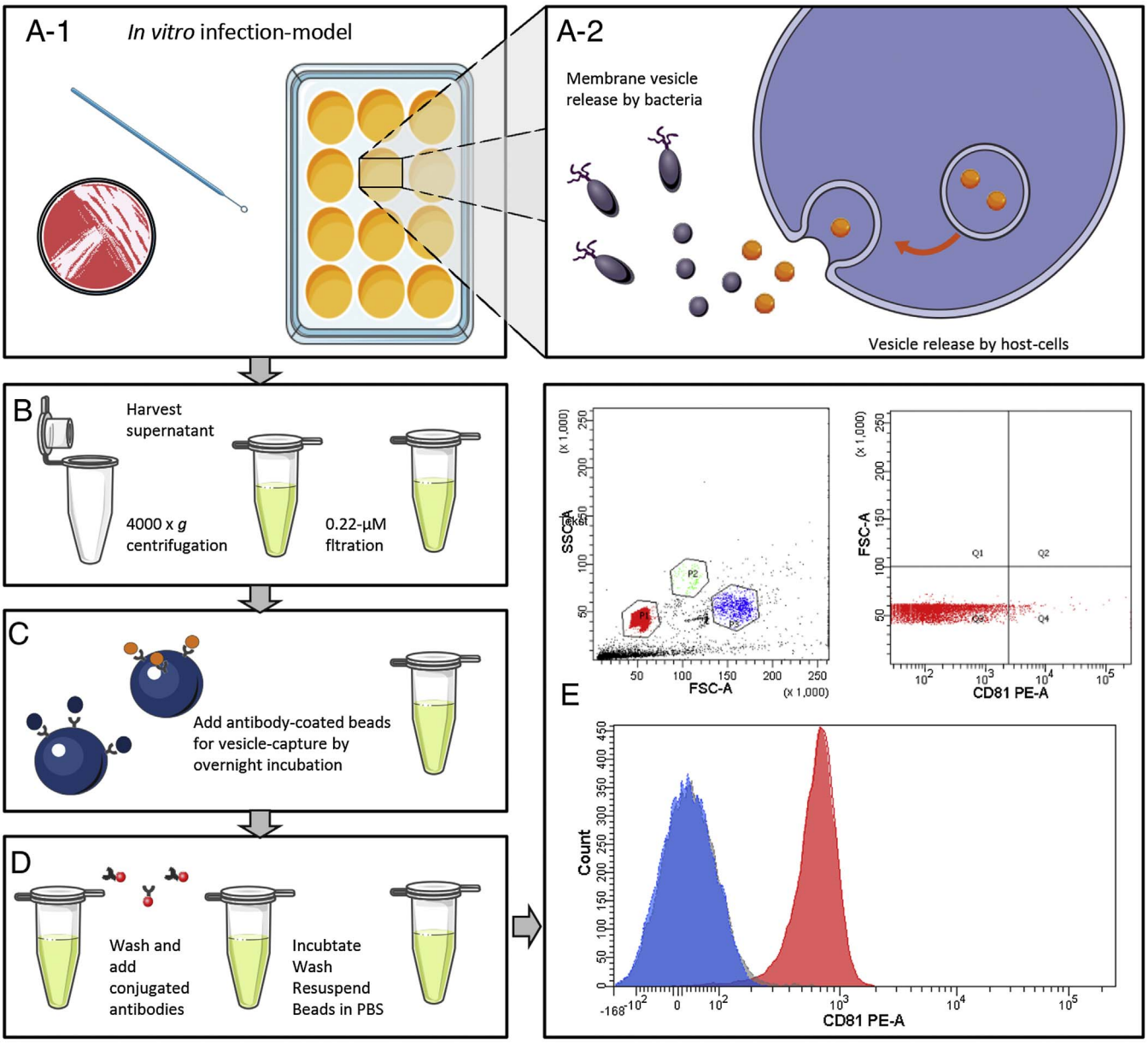

Fig. 6. Workflow and principle of bead-based flow-cytometry for the analysis of complex membrane vesicle populations. Bead-based flow-cytometric analysis for the semi-quantitative analysis of specific membrane vesicle populations within a complex vesicle population, such as released during bacterial infection (A-1/2) . After infection, supernatants are harvested and processed by centrifugation and filtration (B). Then, antibody-coated beads are added and vesicles are captured during overnight incubation (C). Hereafter, the bead-vesicle complexes are washed and stained with conjugated secondary antibodies (D) and subjected to flow-cytometric analysis (E). The beads in gate 1 (gates 2 and 3 contain duplicate and triplicate complexes) are plotted in a scatter plot to determine the % of positive beads in quartile 4, adjusted to 2% using medium control beads. The fluorescence histogram on single beads can then be made, which is done here for an experimental condition based on macrophage vesicles released upon Mrc-infection (red), unconditioned medium (grey), and an isotype control (blue) (E). The relative intensity in arbitrary units (AU) can then be calculated by the multiplying the % of positive beads in quartile 4 with the median fluorescence intensity. This figure was created using Servier Medical Art. (For interpretation of the references to color in this figure legend and text, the reader is referred to the web version of this article.)

duplicates or triplicates). A scatter plot of the fluorescence intensity of single complexes is then used to measure the percentage positive beads. The settings of the quartiles were adjusted using the plots of the unconditioned media for which the percentage positive beads in quartile 4 was set at 2%. Histograms on the beads in quartile 4 can then be used to determine the fluorescence intensity of the beads under experimental conditions, here shown for macrophage-derived vesicles released on infection with Mrc (red), together with histograms for the control conditions (unconditioned medium (grey) and the isotype control (blue)). Calculations are made using tables of statistics to determine the relative concentration in arbitrary units (AU) by multiplication of the percentage and median fluorescence intensity of the positive beads.

4. Discussion and conclusion

In this paper, we present a novel application of the bead-based flow-cytometry assay for the semi-quantitative analysis of specific bacterial and host cell vesicle populations within complex samples, such as obtained after infection.

Membrane vesicle shedding during infection is a putatively important process in host-pathogen interactions. A better understanding of mechanisms for release of membrane vesicles during infection and how these are affected by environmental factors is required.

To assess this is complicated as most membrane vesicles fall within the same size range of 30–300 nm. Conventional techniques that allow vesicle quantification at a single particle level generally have certain

limitations as they may require highly specialized equipment (Hoen et al., 2012; van der Vlist et al., 2012), extensive sample pre-processing, or are not able to distinguish between different vesicle subpopulations (as compared by van der Pol et al., 2014; Witwer et al., 2013).

The application presented in the present study was originally developed by Thery et al. for the analysis of exosomes and has since then shown to be useful to study the processes that drive exosome release (Thery et al., 2006; Ostrowski et al., 2010). We found that the relative vesicle amount determined by flow-cytometry correlated well with the absolute vesicle concentration determined by TRPS demonstrating that this bead-based method is a practical tool for semi-quantitative vesicle detection. We show that there was little variation between separate experiments, making this assay highly reproducible. When this assay is adopted for custom applications we recommend validation of this assay accordingly, since it is imperative to start with the assessment of the antibody reactivity and to test for linearity to establish the concentration range over which this assay can be applied. The upper detection limit is reached when the beads are saturated and display none or little variation in the fluorescent intensity. The lower detection limit is reached when the signal can no longer be distinguished from the background signal. To further decrease the lower limit, we suggest to concentrate the samples. Apart from this, the platforms used in this study show a high variation in the absolute fluorescent intensity among the vesicle-bead complexes from the different bacteria. This variation could have been introduced by a higher affinity of the antibody to one or multiple vesicle-associated antigens per se or by the abundance of the antigen on the vesicle populations. Yet, as this assay can only be used to determine the relative abundance (relative to the control condition and other conditions based on the same platform), this does not affect the eventual interpretation of the results.

In this study, we showed that this method can be used to study the release of specific membrane vesicle subpopulations in complex samples composed of multiple types of vesicles, e.g. following infection of host cells by bacteria. The application of this assay on undiluted and diluted supernatants resulted in a dilution-dependent decrease in relative fluorescence intensity, confirming that this method allows the detection of relative changes in the vesicle concentration. Given the strong correlation between the relative concentrations (bead-based flow-cytometry) and the absolute numbers (TRPS), our data indicate that the methodology described here can be used to infer the absolute vesicle concentration of a specific vesicle population within a heterogeneous vesicle population. Determination of an absolute vesicle concentration can be performed with the use of a reference sample of the given specific vesicle population. So, to assess the release of vesicles by bacteria or host cells during infection, a condition that is solely based on vesicles released by bacteria or cells can be used as a reference.

The advantages of this technique are abundant. Firstly, no specialized and costly equipment is required, making this technique highly accessible. As this technique is based on the “in sample”-capture of vesicles, which also limits the processing time to 3–4 h, the introduction of processing-related artefacts is circumvented. Also, smaller volumes can be used, and processing is eased by the use of 4-μM-sized beads, making this method cheaper and less laborious then most conventional methods. As the flow-cytometric detection is based on the 4-μM-sized beads, the gates can be applied based on the forward and side scatter. Hence, signals that may interfere with other assays such as antibody aggregates, other aggregated proteins and soluble proteins do not complicate the measurements. Moreover, this assay offers a versatile platform that allows the simultaneous analysis of different phenotypic vesicle characteristics as beads can be coated with multiple antibodies. Also, labeling of bead-bound vesicles yields a higher fluorescence intensity in comparison with individual vesicles and thereby allows the detection of less abundant vesicle populations.

Inevitably, this technique also has some disadvantages. By choosing (a set of) specific antibodies to coat the beads, it cannot be excluded that subsets of vesicles which do not express this specific marker will not be recognized. Therefore, when specific subsets of vesicles are to be detected, it is recommended to use a combination of the most common and well accepted membrane vesicle markers (e.g. CD9/63/81 for host cell derived vesicles). Also, it has been shown that the vesicle composition may change when the circumstances change, notably if this applies to an epitope that is targeted by the antibody of choice this can be crucial. This for example has been shown for *P. aeruginosa* where exposure to stressors including oxygen tension, hydrogen peroxide, and gentamycin has been shown to affect the vesicles’ LPS phenotype. In these circumstances, it is important to determine if this has an effect on vesicle capture and detection.

Taken together, this method has been shown a valuable tool in assessing the release of membrane vesicles under a variety of conditions. In case of a homogeneous vesicle population, e.g. from a specific cell line, other techniques like high resolution flow-cytometry, dedicated flow-cytometry, nanoparticle tracking analysis, or TRPS may be found superior, but in more complex samples this method allows a rapid and accurate (semi-quantitative) analysis of specific vesicle subpopulations. Moreover, the versatility and ease of this platform may also be further exploited e.g. for vesicle arrays by using different coating-antibody and conjugated-antibody combinations or by using fluorescent or magnetic beads. Therefore, in our opinion this assay provides a valuable addition to the rapidly growing offer of techniques for vesicle detection as a fast and easy approach for the semi-quantitative assessment of vesicle subpopulations in complex samples.

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