

PROCESS FOR THE PURIFICATION OF OMVS FROM GRAM-NEGATIVE
BACTERIA, AND RELATIVE USES

The present invention relates to a process for the
5 purification of vesicles released from the outer
membrane (OMVs) of Gram-negative bacteria, the OMVs
thus obtained and their relative uses as antigen
carriers in vaccine formulations and as a system for
conveying molecules for therapeutic applications.

10 Outer Membrane Vescicles (OMVs) are double-membrane
vesicular structures released naturally and
spontaneously by all Gram-negative bacteria during
their growth. ([1],[2]).

OMVs consist of a lipid-based spherical structure
15 with a diameter of 20-250 nm and comprise bacterial
outer membrane proteins, lipopolysaccharides (LPS),
phospholipids, cytotoxic factors, periplasmic space
proteins, DNA, RNA and enzymes [3].

OMVs are formed when a portion of the outer
20 membrane with a periplasmic content is selectively
expelled to form spherical vesicles.

The production of OMVs has been observed for a wide
variety of Gram-negative bacteria at all growth stages
and in different growth environments, including
25 infected tissues. Vesicle production is in fact
probably related to the response to bacterial stress.

OMVs can be purified from bacterial cultures with
different methods that mainly involve double tangential
flow filtration (TFF) ([4],[5],[6]). The vesicles,
30 after being purified, are analytically characterized
through an analytical panel that allows their

quantification, dimensional determination, the detection of specific molecules of interest and the quantification of the components of the OMVs themselves [7].

5 OMVs are widely recognized as a very versatile platform for pharmaceutical applications, ranging from vaccines to drug delivery. Numerous studies demonstrate a high level of *in vivo* immunogenicity of the vesicles and their excellent adjuvant properties are also
10 studied and demonstrated ([2],[8]).

The development of a safe and effective vaccine requires considerable resources and time. The imminent post-antibiotic era and the associated antimicrobial resistance (AMR, the ability of microorganisms to
15 survive or grow despite the presence of an antimicrobial agent) may, on the other hand, require the development of a significant number of vaccines within a short period.

In this context, the OMV technological platform
20 combines a natural and optimal antigen presentation with excellent adjuvant properties, making it highly suitable as a vaccine platform [9]. OMVs are vesicles naturally produced by Gram-negative bacteria and endowed with excellent intrinsic immunostimulatory
25 properties based on their composition, particulate nature and bacterial origin. Furthermore, OMVs are suitable for acting as antigen carriers, heterologous proteins and glycan antigens can in fact be easily incorporated into vesicles.

30 The unique capacity of OMVs of conveying molecules through the cell membrane of Gram-negative bacteria makes OMVs suitable for use as natural delivery

vehicles for drugs, biopharmaceuticals and nucleic acids for overcoming the limitations and innate or acquired resistance of bacteria ([10]-[16]).

5 Their immunostimulatory properties can be optimized and their innate reactogenicity can be reduced, using different genetic engineering techniques [17]. These modified OMVs (mOMVs) have proven to be an industrially adaptable and sustainable vaccine production platform at very low costs and with a consequent lower
10 environmental impact in terms of raw material consumption, energy consumption and waste materials to be disposed of.

There are some limitations, however, that affect the production yield of OMVs in OMV production
15 processes from pathogenic and non-pathogenic bacterial species. This has consequences not only in financial terms, in the case of using the platform for large-scale vaccine production, but also in terms of sustainability.

20 Furthermore, due to their intrinsic nature, purified OMVs may give rise to heterogeneous mixtures especially in terms of size, which may have significant consequences in terms of production standardization. The homogeneity of OMV particles therefore provides
25 greater reproducibility in terms of membrane antigen presentation and a greater capacity to serve as a delivery system for heterologous molecules.

Another major obstacle, especially for pathogenic and non-pathogenic *Neisseria* species, relates to the
30 presence of contaminants such as nucleic acids in the purified OMVs. This leads to significant consequences especially in terms of product safety and the research

and development efforts necessary for complying with regulatory requirements in terms of maximum residual DNA concentration present in the final product [18].

Piliou S. et al. 2023 [19] describe a procedure for
5 the production of mOMVs from the *Neisseria cinerea* strain ATCC14685 which involves a double ultracentrifugation step.

The OMVs thus obtained, although having a good degree of homogeneity and dimensions between 30-200 nm,
10 still have a fairly significant degree of contamination by nucleic acids.

The authors of the present invention have now developed a process for the purification of OMVs which involves the use of depth filtration for the removal of
15 bacterial biomass, thus overcoming the various technical problems that affect the production processes of the state of the art.

The innovative use of depth filtration in the bacterial biomass removal process has in fact allowed
20 for the efficient extraction of OMVs, which are not retained by the filter, thus significantly increasing their degree of purity in terms of a more effective removal of nucleic acids which however are selectively retained by the filter. The entire process is
25 drastically simplified by eliminating the expensive and complex cleaning phases of the purification system used in tangential flow filtration (TFF) that weigh on the production processes of the state of the art. The use
30 of tangential flow filtration in fact involves the use of a significant volume of solutions for effecting the filtration processes and the final cleaning of the equipment. The high volume of solutions used leads to

high volumes of purified water to be produced and subsequently disposed of.

The present invention therefore relates to a purification process of vesicles released from the outer membrane (OMVs) of Gram-negative bacteria which comprises the following steps:

- a) microfiltration of the bacterial biomass obtained from the fermentation of Gram-negative bacteria producing OMVs by means of a depth filter (DF);
- b) ultrafiltration of the filtrate obtained from step a) by means of i) tangential flow filtration (TFF), depth filtration (DF), ultracentrifuge and chromatographic filters.

In a preferred embodiment of the present invention said OMV-producing Gram-negative bacteria are wild-type or genetically modified strains. According to a preferred embodiment said Gram-negative bacteria belong to the genus selected from the group consisting of *Neisseria*, *Escherichia*, *Klebsiella*, *Shigella*, *Salmonella*, *Yersinia*, *Haemophilus*, *Pseudomonas*, *Moraxella*, *Bordetella*, *Borrelia*, *Brucella*, *Chlamydia*, *Legionella*, *Vibrio* and *Helicobacter*.

In a particularly preferred embodiment said strains belong to the species *Neisseria cinerea*. Again preferably, said *Neisseria cinerea* strain is *N. cinerea* ATCC14685.

Alternatively, a genetically modified strain of *N. cinerea* can be used for down-regulating or inactivating a gene selected from the group consisting of the *lpxL1* gene, the *RmpM* gene and the *MltA* gene.

According to a further embodiment, the strain of *N. cinerea* is genetically modified to introduce and/or up-

regulate the expression of a gene selected from the group consisting of the fHbp gene and the mutated fHbp gene R41S.

The term "genetically modified" includes genetic
5 modifications introduced by means of recombinant techniques, but also non-recombinant techniques such as, for example, exposure to chemicals, radiation or other agents that modify or damage DNA and the like.

In a preferred embodiment of the purification
10 process according to the present invention, the microfiltration step a) comprises the following sub-steps:

- i) hydration of the depth filter with a low salinity (< 0.2 M) and conductivity (< 20 mS/cm) buffer;
- 15 ii) flushing the bacterial biomass directly from the fermenter to the depth filter and recovering the filtrate;
- iii) flushing the depth filter with a wash buffer to recover the dead volume of the filter and recover the
20 filtrate.

In a preferred embodiment, the depth filter is connected to a peristaltic pump.

According to a further embodiment, the depth filter used for the microfiltration of step a) is a cellulose
25 or polypropylene filter with a retention index within the range of 3-6 μm .

Said filter is preferably a depth filter of recycled cellulose Supracap™ 100.

According to a further embodiment of the
30 purification process of the invention, the buffer used in steps i) and iii) is a phosphate buffer, preferably PBS.

Again according to a further preferred embodiment of the invention, step b) for the TFF ultrafiltration of the filtrate obtained from step a) is effected through a 300 kDa filter.

5 In a preferred embodiment, said TFF ultrafiltration step b) comprises the following sub-steps:

- i) diafiltration with 3 volumes of PBS buffer;
- ii) diafiltration with 8 volumes of high salinity (> 0.5 M) and conductivity (> 50 mS/cm) Tris-HCl
- 10 buffer;
- iii) diafiltration with 2 volumes of PBS buffer;
- iv) final concentration 10:1 v/v of retentate;
- v) collection of the OMVs and sterilization on a 0.22 μ m filter.

15 The present invention further relates to the OMVs produced by *Neisseria cinerea* obtained by means of the purification process described above, characterized by having a ratio quantity of DNA/quantity of OMVs < 1.5 μ g/mg, a polydispersity index of approximately 1 and a

20 hydrodynamic radius ranging from 20 nm to 50 nm. The OMVs thus obtained have a DNA/OMV ratio which is such as to make them suitable for use as a component of vaccines according to the requirements established by the FDA and EMEA guidelines which provide for a DNA

25 content per dose of vaccine of <10 ng.

The present invention also relates to OMVs produced by *Neisseria cinerea* obtained by means of the purification process described above for use in the medical field as antigen carriers, adjuvants in vaccine

30 formulations or for conveying the administration of molecules and biomolecules for therapeutic applications.

The OMVs produced by *Neisseria cinerea* thus produced can in fact be advantageously charged with one or more heterologous molecules, selected from immunologically active agents and pharmaceutical active ingredients.

As used herein, an "immunologically active agent" is an agent capable of inducing and/or positively modulating an immunological response in a subject, alone or in combination with other agents.

The invention also provides for a pharmaceutical composition comprising the OMVs purified from *Neisseria cinerea* obtained by the purification process described above, as an antigen carrier, an adjuvant for use as a vaccine or a vehicle for the administration of molecules and biomolecules for therapeutic applications.

The pharmaceutical compositions comprising the OMVs according to the invention can be administered intraperitoneally, intranasally, subcutaneously, intramuscularly, transdermally, orally, topically or by aerosol. The above pharmaceutical compositions can be provided in the form of aerosol, liquid or suspension.

According to a preferred embodiment the above pharmaceutical composition in the form of a vaccine comprising the OMVs of the invention will be directed against an infection caused by a viral or bacterial pathogen selected from the group consisting of *N. gonorrhoeae*, *N. meningitidis*, staphylococcus, streptococcus, coronavirus, influenza virus, HIV, Zika virus and plasmodium.

Finally, the invention relates to a carrier system of antigens or drugs comprising the OMVs from *Neisseria*

cinerea obtained by means of the purification process described above.

The present invention will now be described for illustrative, but non-limiting, purposes according to a preferred embodiment with particular reference to the attached figures, in which:

- Figure 1 shows the schematics of the purification process of OMVs by tangential flow filtration (panel A) and according to the invention by depth filtration (panel B).
- Figure 2 shows the overlay of the chromatograms with absorbance 280 nm (solid line) and 260 nm (dashed line) of the OMVs purified with TFF (A) and DF (B).
- Figure 3 shows the HPLC chromatograms, absorbance at 280 nm of OMVs isolated using depth filtration (solid line) and TFF (dashed line). The two samples were analyzed at the same OMV concentration and volume.
- Figure 4 shows the MALS chromatograms of OMVs purified by depth filtration (solid line) and by tangential flow filtration (dashed line).

The following illustrative but non-limiting examples of the purification process of OMVs from *Neisseria cinerea* and the advantages obtained are provided for a better illustration of the invention:

EXAMPLE 1: OMV production process from *Neisseria cinerea*

MATERIALS AND METHODS

Revitalization of the bacterial strain and production of the Cell Bank

The bacterial strain used was purchased from ATCC (American Type Culture Collection) and is the strain *Neisseria cinerea* ATCC® 14685.

The strain was received in lyophilized form and was revitalized as indicated hereunder. The lyophilized pellet was rehydrated using a liquid GCBL culture medium ("gonococcal base medium") containing supplements [20]. The cell suspension obtained was used for inoculating a Falcon tube containing liquid GCBL medium which was then placed in an incubator shaker (Inforce HT Minitron) at $37^{\circ}\text{C} \pm 1$ and 150 rpm. Using an inoculating loop, a small amount of suspension was used for plating the culture on two GC agar plates. The plates were incubated at $37^{\circ}\text{C} \pm 1$ in a 5% CO_2 atmosphere (Memmert ICOMed) for 24 hours. After growth on plates, a single colony was selected and used for re-plating on two GC agar plates in order to isolate pure colonies. The 2 plates were incubated at $37^{\circ}\text{C} \pm 1$ in a 5% CO_2 atmosphere for 24 hours. Single colonies grown on these plates were selected and used for inoculating a Falcon tube containing 5 ml of Neisseria medium free of animal-derived components. This Neisseria medium was developed by modifying the Frantz medium commonly used for the growth of *Neisseria meningitidis* strains [21]. The inoculated Falcon was used for producing Research Cell Bank stocks, which are kept frozen at -80°C with a final glycerol concentration of 20%.

The Research Cell Bank was sent to the Laboratory of Molecular Microbiology and Biotechnology (Department of Medical Biotechnology, University of Siena) for bacterial identification. Identification tests comprise the following molecular and biochemical assays: culture on selective and non-selective media, microscopic analysis with Gram's test, oxidase and catalase tests,

identification with VITEK® MS MALDI-TOF BioMérieux and identification by 16S ribosomal RNA sequencing.

Preparation of the inoculum

The inoculation step of the culture is essential in order to produce sufficient bacterial density for starting the process in the fermenter.

The inoculation flasks were prepared as follows: for each flask, a Research Cell Bank vial was thawed in an incubator at $37^{\circ}\text{C} \pm 1$ and used for inoculating a polycarbonate flask with a breakwater (Erlenmeyer) having a total volume of 1000 mL and a vented screw cap (CORNING 431403), containing 200 mL of preheated Neisseria medium.

The 2 flasks were incubated at $37^{\circ}\text{C} \pm 1$ for 9 hours in a shaking incubator at 150 rpm. At the end of the growth, the cultures reached an OD 600nm value of 3.5. This value was measured using a photometer and with plastic cuvettes with a light path length of 1 cm.

Growth in a bioreactor

A fermenter having a total capacity of 3 liters (Sartorius II MO) was used for the growth of the bacterial strain. The fermenter contained 1.5 l of sterile Neisseria medium and was assembled with breakwaters and two Rushton-type impellers with six blades ($\phi = 5.5$ cm) for stirring the culture. One impeller was placed 3.5 cm from the bottom of the fermenter and the other 7 cm from the first.

The operating conditions for the fermentation of the bacterial strain were: temperature at 37°C ; Dissolved Oxygen (DO) 30%; air flow from 1.5 to 3 l/min (from 1 to 2 vvm); stirring frequency from 200 to 1,000 rpm. Air and stirring were set in cascade to maintain

the DO value at 30%.

The pH was set at 7.2 and measured with a sterilizable on-line electrode (Hamilton EasyFerm BIO HB Arc 225). 2M NaOH and 1M H₃PO₄ solutions were used
5 for maintaining the pH value set. The DO concentration was detected with a sterilizable on-line electrode of the optical type (Hamilton VisiFerm DO Ecs 225 H0). The calibration of the 100% DO point was effected under the same operating conditions before inoculating the
10 fermenter.

The culture contained in the inoculating flask was used for inoculating the fermenter. For all tests the volume of inoculum culture to be used was calculated to obtain an initial OD 600 nm in the fermenter of 0.2
15 units. The foam was controlled with the presence of PPG (VWR 297776T) in Neisseria medium at a final concentration of 0.2 g/kg. The fermentation was effected in batch. The OD 600 nm value of the culture was monitored during bacterial growth using a 600 nm
20 photometer and using plastic cuvettes with a light path length of 1 cm.

OMVs were produced during the batch fermentation from the microbial biomass.

The fermentation process was terminated when the
25 culture reached the stationary phase.

This phase can be recognized as the OD 600 nm value measured every hour is unchanged (or lower) compared to the value previously measured. Furthermore, a sudden increase in the DO signal associated with a rapid
30 decrease in the stirring and a reduction in the acid recall for controlling the pH, confirm the stationary phase and therefore the end of the fermentation.

In order to ascertain the actual impact of depth filtration on the biomass removal process, two fermentations of *Neisseria cinerea* ATCC 14685 were effected maintaining identical growth conditions.

5 The two cultures of *Neisseria cinerea* ATCC 14685 reached a final OD 600 nm value equal to 9 after 7 hours from the moment of inoculation of the fermenter.

EXAMPLE 2: Comparison of microfiltration purification processes of OMVs produced by *Neisseria cinerea*

10 The biomasses obtained were subsequently microfiltered using depth filtration (DF) according to the invention and TFF (known technique).

Process A (according to the invention): microfiltration with a depth filter (DF)

15 In order to remove the biomass and extract all the OMVs from the culture broth, a depth filtration (DF) step was used instead of the well-known tangential flow filtration (TFF) step.

20 The depth filter used for microfiltration is a regenerated cellulose Supracap™ 100 with a retention rating of 3-6 µm connected to a peristaltic pump equipped with two flip-top heads series 300 supplied by Watson Marlow (see Figure 1 B).

25 The purification process comprises the following steps:

1. Hydrating the filter with 1 liter of low salinity (< 0.2 M) and conductivity (< 20 mS/cm) phosphate buffer, hereinafter referred to as PBS.
2. Flushing 1.5 liters of biomass into the Supracap™
- 30 100 depth filter directly from the fermenter at a constant flow-rate of 180 ml/min.
3. Flushing the filter with 1.5 liters of PBS buffer

to recover the dead volume of the filter and maximize the recovery of OMVs.

4. The filtrate from steps 1-2 was collected continuously.

5 5. 0.22 μm filtration, effected with Sartorius® Sartolab RF 1000 PES, of the filtered material to ensure the absence of live bacterial cells in the final material.

6. Sterilizing and disposing of the Supracap™ 100
10 filter and whole tubing system.

The system does not require any type of washing, the peristaltic pump does not come into contact with the processed material.

The filtrate collected will be subjected to a
15 further purification step to obtain the necessary degree of purity of the OMVs.

Process B (known technique): microfiltration with tangential flow filtration (TFF)

In order to remove the biomass and extract all the
20 OMVs from the culture broth, the tangential flow filtration (TFF) process of the prior art was also used in parallel.

The TFF system used was a Sartorius Sartoflow® Advanced equipped with a Sartocan® Slice Hydrosart®
25 0.20 μm cassette (see Figure 1 A).

The purification process, in this case, comprises the following steps:

1. Assembling the Sartocan® Slice Hydrosart® 0.20 μm on the TFF system.

30 2. Washing the Sartocan® Slice Hydrosart® 0.20 μm with water until the 0.1 M NaOH (storage solution) is completely removed.

3. Carrying out efficiency tests of the Sartocan® Slice Hydrosart® 0.20 µm, with water, as described by the cassette vendor's protocol.
4. Hydrating the cassette with purification buffer
5 (PBS).
5. Transferring 1.5 liters of biomass from the fermenter to the TFF system tank.
6. Concentrating up to 500 ml and collecting the filtrate in a sterile bag.
- 10 7. Adding 1 liter of PBS.
8. Repeating steps 6-7 3 times, washing the biomass with 3 liters of PBS.
9. Collecting and disposing of the biomass.
10. Washing the Sartocan® Slice Hydrosart® 0.20 µm
15 cassette and TFF system with NaOH.
- The protocol described by the vendor includes 1 hour of washing with 1M NaOH and 1 hour of washing with 0.1M NaOH.
11. Washing the cassette and the TFF system with plenty
20 of water (> 10 liters) and repeating the efficiency test on the cassette as illustrated in point 3.
12. Hydrating the Sartocan® Slice Hydrosart® 0.20 µm with 0.1 M NaOH and storing at 4°C.

The filtrate collected will be subjected to a
25 further purification step by ultrafiltration with TFF to obtain the necessary degree of purity of the OMVs.
Ultrafiltration by TFF for purification of the OMVs

Both of the starting materials (SM) obtained with Process A and B were then subjected to a second
30 purification step from small molecules (amino acids, proteins, small peptides, lipids, nucleic acid fragments, etc. present in the initial fermentation

broth) through an ultrafiltration step with TFF.

The ultrafiltration conditions were the same for both SMs and led to the production of a final product deriving from depth filter microfiltration (hereinafter, FB DF) and a final product deriving from TFF microfiltration (hereinafter, FB TFF).

Each SM was ultrafiltered by tangential flow filtration (TFF) on a Sartoflow® Smart system (Sartorius) equipped with two Sartocore® Slice 200 Hydrosart® cassettes at 300 kDa MWCO.

The type and volumes of the filtration pads, together with the pressures and the recirculation flow, were optimized to efficiently remove all types of impurities and kept unchanged for SMs from depth filtration (SM DF) and SMs from TFF 0.22 µm (SM TFF).

The ultrafiltration process used comprises the following steps:

1. Diafiltration with 3 volumes of PBS buffer.
2. Diafiltration with 8 volumes of high salinity (> 0.5 M) and conductivity (> 50 mS/cm) Tris-HCl buffer.
3. Diafiltration with 2 volumes of PBS buffer.
4. Final concentration 10:1 v/v of the retentate.
5. Collection of the OMVs.

The purified OMVs are finally filtered at 0.22 µm and stored sterile at 2-8°C.

RESULTS

By carrying out the growth and purification process as described above and applying the two different types of microfiltration, considerable differences are revealed in the yield and speed of the entire process, as well as in the finished product, i.e. in the characteristics of the OMVs.

The following Table 1 shows the volumes of buffer, washing solutions (NaOH) and water used for a single experiment and the duration of the process for the two types of microfiltration used starting, in both cases, from a biomass volume equal to 1.5 liters.

Table 1

	Buffer (liters)	NaOH (liters)	Water (liters)	Process duration (min)
TFF	4.5	3	30	350/400
DF	2.5	0	0	60/90

On analyzing these values, the superiority in terms of time, savings and lower quantity of liquids used in depth filtration compared to TFF is overwhelming.

Using DF, in fact, does not require all the preparation, washing and efficiency testing steps of the cassettes that are necessary however in carrying out TFF. This discrepancy, in terms of volumes of process liquids, increases as the fermentation volume increases, making DF much more suitable for scaling up the process on an industrial scale and therefore making the production process more sustainable and with a lower environmental impact in terms of volume of purified water used and volumes of hazardous liquids to be disposed of.

Furthermore, Figure 1, in which the two microfiltration processes are schematized, clearly shows that for DF there is only the need, in terms of equipment or systems, for a peristaltic pump and a system of disposable tubes that allow the biomass and

then the washing buffer to flow into the filter, which is also disposable.

In the case of TFF microfiltration, it is necessary to use a specific device or system suitable for tangential filtration (consisting of a recirculation tank, valves, pressure gauges and a suitable pump) and an additional peristaltic pump for transferring the biomass into the recirculation tank of the TFF system. Depth filtration is therefore a much simpler and also cheaper purification system, reducing the costs of the instrumentation necessary for the process (the cost of a TFF system is significantly higher than a simple peristaltic pump) and exposing the final product to lower risks of contamination during the production steps.

EXAMPLE 3: Characterization of OMVs obtained by a purification process with TFF and with DF

The materials produced and purified as previously described from the strain of *Neisseria cinerea* ATCC 14685 were characterized with various analytical techniques.

MATERIALS AND METHODS

Lowry assay for protein quantification with biciquinonic acid

The Detergent Compatible (DC) Protein Assay II from Bio-Rad was used ([22],[23]). All samples were analyzed according to the supplier's protocol, at a final concentration of 10% w/v SDS, this was used to ensure accessibility of all the membrane proteins present in the vesicles.

The standard curve was prepared using BSA, making each point in duplicate. The curve shows a good

linearity ($R^2 > 0.99$, at 650 nm) within the range of 0.2 - 1.5 mg/ml. The samples were analyzed in triplicate at each dilution factor; the final result is extrapolated from at least 5 mean values. The accuracy of the analytical method was tested by mixing the sample and a point of the curve with a known concentration (these points are called Spike). The recovery of the Spike sample always remains within the range of values 80-120% of the expected value.

10 Picogreen Method

The nucleic acids present in the samples were detected and estimated using a Picogreen assay, purchased from Thermo Fisher (Quant-iT™ PicoGreen™ dsDNA Assay Kit. Life technologies Corporation, Eugene, OR, USA), according to the manufacturer's instructions [24].

The DNA concentration was estimated with respect to a reference curve obtained with the standard calf thymus DNA supplied with the kit. The accuracy of the analytical method was tested by mixing the sample and a point of the curve with a known concentration (these points are called Spike). The recovery of the Spike sample always remains within the range of values 80-120% of the expected value.

25 SEC-HPLC coupled with MALS-QELS in series

The OMV samples were analyzed by SEC-HPLC with Tosoh TSK gel G6000PW (30 cm × 7.5 mm) + G4000PW (30 cm × 7.5 mm) columns in series equilibrated in DPBS (Sigma Aldrich D5652, Dulbecco's PBS), on a Waters e2695 Alliance system equipped with on-line UV absorbance (PDA 2998), fluorescence (FLR 2475) and Wyatt MALS-QELS-RI detectors. An 18-angle Dawn Heleos equipped

with a 660 nm laser source and an integrated 90° fiber optic for QELS acquisitions was used coupled with a DynaPro NanoStar system for on-line dynamic light scattering measurements. An OptiLab Trex instrument was
5 used as a concentration detector (default protein dn/dc = 0.185 ml/g). A volume of 80 μ l of sample with a protein content of 0.5 mg/ml (Lowry) was injected and eluted with a flow-rate of 0.5 ml/min (analysis time 70 min). All the sample dilutions were effected in DPBS
10 and all samples were then filtered at 0.22 μ m before analysis. The performances of the system were controlled for each sample set: the column efficiency was tested by uracil injections at 260 nm (retention time 55.7 min); the accuracy of the determination of
15 the absolute molecular weight and size by MALS was tested with BSA protein. Each sample injection was followed by a blank injection (80 μ L of mobile phase) to verify any impurity elution after the 70 minute chromatographic run. The PDA and FLR data were
20 processed by Empower 3 software. All Wyatt data were collected and processed using ASTRA 7: Zimm or Debye "spherical" models were applied (Zimm model unless otherwise stated). The size of the OMVs was expressed by the mean geometric radius Z , R_z (also known as RMS
25 or gyration radius, R_g), extrapolated from static light scattering (MALS) measurements or by the hydrodynamic radius $R_h(Q)z$ values extrapolated from dynamic light scattering (QELS) measurements.

RESULTS

30 The OMVs were quantified based on the total protein content estimated by the Lowry colorimetric assay.

The residual nucleic acids were quantified by the

PicoGreen fluorimetric assay that quantifies double-stranded DNA.

The following Table 2 summarizes all the concentration values of proteins and nucleic acids present in the microfiltered samples (SM) and in the final products (FB).

Table 2

Type of microfiltration	DNA		Proteins		DNA/OMV ratio
	SM	FB	SM	FB	
TFF (0.2 μ m)	10.42 mg	773.56 μ g	1923.82 mg	199.2 mg	3.88 μ g/mg
DF (3-6 μ m)	8.01 mg	146.85 μ g	2104.89 mg	198.03 mg	1.35 μ g/mg

The data in Table 2 clearly show an advantage in using DF for the removal of bacterial biomass. Already at the SM level it can in fact be noted that the DNA concentration is significantly lower in the starting material obtained after depth filtration (SM DF) with respect to the starting material obtained with TFF (SM TFF). This data is even more evident on comparing the two final products, using the depth filter, in fact, the quantity of nucleic acids is reduced by 5 times. Furthermore, on analyzing the protein quantification of the FB (it directly indicates the yield in terms of OMV concentration) a practically equal yield between the two purification processes can be observed.

The total quantities of DNA and proteins were finally used for calculating the residual DNA/OMV ratio, as required by current regulations: the sample purified by depth filtration (FB DF) shows a very

favourable DNA/OMV ratio (3 times lower) compared to the FB TFF sample. The quantitative analyses effected clearly show that DF drastically reduces the presence of contaminants while maintaining the OMV yield unchanged.

The absence of free soluble proteins that could invalidate the colorimetric assays was confirmed by SEC-HPLC analysis effected on both of the FB final products (Figure 2). The chromatograms (absorbance 280 nm), in fact, show no impurities demonstrating that the ultrafiltration process worked efficiently for both of the starting materials SM. On analyzing the chromatograms with absorbance 260 nm (characteristic absorbance of nucleic acids), on the other hand, it can be observed that in the FB TFF sample (Figure 2A) there are contaminants in the initial part (before 25 minutes) and in the final part (after 35 minutes) that are not present in the FB DF chromatogram (Figure 2B), demonstrating that the sample microfiltered with TFF with the same ultrafiltration process leads to the presence of more nucleic acids in the final purified product with respect to the sample purified with DF. This data confirms the data obtained from Picogreen presented in Table 2.

In Figure 3 the chromatograms (absorbance at 280 nm) of the FB DF and FB TFF samples are superimposed in order to compare the differences, in terms of purity and homogeneity, in the final product. The FB DF presents a chromatogram with a characteristic OMV peak (elution between 25 and 35 minutes) that is symmetrical and clean, typical of a mixture of OMVs that is extremely homogeneous in size and composition. The

chromatogram of the FB TFF on the other hand has a main peak (elution between 25 and 35 minutes) that is asymmetrical and a second secondary peak (elution between 22 and 23 minutes) denoting the presence of two
 5 distinct populations of OMVs and therefore a mixture of vesicles that is much more heterogeneous in size due to the presence of a significant amount of contaminating DNA which, as it is notoriously a sticky molecule, binds to a part of the vesicles, making them appear to
 10 be of a different size from the predominant ones in the main peak. The data extrapolated from the SEC HPLC measurements confirm the validity of the data obtained by Lowry and Picogreen.

The peak assignment of the OMVs eluting in the 25-
 15 35 minute range was confirmed by light scattering: the MALS chromatograms (90 degree LS detector) in Figure 4 show that the OMVs are within the radius range of 10-50 nm. The particle-size distribution of the OMVs and the polydispersity were determined by simultaneous static
 20 (MALS) and dynamic (QELS) light scattering measurements on eluted column fractions: mean values z extrapolated for the gyration radius, R_g (also known as root mean square or rms radius) and hydrodynamic radius, R_h (also known as Stokes radius) are indicated in the following
 25 Table 3.

Table 3

	OMV (FB TFF)	OMV (FB DF)
Hydrodynamic radius	33.292 ($\pm 1.643\%$)	21.493 ($\pm 1.647\%$)
moment	nm	nm
Polydispersity	1.298 ($\pm 0.289\%$)	1.063 ($\pm 0.535\%$)

From the MALS measurements it can be deduced that the OMVs extracted with the two methods have a different size, the vesicles purified with depth filtration have a smaller radius than the OMVs obtained
5 by TFF, but have a lower polydispersity value. This data allows it to be affirmed that with DF a very homogeneous population of OMVs is selected, confirming the data extrapolated from the SEC HPLC measurements.

The analytical characterization data clearly show
10 that using depth filtration for the microfiltration step of bacterial biomass, for the purification of OMVs, has significant advantages. The final nucleic acid concentration is significantly lower (i.e. <1.5 µg/mg), which allows compliance with the limits of the
15 current international regulation on the maximum value of DNA per vaccine dose [25]. In order to reach the legally required nucleic acid values per dose, in the production of vaccines based on OMV platforms on an industrial scale, it is often necessary to introduce an
20 additional nucleic acid digestion step with endonucleases such as benzonase ([5],[26],[27],[28]), extremely expensive enzymes that must also be completely removed from the final product. Depth filtration therefore allows the use of benzonase to be
25 avoided, thus simplifying the entire production process, avoiding the use of additional enzymatic reagents, making it less expensive, more reproducible and producing a safer final product.

DF improves the quality of the final product, in
30 terms of concentration of contaminants, maintaining a very high yield in terms of quantity of OMVs recovered at the end of the production process.

Furthermore, the purification process developed allows an extremely homogeneous population of OMVs to be selected, which makes the product more suitable as a potential antigen carrier for the development of
5 vaccines.

In addition to the advantages listed above, the use of the depth filter significantly reduces production costs and contamination risks, as it is a disposable device that does not require any type of specific
10 equipment/system and the relative preparation and subsequent washing steps (see Figure 1 and Table 1). At the end of the process, the entire system (tubes and filter) used must only be disposed of. Microfiltration with TFF, on the contrary, requires a lengthy and
15 complex washing process with numerous steps with basic solutions (NaOH) at various concentrations, buffer solutions and purified water which significantly increases costs, production time if scaled up to industrial production and the risks of contamination
20 for the product.

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CLAIMS

1. A purification process of vesicles released from the outer membrane (OMVs) of Gram-negative bacteria comprising the following steps:
- 5 a) microfiltration of the bacterial biomass obtained from the fermentation of Gram-negative bacteria producing OMVs by means of a depth filter (DF);
- b) ultrafiltration of the filtrate obtained from step
- 10 a) by means of i) tangential flow filtration (TFF), depth filtration (DF), ultracentrifuge or chromatographic filter.
2. The purification process according to claim 1, wherein said OMV-producing Gram-negative bacteria are
- 15 wild-type or genetically modified strains belonging to the genus selected from the group consisting of *Neisseria*, *Escherichia*, *Klebsiella*, *Shigella*, *Salmonella*, *Yersinia*, *Haemophilus*, *Pseudomonas*, *Moraxella*, *Bordetella*, *Borrelia*, *Brucella*, *Chlamydia*,
- 20 *Legionella*, *Vibrio* and *Helicobacter*.
3. The purification process according to claim 2, wherein said strains belong to the species *Neisseria cinerea*.
4. The purification process according to any of claims
- 25 1-3, wherein said strain of *Neisseria cinerea* is *N. cinerea* ATCC14685.
5. The purification process according to any of claims 1-4, wherein said microfiltration a) comprises the following steps:

- i) hydration of the depth filter with a low salinity (< 0.2 M) and conductivity (< 20 mS/cm) buffer;
- ii) flushing the bacterial biomass directly from the fermenter to the depth filter and recovering the
5 filtrate;
- iii) flushing the depth filter with a wash buffer to recover the dead volume of the filter and recover the filtrate.
6. The purification process according to any of claims
10 1-5, wherein said depth filter is connected to a peristaltic pump.
7. The purification process according to any of claims 1-6, wherein said depth filter used for the microfiltration of step a) is a cellulose or
15 polypropylene filter with a retention index within the range of 3-6 μm .
8. The purification process according to claim 7, wherein said depth filter is a Supracap™ 100 recycled cellulose depth filter.
- 20 9. The process according to any of claims 1-8, wherein said buffer of steps i) and iii) is a phosphate buffer, preferably PBS.
10. The process according to any of claims 1-9, wherein said step b) for TFF ultrafiltration of the filtrate
25 obtained from step a) is effected through a 300 kDa filter.
11. The process according to claim 10, wherein said TFF ultrafiltration step b) comprises the following steps::

i) diafiltration with 3 volumes of PBS buffer;
ii) diafiltration with 8 volumes of high salinity (> 0.5 M) and conductivity (> 50 mS/cm) Tris-HCl buffer;

5 iii) diafiltration with 2 volumes of PBS buffer;
iv) final concentration 10:1 v/v of the retentate;
v) collection of the OMVs and sterilization on a 0.22 μ m filter.

12. OMVs from *Neisseria cinerea* obtained by means of
10 the purification process according to any of claims 3-11, characterized by having a DNA/OMV ratio < 1.5 μ g/mg, a polydispersity index of approximately 1 and a hydrodynamic radius ranging from 20 nm to 50 nm.

13. OMVs from *Neisseria cinerea* according to claim 12,
15 for use in the medical field as an antigen carrier or adjuvant in vaccine formulations.

14. A pharmaceutical composition comprising OMVs purified from *Neisseria cinerea* according to any of claims 12-13, as an antigen carrier or adjuvant for use
20 as a vaccine.

15. An antigen or drug carrier system comprising OMVs from *Neisseria cinerea* according to any of claims 12-13.

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