REPORT ON EXPRESSION AND PURIFICATION OF A DEUTERATED MODEL MEMBRANE PROTEIN: OMPX

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The detailed protocol "Lethier M., Moulin M., Härtlein M., Ebel C. Report on expression and purification of a deuterated model membrane protein: OmpX" is available on the web site of IBS: http://www.ibs.fr. Then search: SSIMPAS.

INTRODUCTION

Membrane proteins correspond to about 30% of the proteome of all organisms. They perform a wide range of essential cellular functions, are involved in a number of genetic diseases and have considerable therapeutic importance: 60 % of drug targets are membrane receptors or ion channels. Membrane proteins are also essential for the virulence of pathogens. The determination of their structures, required for understanding their role and function, designing drugs, etc is often difficult. The structural knowledge concerning membrane proteins remains very limited. The number of available structures increases, but only 250 unique membrane protein structures are known (http://blanco.biomol.uci.edu/Membrane_Proteins_xtal.html) to date (June 2010), a small number as compared to that (≈ 50000) for soluble proteins. Small angle neutron scattering (SANS) can be carried out in solution and can be used to provide *ab initio* low resolution envelops, confirming a 3D structure or addressing conformation changes (Petoukhov & Svergun, 2007). In the case of solubilized membrane proteins, detergent and/or lipid contributions may be masked by specific deuteration of solvent and/or of one of the protein/detergent complex components (Johs *et al.* 2006, Nogales *et al.* 2010, Cardoso *et al.* 2009). Deuteration of the protein part is advantageous for increasing the signal over noise ratio in the scattering curve, studying conformation of a partner within multi-protein complexes. In addition, we are investigating the potentialities of new classes of surfactants (fluorinated or neutral amphiphilic polymer surfactants) designed to improve the stability of solubilized membrane protein assemblies. They are matched in solvent conditions (at a D_2O percentage) close to that of a hydrogenated protein (Gohon *et al.* 2006, Gohon *et al.* 2008, Breyton *et al.*, 2009), which imposes membrane protein deuteration in SANS.

RESULTS

We describe here a protocol for obtaining deuterated at OmpX in large amounts. OmpX is known from X-Ray and NMR studies (Pautsch *et al.* 1999; Vogt and Shultz 1999; Fernandez *et al.*, 2004, Catoire *et al.* 2010). OmpX relatively easily obtained in hydrogenated and deuterated forms (see in the references above). The protocol presented here reports the modifications to known protocols,needed to carry out high cell density fermention at the ILL-EMBL Deuteration Facility (Grenoble, France). The aim is to obtain large amounts. Full deuteration is not required: the required level of deuteration is ≈ 75% to match-out OmpX in 100% D_2O. We have also introduced a His-Tag on the protein for facilitating surfactant exchange procedure.

Our results show:

1: Cell lysis and solubilisation of the inclusion body is tricky with the high density culture in deuterated minimum media.

2 : Introducing a His Tag confers new folding properties: refolding of the protein from inclusion bodies has to be optimised for new construct.

3: Refolding rate is the same for hydrogenated and deuterated forms.

4 : An aggregation propensity is noted for the construct bearing a His-Tag after elution from NiNTA affinity columns.

<u>Step 1</u>: Modification of the plasmid of OmpX for high density cell culture.

- The plasmid encoding OmpX was a gift from Laurent Catoire (IBPC), who obtained it from G.E. Schultz (Germany). The gene lacks the sequence coding for the signal peptide. The truncated gene was inserted into the ampicillin resistant vector pET3b, between the NdeI and BamHI restriction sites.
- Since the requirement for over-expression in D_2O in the Dlab fermentors is a kanamycin-resistant vector, we cloned OmpX into pETM11, between the NcoI and BamHI restriction sites. The vector provides also a poly-histidine-tag at the N-terminal position of OmpX. The His-tag can be eventually removed with the TEV protease. After *E. coli* BL21 (DE3) strain transformation, the expression and purification of OmpX in hydrogenated LB medium was tested, but unfortunately, the protein could not be refolded efficiently (data not shown), most probably because of the presence of the His-tag at the N-ter position.
- We thus cloned OmpX into pET24a+ plasmid, between the XhoI and NdeI restriction sites, this second construction providing a poly-histidine-tag at the C-terminal position of OmpX. A single mutation H100N, which was described to favour protein crystallisation (Pautsch *et al.,* 1999), was added by site-directed mutagenesis. In the following, OmpX and, for the deuterated form, D-OmpX, correspond to proteins from this construct.

<u>Step 2</u>: Purification of hydrogenated OmpX.

E. coli BL21 DE3 strain was transformed, and OmpX was over-expressed in inclusion bodies. After cell breakage, they were recovered and washed by centrifugation as described in (Pautch *et al.*, 1999). As minor modifications, 1: the buffer for re-suspension of the bacterial cells (20 mM Tris pH 8.5) contained DNase I (250 U) and a cocktail tablet of anti-protease (one tablet /50mL solution) from Roche whereas EDTA was removed. 2: a microfluidizer apparatus (Microfluidics M-110P) was used to break the cell instead of sonication. Inclusion bodies solubilisation and refolding steps were performed similarly to (Pautch *et al.*, 1999). However, the His-tagged OmpX refolding in 5% C8POE, was much lower when compared to the protein without His-tag. The yield was ~50% instead of 90-100%.

Screening the detergent for refolding:

Optimization of refolding yield was done following Zhang *et al*. (2008): 33 µL of unfolded OmpX (3.5 mg/mL, 20 mM Tris-HCl pH 8.5, 6 M urea) was diluted 30-fold with 1 mL of 1% (w/v) detergent solution at 25°C. The refolding buffer was Tris-HCl, 20 mM (pH of 7.5, 8.5 and 9.5), 1 mM EDTA *plus* 1% (w/v) of the candidate detergent. The solution was incubated at room temperature for 3 hours under gentle shaking and loaded on a 15 % SDS polyacrylamide gel. The folding efficiency can be analyzed because the migration on SDS PAGE differs for the native and non-folded form (Pautch *et al.*, 1999). As an example, Figure 1 shows clearly that Foscholine-12 and LDAO are good detergent to refold OmpX. Whereas C8POE, which is efficient for the non-His tagged protein, does not allow significant refolding. LDAO was selected for refolding OmpX in large amounts, since less expensive than Foscholine-12.

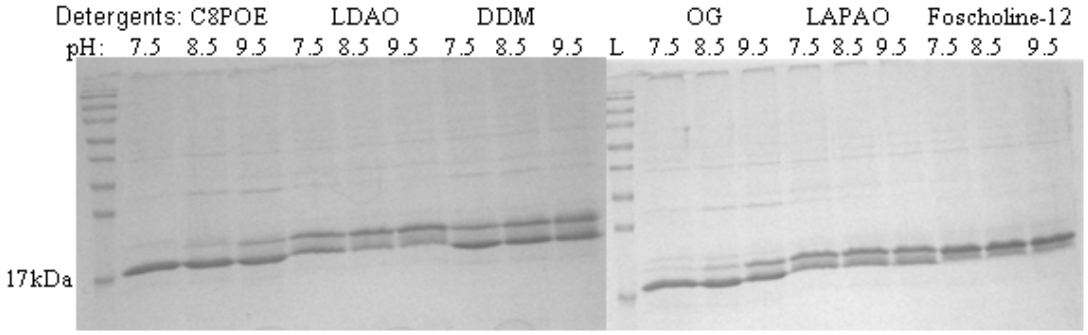


Figure 1: 15% SDS PAGE gel of hydrogenated OmpX refolding test (see eperimental conditionin the text): Ladder: 170, 130, 95, 72, 55, 43, 34, 26, 17, 11kDa; 20 µL of refolding solution + 5 µL loading buffer were loaded. The lower and upper bands correspond respectively to missfolded and folded OmpX forms.

A protocol using LDAO was established to refold OmpX at a yield of ~ 90%. After solubilisation of inclusion bodies with 20 mM Tris-HCl at pH 8.5 and 6 M urea, the solution is centrifuged (48000g 20 min) to remove urea impurities and non-solubilised proteins. Then, at 4°C, 37 mg of unfolded OmpX at a concentration of 3.5 mg/mL (10.5 mL) is added to 6X Vol. (63 mL) of refolding buffer (20 mM Tris-HCl at pH 8.5, 0.6 M arginine and 2 %(w/v) LDAO) at a very low flow rate of ~1 mL/h with vigorous stirring. The resulting OmpX solution remains vigorously stirred at 4°C for ~24 h and is then dialyzed three times, over a total of 24 hours, against three times 400 mL (≈ 5 X Vol.) of 20 mM Tris-HCl pH 8.5, 0.05% LDAO (2 x cmc (critical micelle concentration)). SDS PAGE is used to control the refolding yield. However, because of the large amount of detergent in the solution, protein migration is irregular at those stages.

The solution (≈ 80 mL) is filtered on a 22 µm pore size filter. The protein solution is then loaded on a 5 mL Ni-NTA affinity column (GE healthcare) equilibrated with 25 mL (5 column volumes) of 20 mM Tris-HCl pH 8.5, 0.1 % (w/v) LDAO, 250 mM NaCl. The column is then washed with 25 mL of the same buffer containing 20 mM imidazole, and OmpX is eluted with the same buffer containing 200 mM imidazole (25 mL). Fractions of 1 mL are collected and 50 µL of 100 mM EDTA is immediately added (final concentration 5 mM) to each of the fractions. SDS-PAGE allows determining in a couple of hours the fractions to be pooled (typically 10 mL). The pool is then concentrated to 3 mL by ultrafiltration (10 kDa amicon from Millipore) and the solvent is exchanged to 20 mM Tris NaCl pH 8.5, 100 mM NaCl, 0.1 % LDAO, using a desalting column (10DG-from Biorad). The protein is recovered in a volume of 4 mL. OmpX at this stage is pure and > 90% refolded according to SDS PAGE (Figure 2).

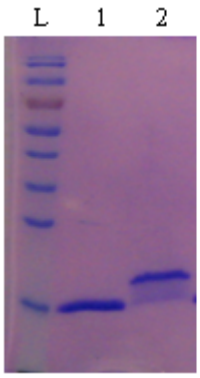


Figure 2: 15% SDS PAGE gel of hydrogenated OmpX purified
Ladder: 170, 130, 95, 72, 55, 43, 34, 26,17, 11kDa
OmpX (0.5mg/mL into 20mM Tris H-Cl pH 8.5, 5mM F6Tac, 100mM NaCl): 5µL + 5µL loading buffer:
1: purified OmpX refolded boiled for 5 min (denaturated)
2: purified ompX refolded not boiled.

Comment:

Adding EDTA is necessary to avoid the aggregation of the His-tagged OmpX. It is actually known that small amounts of Nickel ion can be eluted from the Ni-NTA column and interact with the histidine residues favouring aggregates of some His-tagged proteins (see e.g. Zoonens, 2004). We observed that the solvent after Ni-NTA column has to be quickly exchanged to avoid OmpX aggregation. Aggregation in the absence of EDTA was visually probed (the solution may become cloudy!). The signature of large (>20 nm) aggregates in these solutions could also be found by light scattering measured as a drift of the absorbance in the 300-400 nm range of an UV spectrum.

We tested that the pure and 90 % refolded His-tagged protein may be crystallised. We used the facilities of the High Throughput Crystallization Laboratory at EMBL Grenoble (https://embl.fr/htxlab/) (see Dimasi et al 2007). The pure protein was concentrated at two different concentrations of 10 and 20 mg/mL by ultrafiltration (10 kDa amicon from Millipore). Hampton and Biorad screening conditions were tested for the two concentrations. Crystals were obtained in one solvent condition: 0.1M citric acid pH 4, 20 % (v/v) 2-Methyl-2.4-pentanediol (MPD) (Figure 3).

Figure 3: Picture of OmpX crystals obtained with 0.1M citric acid pH 4, 20% v/v 2-Methyl-2.4-pentanediol

Similar solvent conditions, with 10% instead of 20% MDP were found to provide crystal for the non-His-tagged pure and 100% folded protein (Saab 2009). His tagged OmpX crystals obtained were rapidly soaked in a cryoprotective solution (crystallisation solvent with 60 % MPD) before analysing. They diffracted at 13 A° on the synchrotron ID23-2 line. This result shows that OmpX can be successfully crystallized as a c-ter His-tagged protein, despite the presence of 5-10% miss-folded protein. Improvement of crystal quality is under way.

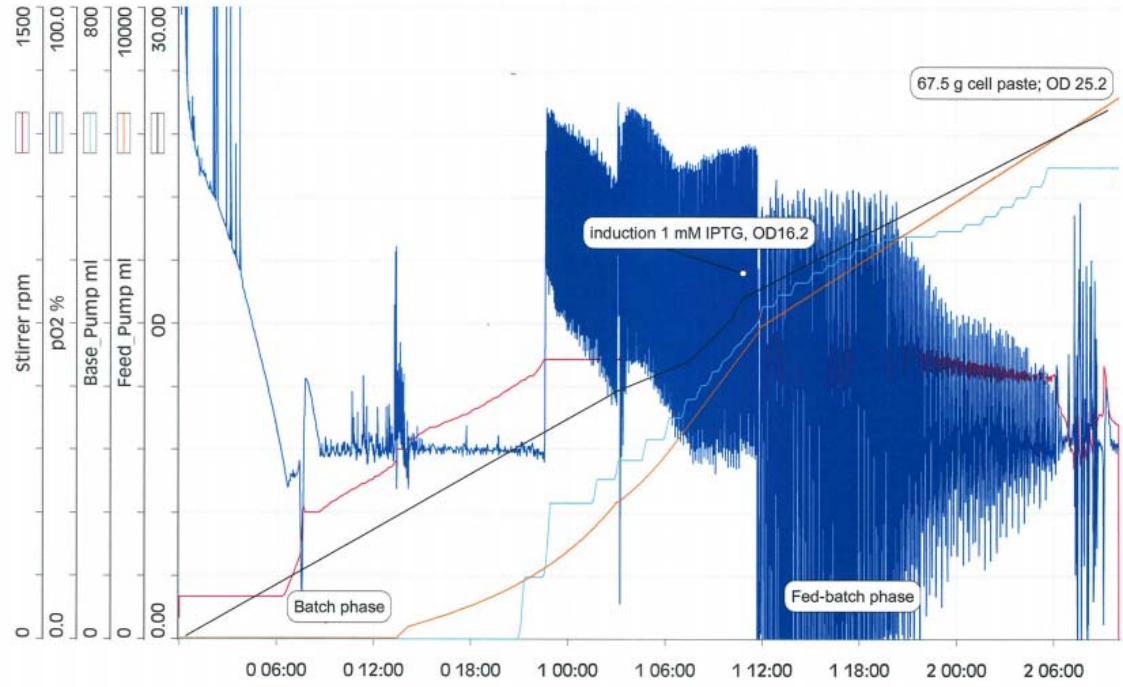
Considering these favourable results, the construction is adapted for the expression in D_2O as well as the protein purification protocol.

<u>Step 3</u>: Express and purify OmpX-His deuterated at 75% for neutron scattering experiment.

The high cell density culture and expression of deuterated OmpX-His was performed at the Deuteration Laboratory. The web site of the laboratory provides accessible protocols: http://www.ill.eu/sites/deuteration/index.htm.

A freshly transformed BL21-DE3-pET24-OmpX clone was used. In order to adapt the strain, the clone was first transferred to hydrogenated filtered "Enfors" minimal medium (6.86 g $(NH_4)_2$ SO_4 + 1.56 g KH_2 PO_4 + 6.48 g Na_2 HPO_4 .$2H_2O$ + 0.49 g $(NH_4)_2$-H-citrate + 5 g glycerol + 1mL (1M Mg SO_4 sterilized by filtration) + 1 mL "trace" metal salt solution (for 1 liter: 0.5 g $CaCl_2.2H_2O$; 16.7 g $FeCl_3.6H_2O$; 0.18 g $ZnSO_4.7H_2O$; 0.16 g $CuSO_4.5H_2O$; 0.15 g $MnSO_4.4H_2O$; 0.18 g $CoCl_2.6H_2O$; 20.1 g Na-EDTA) sterilised by filtration), as described in the section: Protocols for growth media (ILL/EMBL), minimal medium on the web site. Successive cultures were grown at 37°C (~5 successive inoculations in fresh medium). This step takes about one week. Then the pre-adapted strain was inoculated into 85 % deuterated "Enfors" minimal medium. (The component of the "Enfors" minimal medium were diluted into 850mL 100% D_2O. The trace solution was added and the medium was completed with ddH_2O). The strain was adapted as above as successive cultures at 37°C.

The expression was performed in a Labfors System fermenter during three days. Fermentation parameters (e.g. stirrer speed, temperature, pH, pO_2 and gas flow rate) are simultaneously controlled. Nutrient feeding (solution of glycerol at 12 %) is then added, with an increasing feed rate, in the *fed-batch* stage. Induction with 1 mM IPTG was made at $D0_{600nm}$ = 16.2 and the culture was finally stopped at $D0_{600nm}$ = 25 (<u>Figure 4</u>). Cells are harvested by centrifugation at 6000 rpm for 30 min, 4 °C. The recovered cells (67.5 gram) were frozen at -80°C.



<u>Figure 4</u>: Profile of D-OmpX expression:
The horizontal scale gives the duration in days and hours (end at 2 days and ~10hours). The vertical scales give the following parameters: stirrer speed (rpm),0_2 pressure, base pump (controlled the pH); feed pump, and OD.

Protein purification was done with ~1/10 of the whole available cell paste, i.e. ≈ 6 grams. The protocol very similar to that defined for the hydrogenated protein can be used to purify the protein. However, the cell lysis is less easy. The solution of the resuspended cells is more viscous. Thus, a spatula tip of lysozyme (Euromedex) is added to the lysis buffer and the solution is incubated during 20min at room temperature under vigorous stirring before lysis using the microfluidizer apparatus (Microfluidics M-110P). The second indicative difference when compared to the purification of the hydrogenated protein is the solubilisation step of the inclusion bodies in urea. About 25 % of the proteins are lost. Fortunatly, a major part of the lost proteins concerns contaminant proteins. Figure 5 shows that D-OmpX at 17 kDa is mainly solubilised whereas the pellet contains contaminants (e.g. at 36 kDa).

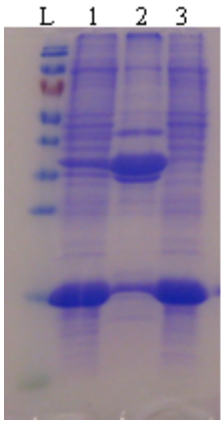


Figure 5: 15% SDS PAGE gel of inclusion bodies solubilisation:

Ladder: 170, 130, 95, 72, 55, 43, 34, 26,17, 11kDa;

1: 5µL total of inclusion bodies solubilised into 6 M urea (12mL at 3.5mg/mL) + 5µL loading buffer.

2: 5µLof pellet resuspended into 2mL 6 M urea (1.4mg/mL) + 5µL loading buffer.

3: 5µL supernatant of inclusion bodies solubilised into 6 M urea (12mL at 2.9mg/mL) + 5µL loading buffer.

We tested the refolding condition with different detergents (Zhang *et al*. 2008) in the same way as we did for the H-protein. Deuterated OmpX presented the same refolding properties (Figure 6, to be compared with Figure 1).

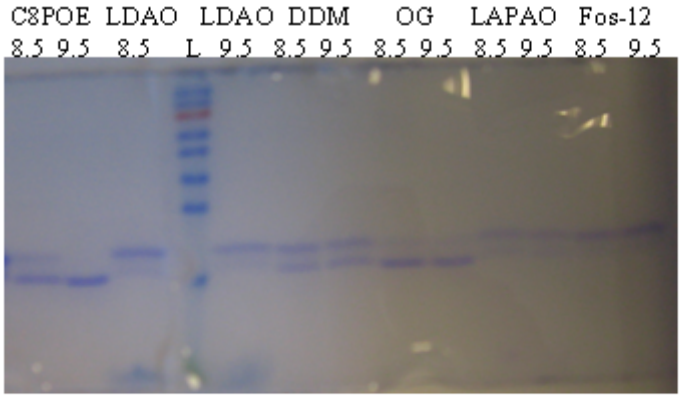


Figure 6: 15% SDS PAGE gel of D-OmpX refolding test:
Ladder: 170, 130, 95, 72, 55, 43, 34, 26,17, 11kDa; 20 µL of refolding solution test + 5 µL loading buffer OmpX with six different detergents (C8POE, LDAO, DDM, OG, LAPAO and Foscholine-12) at two pHs (8.5; 9.5).

Next steps: refolding, purification on Ni-NTA column and desalting were the same as in hydrogenated conditions. The pure D-OmpX obtained was refolded at 90% (Figure 7). From Table 1, the purification yield from the inclusion body step is not significantly different for the hydrogenated and deuterated proteins. MALDI TOF was used to evaluate the deuteration level. OmpX and D-OmpX shows some heterogeneity with three closed peaks separated by 186 Da, with two main peaks being 17411 and 17597 for OmpX, and 18006 and 18196 Da for D-OmpX, to be compared to the theoretical values of 17556 and 18407 (100%

D-OmpX is hydrogenated solvent), respectively. From the difference between these values, the deuteration level is estimated at 70.4%.

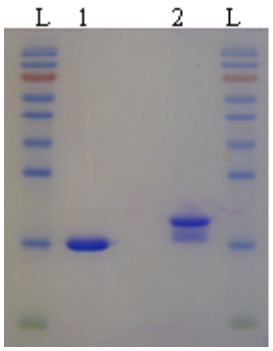


Figure 7: 15% SDS PAGE gel of purified deuterated OmpX :
Ladder: 170, 130, 95, 72, 55, 43, 34, 26,17, 11kDa.
D-OmpX: 3.5mg/mL into 20mM Tris H-Cl pH 8.5, 0.1% LDAO, 100mM NaCl: 1µL+3µL loading buffer.
1: purified D-OmpX refolded and boiled for 5 min (denaturated).
2: purified D-OmpX refolded not boiled.

Steps Constructions	Inclusion bodies	Solubilisation in 6M Urea (yield calculated from 40 mg inclusion bodies)	Refolding *	Purification (Hi-Trap Q/Ni-NTA)	Final yield
pET 3b-OmpX	200 mg (a)	100%	90-100%	40%	40%
pET 24a-OmpX Hydrogenated	150 mg (a)	100%	90%	32%	32%
pET 24a-OmpX Deuterated	200 mg (b)	80%	90%	42%	33%

Table 1: Comparison of OmpX purified yield between the different constructs and hydrogenated / deuterated forms. Values were scaled for 1L of culture * Refolding yield was analysed on 15% Acrylamide SDS-PAGE from the comparison of protein heated and non-heated protein. (a) Inclusion bodies purified from 1L of LB culture. (b) Inclusion bodies purified from 6g (1/10 cell paste) of high cell density culture in fermenter.

Crystallisation of deuterated OmpX:

Manual screening was attempted in a variety of conditions which were successful for the hydrogenated proteins (see above and Saab 2009). Three crystals were obtained without particular optimisation, e.g. in 0.1M citric acid pH 4, 20% v/v 2-Methyl, 2, 4-pentanediol. Their characterisation and optimisation is under way.

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References:

Breyton, C., F. Gabel, M. Abla, Y. Pierre, F. Lebaupain, G. Durand, J. L. Popot, C. Ebel and B. Pucci (2009). "Micellar and biochemical properties of (hemi)fluorinated surfactants are controlled by the size of the polar head." Biophys J **97**(4): 1077-86.

Catoire, L. J., M. Zoonens, C. van Heijenoort, F. Giusti, E. Guittet and J. L. Popot (2010). "Solution NMR mapping of water-accessible residues in the transmembrane beta-barrel of OmpX." Eur Biophys J **39**(4): 623-30.

Dimasi, N., D. Flot, F. Dupeux and J. A. Marquez (2007). "Expression, crystallization and X-ray data collection from microcrystals of the extracellular domain of the human inhibitory receptor expressed on myeloid cells IREM-1." Acta Crystallogr Sect F Struct Biol Cryst Commun **63**(Pt 3): 204-8.

Fernandez, C., C. Hilty, G. Wider, P. Guntert and K. Wuthrich (2004). "NMR structure of the integral membrane protein OmpX." J Mol Biol **336**(5): 1211-21.

Gohon, Y., T. Dahmane, R. W. Ruigrok, P. Schuck, D. Charvolin, F. Rappaport, P. Timmins, D. M. Engelman, C. Tribet, J.-L. Popot and C. Ebel (2008). "Bacteriorhodopsin/amphipol complexes: structural and functional properties." Biophys. J. **94**(9): 3523-37.

Gohon, Y., F. Giusti, C. Prata, D. Charvolin, P. Timmins, C. Ebel, C. Tribet and J. L. Popot (2006). "Well-defined nanoparticles formed by hydrophobic assembly of a short and polydisperse random terpolymer, amphipol A8-35." Langmuir **22**(3): 1281-90.

Johs, A., M. Hammel, I. Waldner, R. P. May, I. Laggner and R. Prass (2006). "Modular structure of solubilized human apolipoprotein B-100 Low resolution model revealed by small angle neutron scattering." J. Biol. Chem. **281**(28): 19732-39.

Nogales, A., C. Garcia, J. Perez, P. Callow, T. A. Ezquerra and J. Gonzalez-Rodriguez (2010). "Three-dimensional model of human platelet integrin alphaIIb beta3 in solution obtained by small angle neutron scattering." J Biol Chem **285**(2): 1023-31.

Nogales, A., C. Garcia, J. Perez, P. Callow, T. A. Ezquerra and J. Gonzalez-Rodriguez (2010). "Three-dimensional model of human platelet integrin alphaIIb beta3 in solution obtained by small angle neutron scattering." J Biol Chem **285**(2): 1023-31.

Pautsch, A., J. Vogt, K. Model, C. Siebold and G. E. Schulz (1999). "Strategy for membrane protein crystallization exemplified with OmpA and OmpX." Proteins **34**(2): 167-72.

Petoukhov, M. V. and D. I. Svergun (2007). "Analysis of X-ray and neutron scattering from biomacromolecular solutions." Curr Opin Struct Biol **17**(5): 562-71.

Saab, S. (2009). Rapport de maitrise: Purification et cristallisation d'OmpX et échange de détergent contre un tensio-actif fluoré, le F6-TAC., Université Joseph Fourrier, Grenoble, France.

Vogt, J. and G. E. Schulz (1999). "The structure of the outer membrane protein OmpX from Escherichia coli reveals possible mechanisms of virulence." Structure **7**(10): 1301-9.

Zhang, Q., R. Horst, M. Geralt, X. Ma, W. X. Hong, M. G. Finn, R. C. Stevens and K. Wuthrich (2008). "Microscale NMR screening of new detergents for membrane protein structural biology." J Am Chem Soc **130**(23): 7357-63.

Zoonens, M. (2004). Thèse: Caractérisation des complexes formés entre le domaine transmembraniare de la protéine OmpA et des polymères amphiphiles, les amphipols. Applications à l'étude structurale des protéines membranaires par RMN à haute résolution Paris, Université Pierre et Marie Curie, Paris, France.