Preparation of Bacterial Outer Membrane Vesicles for Characterisation of Periplasmic Proteins in Their Native Environment

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[Abstract] Bacterial outer membrane vesicles (OMVs) are naturally formed by budding from the outer membrane of Gram-negative bacteria. OMVs consist of a lipid bilayer identical in composition to the original outer membrane and contain periplasmic content within their lumen. Enriched with specific envelope proteins, OMVs make for an excellent native-like platform to study these proteins *in-situ* using biophysical methods. Here, we describe in detail the preparation of OMVs from *Escherichia coli*, which are luminally enriched with periplasmic proteins and uniformly labeled with stable isotopes (^{2}H and ^{15}N), suitable for the subsequent characterisation of proteins at atomic resolution in their native environment by solution-state NMR spectroscopy. The ability to perform structural studies of periplasmic components *in-situ* clears the way to reaching an in-depth understanding of the functional and mechanistic details of this unique cellular compartment.

Keywords: Outer membrane vesicles, Gram-negative bacteria, Periplasm, NMR spectroscopy, *In-situ* protein studies

[Background] The periplasm of Gram-negative bacteria is a quite remarkable cellular compartment. This space, incarcerated between the inner and outer bacterial membrane, contains proteins at an extraordinarily high concentration exceeding 300 mg ml^{-1} (Oliver, 1996) and, in the absence of cellular sources such as ATP, functions almost energetically independent from its cytosolic counterpart. So far, structural knowledge about periplasmic proteins has been exclusively obtained using purified proteins isolated from their native environment. Thus, any structural and functional influence this special environment might impose on a protein are lost during purification. Efforts to study periplasmic proteins *in-situ* using biophysical methods such as *in-cell* NMR spectroscopy have been hampered by the low volume ratio of the periplasm, which contributes only 5-10% to the total bacterial volume (Brass *et al.*, 1986).

We recently described a new method to characterize proteins within bacterial outer membrane vesicles (OMVs) using solution-state NMR spectroscopy (Thoma and Burmann, 2020). OMVs, which are naturally released by Gram-negative bacteria, contain periplasmic content in their lumen and can be non-destructively purified from bacterial growth cultures (Chutkan *et al.*, 2013; Schwechheimer and Kuehn, 2015). Thus, OMVs represent an excellent platform to study periplasmic proteins, enriched in the luminal space, together with their natural environment. To be amendable for NMR studies, OMVs

are uniformly labeled with stable isotopes (^{2}H and ^{15}N) and prepared in large quantities. Our approach proved particularly useful for the study of soluble periplasmic proteins within this environment, as the membrane components of OMVs remain largely invisible to solution-state NMR, due to slower molecular tumbling rates.

One key factor for obtaining large amounts of luminally enriched OMVs is the use of a suitable expression strain. Widely used expression hosts such as *E. coli* BL21(DE3) do not produce sufficient amounts of OMVs to be feasible for these types of experiments (Thoma *et al.*, 2018). In contrast, strains lacking the major outer membrane protein OmpA have been shown to have a 26-fold increased OMV production (Schwechheimer *et al.*, 2014). Moreover, deletion of OmpA omits possible background signals originating from its soluble peptidoglycan binding domain in NMR experiments (Thoma and Burmann, 2020). In our experimental set-up we relied on the strain BL21(DE3)ΔompA, which was kindly provided by Guido Grandi (Fantappiè *et al.*, 2014) and which reproducibly yielded high amounts of luminally enriched OMVs. However, other alternatives such as BL21ΔA are available (Meuskens *et al.*, 2017) or could easily be tailor-made by deletion of the *ompA* gene in the expression strain of choice.

Another critical factor for obtaining large amounts of suitable OMVs is the time point, at which the bacteria are removed from the growth media. Bacterial growth should be maintained as long as possible, since the amount of OMVs present in the culture supernatant correlates directly with the cell density and consequentially with the length of the growth period. However, OMVs should be collected before bacterial growth ceases and cultures enter the stationary phase, because this will substantially increase contaminations with cytoplasmic proteins, inner membranes, and cell debris resulting from stress-induced explosive cell lysis (Turnbull *et al.*, 2016; Thoma *et al.*, 2018). Depending on whether cells are grown in H$_2$O or D$_2$O based media this stage is typically achieved after 3 to 5 h, respectively. Since bacterial growth is not only retarded by the use of D$_2$O but also underlies various influences such as the strain and plasmid used for expression, it is advisable to determine the growth behavior of the bacterial cultures prior to OMV preparation. Whereas deuteration is not strictly necessary for small to medium sized proteins (< 25 kDa)，luminally enriched OMVs prepared from cells grown in D$_2$O based media typically yield NMR spectra of higher quality due to a decrease in background signals and a smaller NMR line-width (Thoma and Burmann, 2020).

Materials and Reagents

1. 0.22 µm vacuum filtration unit (such as Steritop Quick Release, EMD Millipore, catalog number: S2GPT05RE)
2. 0.45 µm vacuum filtration unit (such as Stericup-HV, EMD Millipore, catalog number: SCHVU11RE)
3. ≥ 50 ml syringe (such as Fisherbrand Sterile Syringes, Thermo Fisher Scientific, catalog number: 15899152)
4. 0.45 µm syringe filter (such as Millex-HP, EMD Millipore, catalog number: SLHP033R)
5. Microscale NMR tubes (such as 5mm Shigemi Tube Set, Shigemi, catalog number: BMS-005B)

6. Tryptone (Sigma-Aldrich, catalog number: T9410)

7. Yeast Extract (Sigma-Aldrich, catalog number: Y1625)

8. NaCl (Sigma-Aldrich, catalog number: S7653)

9. Na_2HPO_4 (Sigma-Aldrich, catalog number: S7907)

10. KH_2PO_4 (Sigma-Aldrich, catalog number: P0662)

11. KCl (Sigma-Aldrich, catalog number: P9333)

12. $CaCl_2 \cdot 2H_2O$ (Sigma-Aldrich, catalog number: C5080)

13. $MgCl_2 \cdot 6H_2O$ (Sigma-Aldrich, catalog number: M2670)

14. $^{15}NH_4Cl$ (Sigma-Aldrich, catalog number: 299251)

15. D_2O (Sigma-Aldrich, catalog number: 617385)

16. D-Glucose (Sigma-Aldrich, catalog number: G7528)

17. $MgSO_4$ (Sigma-Aldrich, catalog number: M7506)

18. Thiamine (Sigma-Aldrich, catalog number: T1270)

19. Isopropyl-β-D-thiogalactoside (IPTG; Sigma-Aldrich, catalog number: I6758)

20. LB-medium (Lennox) (see Recipes)

21. M9 medium (see Recipes)

22. Dulbeccos phosphate buffered saline with added salts (DPBSS)/NMR sample buffer (see Recipes)

Equipment

1. Shaking incubator (such as New Brunswick Innova® 42, Eppendorf AG, catalog number: M1335-0012)

2. 500 ml baffled culture flask (such as ROTILABO baffled flasks, Carl Roth, catalog number: LY96.1)

3. 5,000 ml baffled culture flask (such as ROTILABO baffled flasks, Carl Roth, catalog number: PK29.1)

4. Cell density meter (such as GE Healthcare Ultrospec, Thermo Fisher Scientific, catalog number: 10704417)

5. High speed centrifuge (such as Avanti J-series, Beckman Coulter, catalog number: B22989)

6. High speed large-volume fixed angle rotor (such as J-LITE JLA-16.250, Beckman Coulter, catalog number: 363934) equipped with suitable centrifugation bottles (such as 250 ml Polycarbonate Bottle, Beckman Coulter, catalog number: 356013)

7. High speed small-volume fixed angle rotor (such as JA-25.50, Beckman Coulter, catalog number: 363055) equipped with suitable centrifugation tubes (such as 50 ml Polycarbonate Bottle, Beckman Coulter, catalog number: 357000)

8. Vacuum pump, sufficiently strong for use with vacuum filtration units (ultimate vacuum ~0.01 mPa)

9. Vortex mixer (such as Vortex-Genie 2, Scientific Industries, catalog number: SI-0246)

10. Microvolume spectrophotometer (such as Nanodrop One, Thermo Fisher Scientific, ND-ONE-W)

11. Gel electrophoresis system for SDS page (such as Mini-PROTEAN Tetra, Bio-Rad, catalog number: 1658004) including power supply, polyacrylamide gels, and necessary buffers and staining solutions (refer to manufacturers specifications for details)

Software

1. Any spreadsheed or scientific graphing software allowing basic calculations (Excel, Numbers, LabPlot, Origin, *etc*.)

Procedure

A. Gene cloning and transformation

The complete gene coding for the protein of interest including its preceding periplasmic export signal should be cloned into a suitable plasmid backbone of the pET expression system (such as pET21a). Thereby, additional features such as affinity tags are not necessary and should be omitted. The final plasmid should be transformed into an OMV-overproducing strain such as BL21(DE3)ΔompA (Fantappiè *et al.*, 2014). These steps can be carried out following standard molecular biology techniques. The description of detailed cloning and transformation procedures lies outside the scope of this protocol.

B. Preparation of growth media and buffers

Per sample requirements are: 5 ml LB medium, 1 L M9 medium, 25 ml DPBSS, 0.2 ml NMR sample buffer (see Recipes for formulations). Supplement growth media with appropriate antibiotics for selection of the strain/plasmid used for expression. When using the pET expression system, use Ampicillin to a final concentration of 100 μg ml^{-1} or Kanamycin to a final concentration of 50 μg ml^{-1}. Stock solutions of antibiotics (Ampicillin: 100 mg ml^{-1}; Kanamycin: 50 mg ml^{-1}) and IPTG (1 M) can be prepared in H_2O/D_2O, corresponding to the solvent used for growth media.

C. Bacterial growth

1. Inoculate 5 ml LB medium with a single colony.

2. Grow shaking at 37 °C for 10 h.

3. Inoculate 100 ml M9 in a 500 ml baffled culture flask using 1 ml of the culture grown in LB medium.

4. Grow shaking at 37 °C overnight (~80 rpm, 25 mm orbit).

5. Add the entire preculture to 900 ml M9 in a 5,000 ml baffled culture flask.

6. Grow shaking at 37 °C (~80 rpm, 25 mm orbit).

7. Check the optical density OD_{600} every 30 min.

8. When the optical density reaches OD_{600} ~0.4 add IPTG to a final concentration of 0.2 mM to induce protein expression.

9. Continue to monitor the optical density OD_{600} every 30 min.

10. When bacterial growth ceases (after ~3 h in H_2O/~5 h in D_2O based media, see Figure 1) remove the cells by centrifugation at 10,000 $x\ g$ for 10 min at 10 °C.

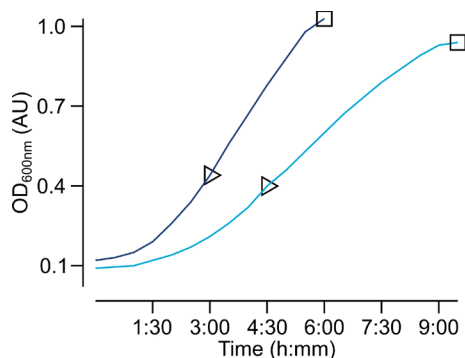11. Filter the supernatant with 0.45 µm vacuum filtration units. The filtered supernatant can be stored at 4 °C for up to 24 h.

Figure 1. Media-dependent growth behavior. Exemplary growth curves in H_2O based (dark blue) and D_2O based (light blue) M9 minimal media. Triangles mark the time of induction, squares mark the time of harvest.

D. OMV Preparation

1. Collect OMVs from the filtered supernatant by centrifugation at 38,400 $x\ g$ for 2 h at 10 °C. After centrifugation, centrifuge bottles should contain a small but clearly visible whitish, slightly cloudy pellet (Figure 2A).

2. Immediately discard the supernatant (OMV pellets will begin to suspend shortly).

3. Resuspend OMV pellets in a total volume of 25 ml DPBSS by pipetting.

4. Filter the OMV suspension with a 0.45 µm syringe filter.

5. Collect OMVs by centrifugation using a single centrifuge tube at 74,500 $x\ g$ for 45 min at 10 °C (Figure 2B).

6. Immediately discard the supernatant.

7. Resuspend the OMV pellet in 200 µl NMR sample buffer by gentle vortexing.

8. Save 2 µl of the OMV suspension for subsequent analysis and transfer the remaining suspension to a microscale NMR tube (Figure 2C). OMV suspensions are in our hands stable at 4 °C for up to 2 weeks.
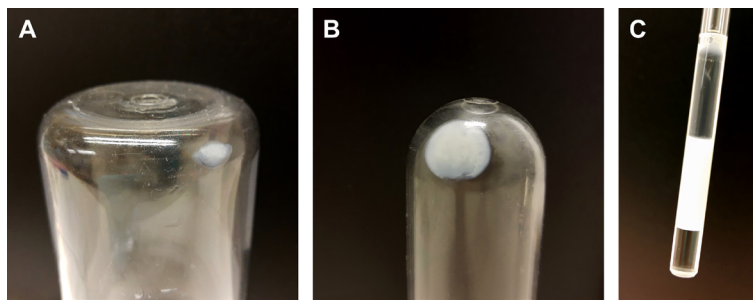
Figure 2. Preparation of OMVs. OMV pellets after the first (A) and second centrifugation (B), and resuspended in NMR buffer in a 5 mm Shigemi NMR tube (C).

E. OMV Analysis

1. Dilute 2 μl of the final OMV suspension with 18 μl DPBSS.

2. Record a UV-Vis spectrum of the dilute OMV suspension using a microvolume spectrophotometer (range: 220-360 nm) and export the spectrum data for further analysis.

3. Correct the exported spectrum for the light-scattering contribution of the OMVs and estimate the protein concentration from the absorbance at 280 nm (see Data analysis and Figure 3A). OMV preparations should contain >15 mg/ml total protein to yield NMR spectra of good quality.

4. Prepare two samples for SDS page using denaturing Laemmli Sample Buffer, containing ~6 μg total protein each.

5. Heat-denature one of the samples at 98 °C for 15 min, keep the second sample at room temperature.

6. Analyze samples by SDS page. Samples of protein-enriched OMVs should result in two prominent bands. One band corresponding to the major porins OmpF/OmpC present in the OMV membrane runs at 37 kDa in the heat-denatured sample and results in a contorted, ladder-like pattern between 60 and 70 kDa in the non-heated sample (Figure 3B). A second band, usually not subject to heat-modifiability, should be present at the weight of the overexpressed protein of choice, indicating successful enrichment in the OMVs (Figure 3B).
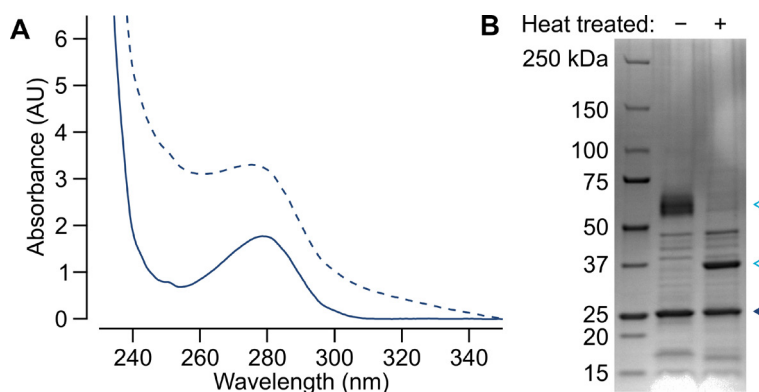

Figure 3. Analysis of OMVs by spectrophotometry and SDS-page. A. Exemplary UV-Vis spectrum of a dilute OMV suspension before (dashed line) and after light-scattering correction

(solid line). B. Exemplary SDS page gel of OMVs enriched with a periplasmic protein (dark blue triangle). Major porins OmpF/OmpC are marked by the light blue open triangles.

Data analysis

The spectrophotometric estimation of the protein concentration in a sample based on absorbance measurement at a wavelength of 280 nm is a simple, fast, and routinely used method. However, UV-Vis spectra recorded of OMVs contain a strong light-scattering component. In order to estimate the protein concentration within an OMV sample, light-scattering due to the vesicles needs to be corrected. A light-scattering corrected UV-Vis spectrum can be obtained using the following equation, originally used to estimate the nucleic acid content of viruses (Porterfield and Zlotnick, 2010):

$$A_{corrected,\lambda} = A_\lambda - \left(\frac{A_{340} - A_{360}}{(340nm)^{-4} - (360nm)^{-4}} \right) \lambda^{-4} - A_{340} + \left(\frac{A_{340} - A_{360}}{(340nm)^{-4} - (360nm)^{-4}} \right) 340nm^{-4}$$

The total protein concentration in the OMV sample can be estimated from the corrected value of A_{280}, assuming 1 Abs = 1 mg ml^{-1} for the mixture of proteins present in OMVs. Due to the presence of nucleic acids in OMVs (Brown *et al.*, 2015), which strongly influences the absorbance at 280 nm, it is useful to estimate the amount of nucleic acids in the sample from the A_{260}/A_{280} ratio of the light-scattering corrected spectrum, which can be done using the following empirical equation (Glasel, 1995):

$$[\%N] = \frac{11.16 \left(\frac{A_{260}}{A_{280}} \right) - 6.32}{2.16 - \left(\frac{A_{260}}{A_{280}} \right)}$$

Typically, protein-enriched OMVs contain ≤ 3% nucleic acids. If nucleic acids are detected ([%N] > 0), the total protein concentration in the OMV sample can be estimated from the corrected values of A_{280} and A_{260}, using the following equation (Layne, 1957):

$$[P] = 1.55 \, A_{280} - 0.76 \, A_{260}$$

Notes

Typically a sufficient amount of enriched OMVs can be obtained from a growth culture volume of one liter using the culture conditions described above. Nevertheless, it should be noted that bacterial growth in D$_2$O based media can be less reproducible than in H$_2$O based media, occasionally resulting in irregular growth behavior and/or lower yields. Moreover, it should be noted that the overexpression of proteins in the periplasmic space can result in cytotoxicity (Schlegel *et al.*, 2013), which might require specific adaption of the induction levels to the expression system used.

Recipes

1. LB-medium (Lennox)

   10 g/L Tryptone

   5 g/L Yeast extract

   5 g/L NaCl

   Dissolve in H_2O and sterilize by autoclaving

2. M9 medium

   6 g/L Na_2HPO_4

   3 g/L KH_2PO_4

   1 g/L $^{15}NH_4Cl$

   0.5 g/L NaCl

   2 g/L D-Glucose

   1 ml/L $CaCl_2$ (0.1 M) solution*

   1 ml/L $MgSO_4$ (1 M) solution*

   0.1 ml/L Thiamine (10 mg/ml) solution*

   Dissolve solid components in H_2O/D_2O, add salt/Thiamine solutions and sterilize by filtration using 0.22 μm vacuum filtration units.

   Note: Prepare stock solutions in H_2O/D_2O, respectively.

3. Dulbeccos phosphate buffered saline with added salts (DPBSS)/NMR sample buffer:

   1.15 g/L Na_2HPO_4

   0.2 g/L KH_2PO_4

   8 g/L NaCl

   0.2 g/L KCl

   0.45 ml/L $CaCl_2$ (1 M) solution

   0.25 ml/L $MgCl_2$ (1 M) solution

   Dissolve solid components in H_2O*, add salt solutions and sterilize by filtration using 0.22 μm vacuum filtration units.

   Note: For NMR sample buffer, dissolve in H_2O to 90% final volume, then add 10% D_2O.

Acknowledgments

This protocol was originally described in Thoma and Burmann (2020).

Competing interests

The authors declare no competing interest.

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