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A post-insertion strategy for surface functionalization of bacterial and mammalian cell-derived extracellular vesicles

Linglei Jiang [a], Joen Luirink [b,c], Sander A.A. Kooijmans [a], Kok P.M. van Kessel [d], Wouter Jong [c], Max van Essen [a], Cor W. Seinen [a], Steven de Maat [a], Olivier G. de Jong [a], Jerney F. F. Gitz-François [a], Wim E. Hennink [e], Pieter Vader [a,f], Raymond M. Schiffelers [a,*]

[a] CDL Research, Division LAB, UMC Utrecht, Utrecht, the Netherlands Faculty of Medicine, Utrecht University, Utrecht, the Netherlands
[b] Department of Molecular Microbiology, Amsterdam Institute for Molecules Medicines and Systems (AIMMS), Vrije Universiteit, Amsterdam, the Netherlands
[c] Abera Bioscience AB, Solna, Sweden
[d] Medical Microbiology, UMC Utrecht, Utrecht, the Netherlands
[e] Pharmaceutics, Utrecht Institute for Pharmaceutical Sciences, Faculty of Science, Utrecht University, Utrecht, the Netherlands
[f] Laboratory of Experimental Cardiology, UMC Utrecht, Utrecht, the Netherlands

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ABSTRACT

Extracellular vesicles (EVs) are nanoparticles which are released by cells from all three domains of life: Archaea, Bacteria and Eukarya. They can mediate cell-cell communication by transferring cargoes such as proteins and nucleic acids between cells. EVs receive great interest in both academia and industry as they have the potential to be natural drug carriers or vaccine candidates. However, limitations to their clinical translation exist as efficient isolation, loading, labelling and surface-engineering methods are lacking. In this article, we investigate a 'post-insertion' approach, which is commonly used in the functionalization of liposomes in the pharmaceutical field, on two different EV types: mammalian cell-derived EVs and bacteria-derived EVs. We aimed to find an easy and flexible approach to functionalize EVs, thereby improving the labelling, isolation, and surface-engineering.

1. Introduction

Extracellular vesicles (EVs) are nanosized phospholipid bi-layered membrane-bound structures which can be released by cells from the three different domains of life: Archaea, Bacteria and Eukarya. The main components of EVs are proteins, lipids and nucleic acids. EVs are important signaling entities that can mediate intercellular communication by transfer of their macromolecular cargo. As a result they also represent a promising drug carrier platform due to the fact that they can protect encapsulated drugs (such as protein and RNA) from degradation. In addition, they have the potential to target specific tissues such as solid tumors or sites of myocardial infarction via the enhanced permeability and retention effect (EPR). [1,2] In addition, EVs hold therapeutic potential as vaccine candidates. For example, immune-stimulatory molecules such as MHC class I and II complexes are present on the surface of dendritic cells (DCs), as well as on the surface of their EVs. [3] In addition, pathogen-associated molecular pattern molecules (PAMPs) are present on the surface of bacteria, and these PAMPS are therefore also present on the surface of EVs shed from these bacteria [4,5]. As EV vaccines do not replicate, such a bacteria-derived vaccine is safer for clinical use when compared with attenuated bacterial vaccines.

However, challenges do exist to advance EV research. For example, an efficient labelling approach is lacking to study the biological roles of EVs in physiology and pathophysiology. Traditional labelling methods using lipidic dyes (e.g. PKH, DiO, DiR) might result in undesirable dye aggregates and hence may introduce 'labelling artifacts'. Genetic modification of donor cells for labelling purposes (e.g. fusing the Palm-tdTomato fluorescent marker to the canonical EV surface protein CD63) may limit the tracking to certain subtypes of EVs [6]. In addition, current isolation approaches are usually time-consuming and laborious which hinder the further scale-up in manufacture. Together, these challenges necessitate the development of a platform that would allow efficient EV labelling, rapid isolation and surface-engineering.

A membrane post-insertion approach could be considered as an attractive strategy for EV modification.'Post-insertion' is a simple and flexible method commonly used for producing ligand-coupled

* Corresponding author.
E-mail address: r.schiffelers@umcutrecht.nl (R.M. Schiffelers).

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liposomes. In this method, ligands of interest are firstly covalently linked to polyethylene glycol (mPEG)-lipid micelles, and afterwards te modified micelles are incubated with the liposomes of choice, after which the micelles transfer into the liposomal bilayer. [7]

Previous reports have shown that by incubating mammalian cells with distearoyl phophatidylethanolamine-poly(ethylene glycol)-biotin (DSPE-PEG-biotin) or DSPE-PEG-folate (DSPE-PEG-FA) micelles, also leads to spontaneous incorporation into the plasma membrane. As a result, EVs shed from these donor cells are also modified. [8,9] Using this 'post-insertion' approach, decoration of the EV membrane with biotin as well as FA is enabled. Several applications and advantages of this approach can be envisioned: 1) as DSPE-PEG lipids form micelles in aqueous solutions, it is unlikely to aggregate compared with traditional lipidic dyes; 2) EVs modified with PEG anchors have been shown to display prolonged circulation times in vivo and thus have increased opportunity to accumulate in target tissue [10]. 3) Successfully biotinylated EVs can bind to a number of streptavidin-conjugated entities such as streptavidin-dye conjugates, streptavidin-coated magnetic beads, and streptavidin-coupled antigens/antibodies, which offers opportunities for specific labelling, rapid isolation (compared with traditional ultracentrifugation methods) and flexible modification of the surface of EVs. In addition, successful incorporation of targeting moieties onto the surface of EVs can enhance their targeting, for example to the FA receptor highly expressed cells, for example, tumor cells. Previous reports show an efficient post-insertion labelling of two mammalian cell types under a single culture condition [8]. Here we investigated the robustness of the labelling in additional mammalian cell lines grown under different conditions. In addition, as bacteria-derived vesicles have emerged as a promising natural nanomedicine platform for vaccination strategies, we also tested the same modification approach here. This is, to our knowledge, the first time that a 'post-insertion' strategy is being used for bacterial outer membrane vesicles (OMVs).

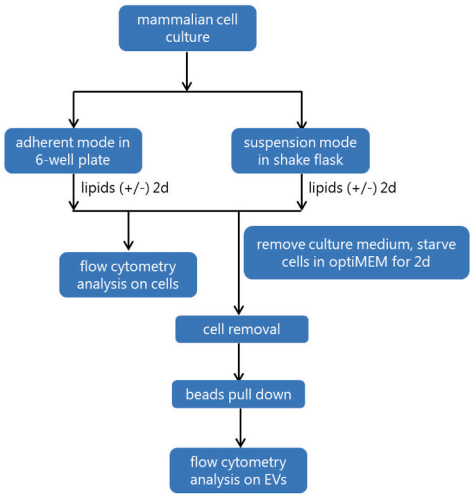
2. Results

2.1. Post-insertion labelling efficiency of MDA-MB-231 and HEK 293 T cells can be improved when cells are cultured in suspension mode and for longer periods of time

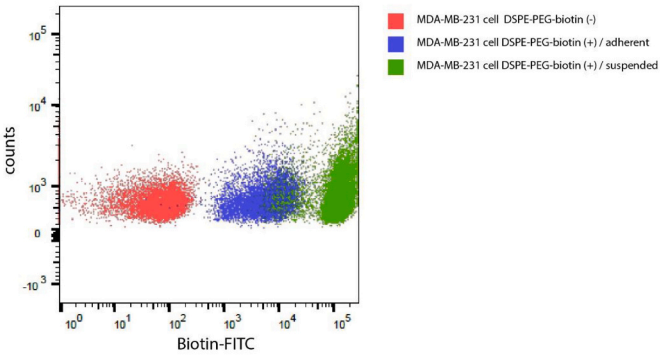
In order to improve the strategy for mammalian cells, two different cell types, i.e. a breast cancer cell line MDA-MB-231 and a human embryonic kidney cell line HEK 293 T, were cultured with/without 5 μg/mL DSPE-PEG-biotin lipid micelles in either adherent mode or suspension mode for 2 and 5 days, respectively. After 2-day culture, cells were collected after removing the free lipid micelles by centrifugation and filtration. The collected cells were then further divided to two groups. One group of cells were subjected to fluorescent-labelling with streptavidin-FITC and then analyzed by flow cytometry, the other group of cells were further cultured in EV-free OptiMEM medium for additional 2 days to produce EVs for further analysis. (Fig. 1a). Both MDA-MB-231 and HEK 293 T cells exhibited a more pronounced fluorescent shift when they were cultured in suspension mode compared to adherent mode (Fig. 1b and c), which suggests that a higher labelling efficiency can be achieved when the cells are cultured in suspension. After culturing for 5 days in adherent mode, both MDA-MB-231 and HEK 293 T cells showed more fluorescent labelling than the cells incubated for 2 days (Fig. S1a and S1c), which suggests the lipids assemble into the membrane of cells in a time-dependent manner. This time-dependent increase was also seen in MDA-MB-231 cells when cultured in suspension mode (Fig. S1b).

2.2. Post-insertion labelling efficiency of MDA-MB-231 cells-derived EV can be improved when cells are cultured in suspension mode but not HEK 293 T cells-derived EV

Next, to investigate the labelling efficiency of EVs derived from these



(a)



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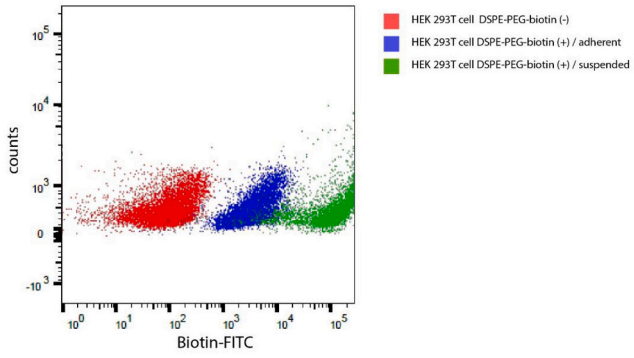


(c)

Fig. 1. Schematic diagram of cell cultured in adherent mode and suspended mode (a). Flow cytometic quantification of biotin on the surface of MDA-MB-231 cells (b) and HEK 293 T cells (c) cultured in adherent and suspended mode for 2 days.

cells, both cell types were first cultured in adherent or suspended mode in the presence of DSPE-PEG-biotin after which they were cultured in serum-free OptiMEM medium for 2 days. As CD63 is a general protein marker expressed on the mammalian EVs, a CD63-magnetic bead pull-down assay was performed. In this assay, the CD63-expressing EVs are bound to the magnetic CD63-antibody-carrying beads. CD63-positive EVs that are bound to the beads can be enriched on a magnetic plate within 5 mins. The captured EVs were then fluorescently labeled with

streptavidin-FITC conjugate. (Fig. 4a).

For MDA-MB-231 cells, when cultured in adherent mode, their EVs showed no labelling based on FACS analysis (Fig. 2a). In contrast, for cells cultured in suspended mode, EVs display clear fluorescent labelling (Fig. 2b). This suggests that the suspension culture can contribute to a higher EV labelling efficiency by this post-insertion strategy for this cell type. In contrast, EVs from HEK 293 T cells show a more pronounced fluorescent label in adherent mode than suspended mode (Fig. 2c and d). Combined, cell culturing conditions strongly influence the post-insertion efficiency in both mammalian cells and their EVs. For cells, higher labelling efficiency is achieved for the HEK 293 and MDA-MB-231 cells when cultured in suspension mode compared with the adherent mode reported previously. The labelling efficiency for EVs does not correlate with the labeling efficiency of the parental cells and optimal labeling conditions require experimental optimization.

2.3. *Salmonella bacterial surface is not modified by a post-insertion strategy*

Bacteria-derived EVs (often referred to as outer-membrane vesicles (OMVs) have triggered great interest for vaccination applications. Compared to their mammalian counterparts, bacteria-derived EVs appear easier to scale-up. In addition, the endogenous PAMPs are efficient triggers for an immune response. The first FDA-approved OMV Bexsero® is a *Neisseria meningitidis*-derived OMV used for protection against meningococcal infections. [11] Within such context, an engineered *Salmonella enterica serovar* typhimurium SL3261 strain with hypervesiculation properties due to deletion of two genes (*ΔTolR*, *ΔTolA*) genes that encode Tol-pal proteins which link peptidoglycan layer and outer membrane layer) and low-toxicity due to a single gene deletion (*ΔmsbB* a gene that encodes a transferase important for LPS biosynthesis) was selected in this study. It represents an interesting platform which can be used to display a range of antigens. [12] The post-insertion strategy may be an attractive approach to decorate the surface of these OMVs.

Bacteria were cultured in medium containing 40 μg/mL DSPE-PEG-Biotin and 5 μg/mL DSPE-PEG-FA for 3 days, after which membrane modification was assessed by FACS and immuno-Electron Microscopy (EM). No labeling was detected on FACS (data not shown) and also no DSPE-PEG-biotin nor DSPE-PEG-FA modification was observed on the membrane surface of bacteria when ultra-thin section slides of the bacteria were examined with highly sensitive immune EM (Fig. 3a and b). We did observe FA-associated gold particles in the bacteria's cytoplasm (Fig. 3b), however, these likely reflect endogenous FA storage [13].

As a positive control, LPS gold-labelling was observed on the surface of bacteria (Fig. 3c). Together, these data suggest that lipids DSPE-PEG-biotin/ DSPE-PEG-FA were not inserted into membranes of bacteria.

2.4. *Salmonella-derived OMVs can be modified by post-insertion strategy independent of bacteria cells*

To check the post-insertion efficiency on the OMVs released by those bacteria. Bacterial cells were removed by centrifugation and filtration, and OMVs were isolated from the filtrate by ultracentrifugation. A similar magnetic bead pull-down experiment as shown above for the mammalian EVs was performed on the isolated OMVs sample. Specifically, streptavidin-coated magnetic beads were used to capture DSPE-PEG-biotin positive OMVs. Subsequently, a one-step immuno-staining with streptavidin-FITC was performed (Fig. 4b strategy 1). Flow cytometry results show that DSPE-PEG-biotin is indeed associated to the OMVs bound by the magnetic beads (Fig. 5a). To rule out the possibility that biotinylated micelles were pulled-down, two two-step staining strategies were investigated. For these strategies (Fig. 4b strategy 2 and

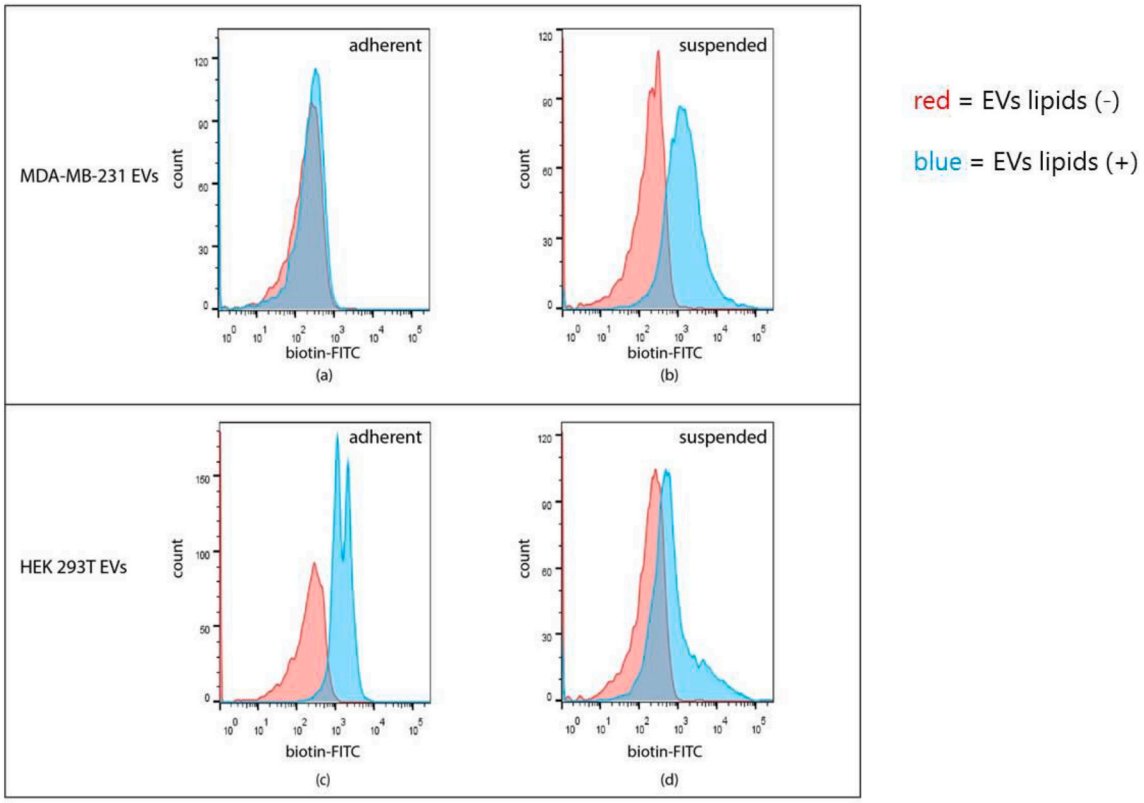
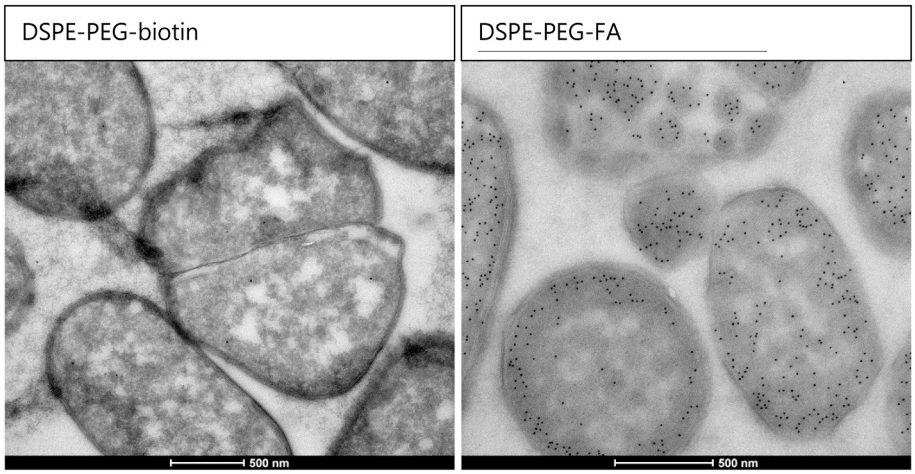


Fig. 2. Quantification of biotin on the surface of EVs shed from MDA-MB-231 2d cultured in adherent mode (a); suspended mode (b) and on the surface of EVs from HEK 293 T cells cultured in adherent mode (c); suspended mode (d). EVs were pulled down by a CD63-dynabeads. EVs from cells cultured without DSPE-PEG-biotin are shown in red and with DSPE-PEG-biotin in blue.



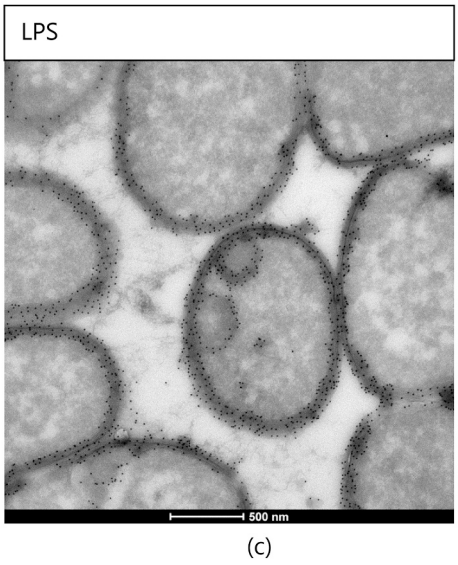


Fig. 3. Immunogold labelling of bacteria Ultra-thin section slides. (a) No anti-biotin-collidal gold labeling is observed for DSPEG-PEG-biotin incubated bacteria nor is (b) anti-FA-colloidal gold membrane labeling observed for bacteria incubated with DSPE-PEG-FA. Intracellular FA staining is seen (c). As a positive control, extensive membrane labeling of anti-LPS-colloidal gold was observed. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3), we detect other components present on the OMVs, thus FA moiety and LPS, than we used for the pull-down. We firstly check the presence of DSPE-PEG-FA which has similar structure of DSPE-PEG-biotin. Since the hypothesis for DSPE-PEG-biotin post-insertion mechanism is that its hydrophobic tail could insert onto the phospholipids bi-layer of OMVs, similar lipids should do the same trick. Next, if the OMVs were successfully captured by the beads, a general OMV marker should also be present. In our study, OMVs were captured using streptavidin-coated magnetic beads. Subsequently, the presence of FA was detected using an anti-folic acid antibody as primary antibody (Fig. 4b strategy 2); Alternatively, an anti-LPS antibody was used as the primary antibody (Fig. 4b strategy 3). The beads were subsequently counterstained with goat anti-mouse IgG APC conjugate and analyzed by flow cytometry. Both FA and LPS immunofluorescence were detected, which indicates the presence of all three epitopes on the OMVs. (Fig. 5a-b). Immuno-EM further confirmed this observation (Fig. 6a-b). As an additional supporting evidence, after OMVs being enriched by the magnetic beads, lysis buffer was added to lyse the samples associated with the beads. A pronounced staining of outer membrane protein C (OmpC), a protein abundantly present on the membrane of gram-negative bacteria, was observed by immunoblot methods (Fig. 6c). Together these data indicate that although the bacterial cell themselves cannot be labeled using a post-insertion strategy, this strategy works for the OMVs derived from the bacteria and both DSPE-PEG-Biotin/DSPE-PEG-FA was assembled onto OMVs successfully.

To further investigate whether OMV post-insertion is independent of the presence of the parental cells, bacteria were removed and the OMV-containing medium was filtered with 0.45um filters. After this, the filtrate was incubated with DSPE-PEG-biotin/FA. Interestingly, similar labeling efficiencies were observed as in the presence of the bacteria (Fig. 5d-f), which suggests the post-insertion process occurs independent of the parental cells from which the OMV were derived.

3. Conclusion and discussion

In summary, our data show that both mammalian cells and EVs and bacteria-derived EVs can be readily modified by a post-insertion strategy. In addition, we demonstrate that the labelling efficiency of mammalian cells can be improved by culturing them in suspension mode. We also discovered differences between the modification process between mammalian-derived EVs and bacteria-derived EVs.

In the mammalian system, lipids first assemble into the donor cell membrane, and, as a result, EVs shed from those cells will also be modified. At present it is unclear how the ratio between exosomal

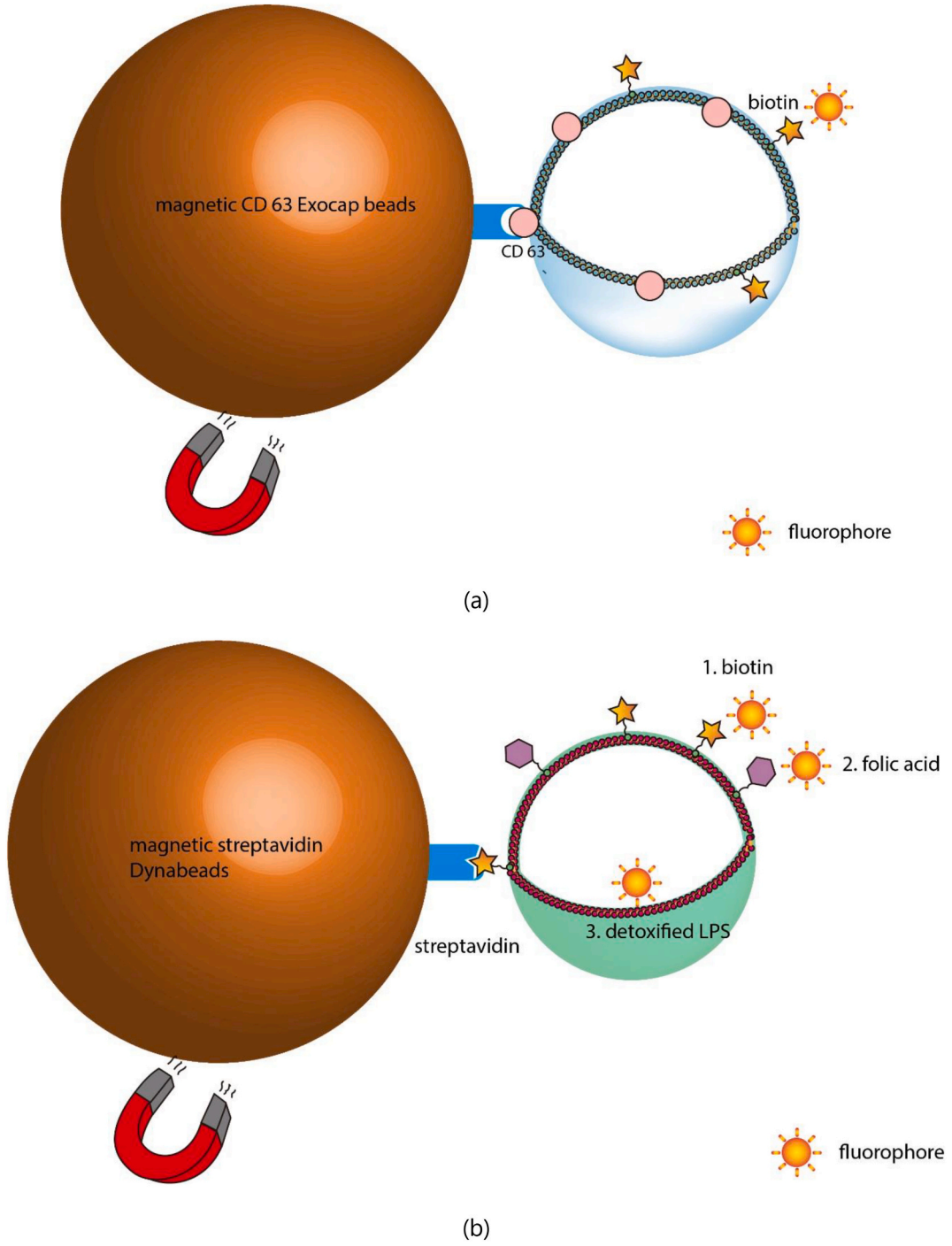


Fig. 4. Bead pull-down strategies for mammalian cells-derived EVs (a) and bacteria-derived OMVs (b). Mammalian EVs are pulled by CD 63 Exocap beads firstly and then stained with streptavidin-FITC; Different detection strategies used to confirm the successful engineering of OMVs. OMVs were firstly pulled down by streptavidin magnetic beads, and the beads were 1) incubated with biotin-fluorophore conjugate; 2) incubated with mouse anti-folic acid antibody, and then incubate with rabbit anti-mouse fluorophore; 3) incubated with mouse anti-LPS antibody, and then incubate with goat anti-mouse IgG APC.

labeling and microvesicle labeling is for this post-insertion strategy. It can be anticipated that the microvesicle labeling is more straightforward as it is not dependent on internalization and sorting.

As for bacteria, no insertion was detectable onto the surface of bacterial cells. This may be due to the fact that the outer membrane contains heavily glycosylated lipids which may have sterically shielded the surface of the cell. In contrast, lipids were found to more readily insert into the membrane of the vesicles directly. This may be due to a different lipid composition of the OMVs compared to the membrane form which they originate or the physicochemical characteristics of the OMVs. As OMVs represent a high curvature structure these often have lipid packing defects that may facilitate insertion [14]. Taken together we propose different post-insertion mechanisms between mammalian EVs and bacterial OMVs. For mammalian EVs, cells incorporate the exogenous lipids into the cell membrane. The EVs shed from those modified cells are subsequently modified as well. As for bacteria, the exogenous lipids inserted into OMVs directly independent of the parental cells (Fig. 7).

This lipid post-insertion strategy offers an easy and rapid approach to modify the surface of both mammalian cells and bacteria-derived EVs

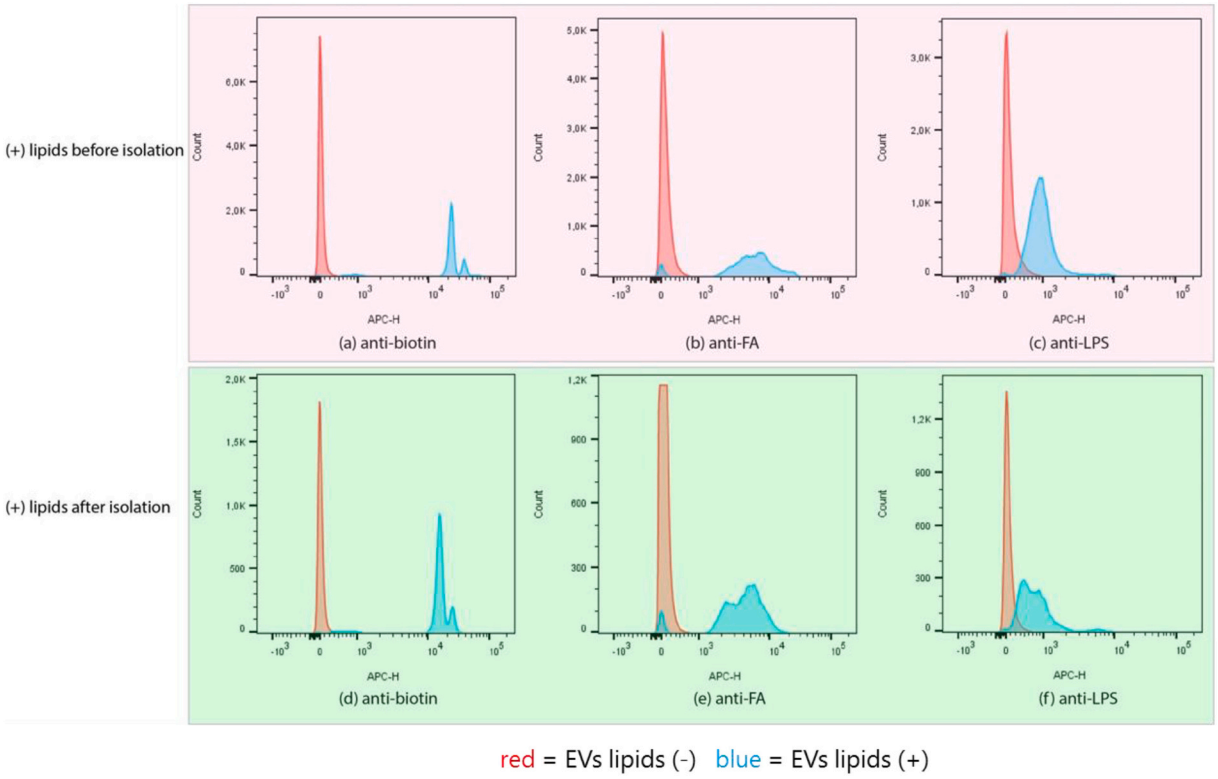


Fig. 5. Quantification of the DSPE-PEG-biotin, DSPE-PEG-FA, LPS on the surface of compositions pulled down by the streptavidin-magnetic beads when lipids were added during the bacteria culture (a)-(c) and when lipids were added to the OMVs-containing medium only (d)-(f).

when compared with traditional genetic engineering approach. However, the in vitro and in vivo function of these lipids-modified EV are not known yet, future study will address this issue.

4. Materials and methods

4.1. Chemicals and reagents

Mouse monoclonal anti-folic acid (F5766), Optiprep density gradient medium (D1556) and Halt Protease and Phosphatase Inhibitor Cocktail (ABCA3796199) were purchased from Sigma-Aldrich chemie. Anti-Salmonella typhimurium 0–4 antibody [1E6](AB8247) was purchased from Abcam. DSPE-PEG-2 K-biotin (880129) was purchased from Avanti Polar Lipids, Inc. DSPE-PEG-2 K-folate were purchased from Nanosoft polymers. Polyclonal Goat anti Mouse IgG APC (405308) was purchased from Biolegend. RIPA buffer (20–188) was purchased from Merck Millipore. Alexa Fluor 647-conjugated streptavidin was purchased from Jackson Immuno Research. And rabbit anti-Omp C antibody is in-house from Department of Medical Microbiolgy, UMC Utrecht. Dulbecco's modified eagle medium (DMEM, 42430–025), OptiMEM medium (51985) and Free-Style 293 medium (12338) was purchased from Thermofisher Scientific.

4.2. Mammalian cell culture

For adherent mode cell culture experiment, MDA-MB-231/HEK 293 T cells were seeded at the density of 2×10^5 in 2 mL medium (DMEM supplemented with 10% fetal bovine serum and 1% Penicillin-Streptomycin) in 6-well plate, and cultured with/without the addition of DSPE-PEG-biotin (5 μg/mL), and cultured in a normal incubator maintained at 37 °C and 5% CO_2.

For suspended mode cell culture experiment, MDA-MB-231/HEK 293 T cells were seeded at the density of 2×10^5 in 2 mL medium, and culture in a shaker embedded inside the incubator.

Cells were collected and stained for 15 min at room temperature with Streptavidin-Alex488 conjugate (final conc. 1μg/mL in 1% FBS-containing PBS) and analyzed by BD FACS Canto II™. SPHERO™ Rainbow Calibration Particles (Spherotech) are used to determine the voltages for the uniform calibration of the flow cytometers.

4.3. Mammalian cells-derived EVs isolation and bead assay

For EVs isolation, cells were cultured in adherent and suspended mode as mentioned before, and washed and continued culturing with OptiMEM™ for 2 days. Remove the cells by serial centrifugation steps, firstly 300 *g* for 5 mins and then 2000 g for 15 mins at room temperature. The EVs in the supernatant was captured by CD63 ExoCap beads (JSR life sciences) overnight at 4°*C*, and the beads were subsequent counterstained with Streptavidin Alexa-488 and analyzed by BD FACSCanto™.

4.4. Preparation of LB-0 medium

10 g bacto tryptone (Oxoid LP0042) and 5 g yeast extract (Oxoid LP0021) were added in 1 L milliQ water and autoclaved at 121°*C* for 15 min.

4.5. Bacteria culture and OMVs isolation

Streaked bacteria from glycerol stock and inoculated in 2.5 mL LB-0 medium (2 mM $MgCl_2$, 2 mM $CaCl_2$ LB-0, 0.2% glucose, 25 μg/mL kanamycin) at 30°*C* overnight, and scale up of the overnight culture in fresh LB medium (subculture ratio 1:40) with the addition of DSPE-PEG-biotin (40 μg/mL) and DSPE-PEG-FA (5 μg/mL) for 3 days.

Bacteria were removed by centrifuging the culture medium at 2000 g for 10 min at 4°*C*. And the supernatant was filtered through 0.45*μ*m filter with syringe. The filtered medium was then subject to ultracentrifugation at 48000 rpm at 4°*C* for 1 h and 15 mins (50.2Ti rotor,

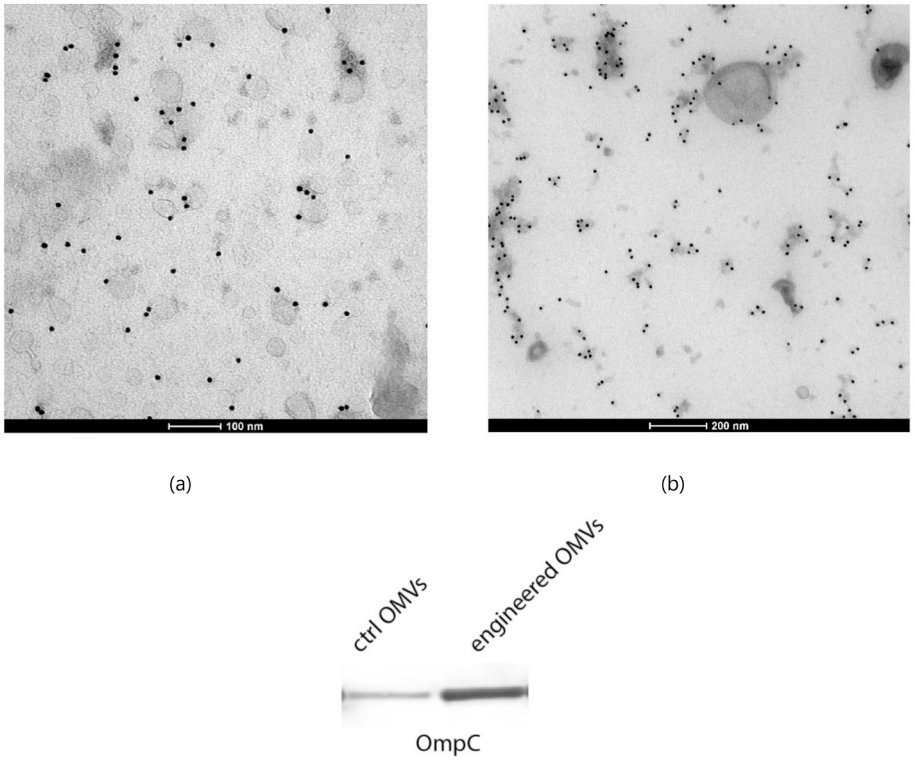


Fig. 6. Immuno-gold labelling of DSPE-PEG-biotin (a) and DSPE-PEG-FA (b), PBS was used as an internal negative control in this experiment; OmpC protein immuno-blot of control OMVs and engineered OMVs (c). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Beckman). The crude pellet were resuspened in 60% iodixanol solutions and then subject to gradient ultracentrifugation (60%, 55% 50%, 45%, 40%, 35%, 30%, 25%, 20%, 15%, 10%, 5% and 0%) (SW40Ti rotor, Beckman) at 39000 rpm at 4°*C* for 14 h. 3 fractions (15%, 20%, 25%) were pooled and concentrated by 100KD centrifugal filter (Amicon). The concentrate was washed with PBS for 3 times to remove the excess iodixanol.

4.6. Bacteria ultra-thin section

Ultrathin bacteria slides are prepared with a diamond knife at −120°*C* on a Leica UCT Ultracut (cryo sectioning). Bacteria section were picked up with looping device filled with mixture of methylcellulose and sucrose. Samples are incubated with freshly carbon coated formvar grid (Hexagonal Cu and processed following the Tokuyasu method. Firstly, samples are fixated in PBS solutions containing 2% paraformaldehyded and 0.2% glutaraldehyde for 1 h at room temperature. Then washed with PBS. Afterwards, samples are added to warm 12% gelatin solution and pelleted by centrifugation. Rapid cooling of the sample-containing gelatin are performed by placing it on ice, which results in the solidification of the gelatin. Pellet of fixated bacteria in solidified gelatin is retrieved from eppendorf vial and cut with a clean razor blade into small cubic blocks (~1 mm^3). Sucrose infiltration steps are performed by incubating the gelatin blocks in 2.3 M sucrose PBS solution overnight in a carrousel.

Samples were incubated with freshly carbon-coated grids. Grids with samples were incubated on several droplets of 40°*C* phosphate buffer. Then inactivate the fixative with 0.15% Glycine. Blocking step was performed with 1% BSA-containing PBS. Immuno- labeling were performed with the rabbit anti-biotin antibody (1:10000)/ mouse anti-folic acid antibody (1:200)/ mouse anti-LPS antibody (1:200), staining and embedding in Uranyl Oxalate/Uranyl Methylcellulose solution. Tecnai 20 Twain MK2 (5022/11) was used for acquiring images.

4.7. OMVs immuno-gold labelling and TEM imaging

For folic acid immuno-gold labelling, OMVs in PBS were adsorbed to carbon-coated formvar grids for 10 mins at room temperature, washed 3 times in PBS droplets, block in 1% BSA for 10 min and incubate with mouse anti-folic acid antibody (1st Antibody) for 20 min, wash 3 times in 1% BSA and then incubate with rabbit anti-mouse antibody (1:250 dilution, bridging antibody from UMC Utrecht, the Netherlands) for 10mins. Intensive washing steps were performed (7 times in PBS and 1 time in 1% BSA), and incubate in 10 nm protein A-gold for 15 mins and fixated in 2% paraformaldehyde and 0.2% glutaraldehyde-containing PBS. After 8 times washing with milliQ water, grids were embedded in methyl cellulose uranyl-acetate (pH 4). For biotin immuno-gold labelling, similar steps as folic acid labelling were used, and rabbit anti-biotin antibody (1:10000 dilution, UMC Utrecht, the Netherlands) was used and the bridging step was omitted. Grids were imaged using a Tecnai T12 transmission electron microscope (FEI, Eindhoven, The Netherlands).

4.8. Beads pull down and flow cytometry

In a 96-well round-bottom plate, 0.2 μL Dynabeads™ M-280 Streptavidin (11205D, Thermofisher) per well were incubated with samples for 2 h at room temperature while shaking. Using the magnetic plate to pull down the beads, and remove the sup ernatant. Beads were blocking in 1% BSA, and afterwards incubate with 50uL diluted mouse anti-folic acid antibody (1:200 dilution)/ mouse anti-*Samonella* antibody (1:200) for 1 h at room temperature. After the removal of 1st antibody and

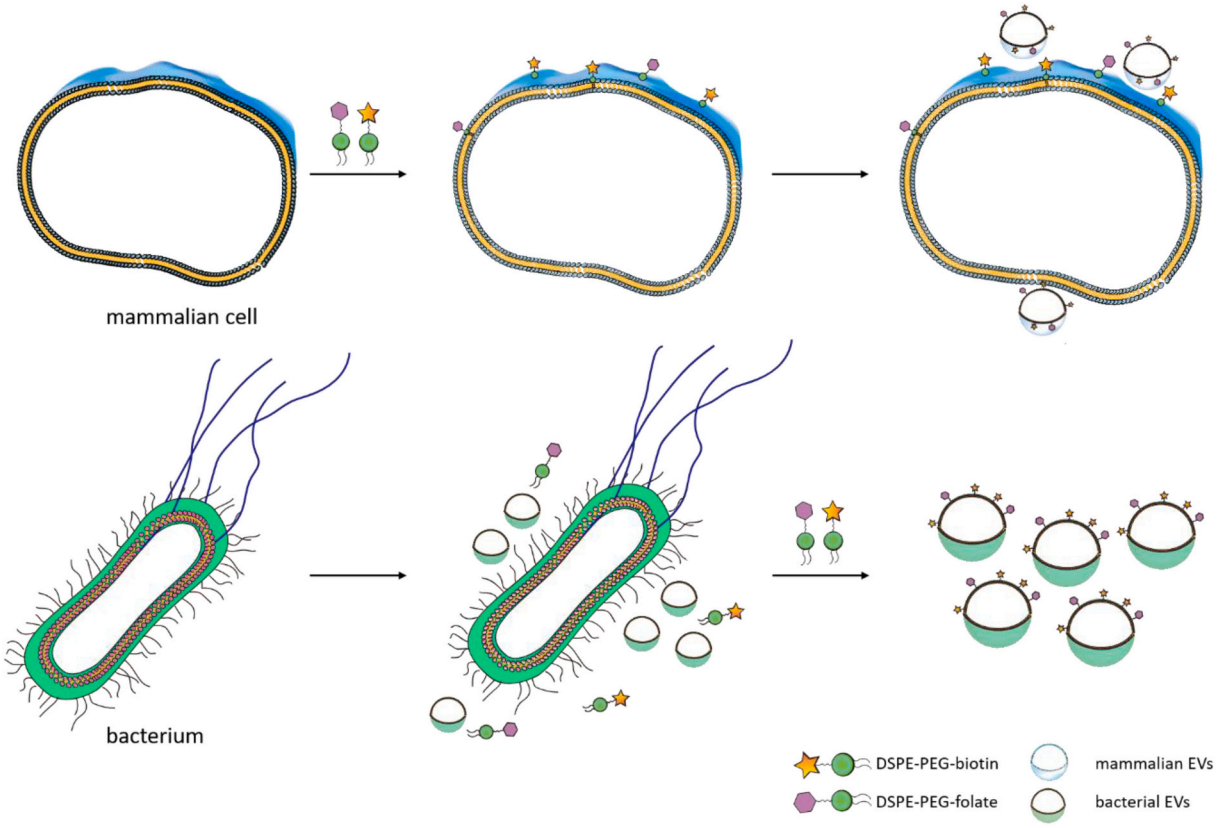


Fig. 7. Lipids modification mechanism involved in mammalian-derived vesicles (upper row) and bacteria-derived vesicles (bottom row).

washing, add Polyclonal Goat anti Mouse IgG APC (405,308 Biolegend, 1:500 dilution) and incubate for 1.5 h at room temperature while shaking (keep plate in dark). Wash away the excess fluorophore and resuspend the beads in 200μL 1% BSA solution. Flow cytometry analysis was performed with FACSCanto II™ (BD science, USA). And for biotin analysis, Alexa Fluor 647-conjugated streptavidin (1:600) was incubated with beads for 1 h at room temperature.

4.9. SDS-PAGE and Western blot

To reach the maximum pull down efficiency of the OMVs in the sample, 0.5μL beads per well were added, and after incubating with beads for 2 h at room temperature while shaking. The supernatant was removed, and beads were washed 3 times with PBS. And lyse the OMVs with 1× RIPA buffer supplemented with 1× protease inhibitor cocktail. Samples were mixed with reducing sample buffer containing dithiothreitol (DTT), heated to 95 °C for 10 mins and subjected to electrophoresis over 4–12% Bis–Tris polyacrylamide gels (Thermo Fisher Scientific). Gels were stained with PageBlue Protein Staining Solution (Thermo Fisher Scientific) according to manufacturer's instructions. Alternatively, proteins were blotted on Immobilon-FL polyvinylidene difluoride (PVDF) membranes (Merck Millipore, Amsterdam, The Netherlands), which were subsequently blocked with 50% *v*/v Odyssey Blocking Buffer (LI-COR Biosciences, Leusden, The Netherlands) in Tris buffered saline (TBS). Antibody incubations were performed in 50% v/v Odyssey Blocking Buffer in TBS containing 0.1% v/v Tween 20 (TBS-T). Primary antibody are in-house rabbit anti-OmpC antibody (dilution 1:50). And secondary antibody is Alexa Fluor 680-conjugated anti-rabbit antibodies (Thermo Fisher Scientific, A-21076, 1:10000). Imaging was performed on an Odyssey Infrared Imager (LI-COR Biosciences, Leusden, the Netherlands) at 700 nm.

Author statement

Linglei Jiang, Max van Essen, Cor Seinen, Jerney Gitz-François, Sander A.A. Kooijmans were responsible for experimental work, data processing and data analysis,

Linglei Jiang, Joen Luirink, Kok P. M. van Kessel, Wouter Jong, Steven de Maat, Olivier G. de Jong, Pieter Vader and Raymond M. Schiffelers were responsible for the conception and design of the work and interpretation of the data and drafting the manuscript.

Declaration of Competing Interest

J.L. and W.S.P.J. are involved in Abera Bioscience AB, which aims to exploit outer membrane vesicle-based delivery technologies.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbagen.2020.129763.

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