The Use of Blue-Native PAGE in the Evaluation of Membrane Protein Aggregation States for Crystallization

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Running title: Evaluating Monodispersity by Blue-Native Gel

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Abbreviations: BN-PAGE, Blue-Native Polyacrylamide gel electrophoresis; Cymal, 7:7-cyclohexyl-1-heptyl-β-D-maltoside; DDM, n-dodecyl-β-D-maltoside; DLS, dynamic light scattering; FOS-Choline-12, n-dodecylphosphocholine; LDAO, Lauryldimethylamine oxide; PDC, protein-detergent complex; $Rsbc_1$, cytochrome bc_1 complex from R. sphaeroides; SEC, size exclusion chromatography; SDS, sodium dodecylsulfate; SMC, sucrose monocaprate; TMH, transmembrane helix.

Synopsis: Monodispersity obtained by the Blue Native PAGE gel for membrane proteins correlates well with their propensity to crystallize. The results from BN-PAGE are more informative on the sample aggregation states in solution than other techniques such as Dynamic Light Scattering and Size Exclusion Chromatography. Also, BN-PAGE is particularly useful for efficient detergent selection for membrane protein crystallization.

Abstract

Crystallization has long been one of the bottlenecks in obtaining structural information at atomic resolution for membrane proteins. This is largely due to difficulties in obtaining high-quality protein samples. One frequently used indicator of protein quality for successful crystallization is the monodispersity of proteins in solution, which is conventionally obtained by size exclusion chromatography (SEC) or by dynamic light scattering (DLS). Although useful in evaluating the quality of soluble proteins, these methods are not always applicable to membrane proteins either because of the interference from detergent micelles or because of the requirement for large sample quantities. Here, we report the use of Blue Native-PAGE (BN-PAGE) to assess aggregation states of membrane protein samples. We demonstrate a strong correlation between the monodispersity measured by BN-PAGE and the propensity for crystallization of a number of soluble and membrane protein complexes. Moreover, we show that there is a direct correspondence between the oligomeric states of proteins as measured by BN-PAGE and those obtained from their crystalline forms. When applied to a membrane protein with unknown structure, BN-PAGE was found useful and efficient for selecting well-behaved proteins from various constructs and in screening detergents. Comparisons of BN-PAGE with DLS and SEC are provided.

1. **Introduction**

It has been estimated that 20-30% of all genes in most genomes encode membrane proteins (Krogh *et al.*, 2001). In multicellular organisms, membrane proteins are involved in a wide range of fundamentally important biological functions including receptors that transduce signals to regulate cellular functions and cell-cell communications, transporters and channels that function in exchange, transportation and detoxification of nutrients and toxic materials, membrane protein complexes that are part of the cellular respiratory chain in the processes of cellular energy conservation, and other regulators and enzymes (See reference (Sakai & Tsukihara, 1998) for review). Because of their crucial roles in numerous cellular functions and accessibility afforded by their frequent surface localization, transmembrane proteins are among the most desirable targets for drugs (Jimonet & Jager, 2004, Schnur *et al.*, 2006). Despite the vast amount of information from biochemical, biophysical and molecular biological studies, our understanding of the mechanisms of functions at sub-molecular resolution for many membrane proteins has been severely hindered by the paucity of structural information, which is clearly demonstrated by the disproportion in the number of deposited membrane protein structures in the Protein Data Bank (PDB) compared to that of soluble proteins (White, 2004). As of the end of 2007, there were barely a hundred unique membrane protein structures in the PDB, but tens of thousands of soluble protein structures.

An integral membrane protein, in order to be crystallized, must first be extracted from its lipid membrane environment by the use of appropriate detergents and then purified. Purified proteins are usually treated as soluble proteins for crystallization. In general, proteins that are monodispersed, i.e. present in a single oligomeric state in

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solution, are more likely to crystallize (D'Arcy, 1994, Zulauf & D'Acry, 1992). Close

monitoring of the oligomeric state of a protein during its purification is therefore highly

encouraged. The extent of monodispersity versus polydispersity of a protein in solution can

be measured with biochemical or biophysical techniques such as the size exclusion

chromatography (SEC) or dynamic light scattering (DLS) (Lemieux *et al.*, 2002).

However, these methods have not been applied routinely to membrane protein

preparations, perhaps due to interference of detergent micelles in solution. Unlike soluble

proteins, detergent-solubilized integral membrane proteins are covered with a large number

of detergent molecules; they exist as protein-detergent complexes (PDC) in solution.

Additionally, a membrane protein solution should be considered a mixture of PDC and

detergent micelles, existing in a complicated equilibrium. The multi-component

composition of a membrane protein solution complicates the use of SEC or DLS as tools to

measure monodispersity. In practice, these methods often require considerable amounts of

membrane proteins to run, which may be a limiting factor when a large number of

detergents have to be screened. Furthermore, some of these methods, such as SEC, require

large volumes of solutions, hence, demand large quantity of expensive detergents.

The Blue Native Polyacrylamide Gel Electrophoresis (BN-PAGE) method has been

established as a powerful tool to analyze molecular sizes of protein complexes for both

soluble and membrane proteins (Reisinger & Eichacker, 2006, Swamy *et al.*, 2006, Wittig

et al., 2006). Unlike SDS-PAGE, BN-PAGE uses a specific dye to charge the protein or

protein complex without denaturing the protein or disrupting the protein complex during

electrophoresis. In this work, we report an extension of the application of BN-PAGE for

the evaluation of monodispersity of membrane protein preparations. We show that there

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exists a strong correlation between a protein's monodispersity measured by BN-PAGE and

its ability to form crystals. The oligomeric states shown by BN-PAGE also correspond

well with those obtained in crystals. The method was furthermore applied to screening for

monodispersity of a membrane protein of unknown structure in our attempts to identify the

most suitable combination of protein construct and detergent for crystallization. The results

demonstrate that BN-PAGE is a useful, convenient, and economical tool for evaluating

protein monodispersity in solution, providing guidance for working with membrane protein

crystallization.

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2. Experimental procedures

2.1. Materials

Purified cytochrome bc_1 complexes from bovine heart mitochondria and from photosynthetic bacterium *R. sphaeroides* were gifts from Prof. C.-A. Yu of Oklahoma State University, which were purified according to published procedures (Yu & Yu, 1980, Esser *et al.*, 2008). Human AAA protein p97/VCP, both the full-length and the N-D1 fragment, were purified in-house based on the known protocol (Dai & Li, 2001). *E. coli* AAA protein ClpA was also purified according to published methods (Guo *et al.*, 2002). All other chemicals were obtained commercially at highest grades possible.

2.2. Cloning, expression and purification of CopB

The DNA fragment for the full-length CopB was amplified from genomic DNA of *A. fulgidus* (ATCC 49558D-5) with the forward primer 5'-TACGCCATGGTAAAGGATACTTATATCTCT-3' and the reverse primer 5'-AATTAAGCTTGCTTCTGAGCTTTGCCTGGT-3'. After digestion with endonucleases *Nco*I and *Hind*III, the DNA was inserted into the pBAD-his vector (Invitrogen, Carlsbad CA), creating the expression plasmid (pBAD-*Af*CopB) for the full-length CopB. *E. coli* strain LMG194 (Invitrogen, Carlsbad CA) harboring pBAD-*Af*CopB was cultured in Luria Broth (LB) at 37°C until the OD_{600} reached 0.8, at which point L-arabinose was added to a final concentration of 0.005% (w/v). After 3 further hours of incubation, cells were collected by centrifugation and lysed using the French Press at 15,000 psi in Buffer A (25 mM Tris, pH 8.0, 100 mM sucrose, 1 mM PMSF, 2 mM EDTA and 1 mM DTT). Cell debris was removed by centrifugation at 7800 ×g for 30 minutes and the crude membrane fraction was collected by centrifugation at 138,000 ×g for 1.5 hours and stored at -80°C in

Buffer A supplemented with 100 mM NaCl. For CopB purification, frozen membranes were thawed and solubilized by adding drop-wise n-dodecyl-β-D-maltoside (DDM, Anatrace, OH),) to a final concentration of 1% (w/v, same below except otherwise stated). The detergent extract was loaded onto a Ni-NTA (Qiagen, Germantown MD) column pre-equilibrated with the loading buffer (25 mM Tris HCl, pH 8.0, 100 mM sucrose, 10 mM NaCl, 0.2% (w/v) Lauryldimethylamine oxide (LDAO, Anatrace, OH)) supplemented with 10 mM imidazole. Following two washing steps with the loading buffer supplemented with 20 and 40 mM imidazole, respectively, the protein was eluted with the loading buffer containing 300 mM imidazole. The sample was then applied to a Superdex 200 column (10/30 GE Healthcare) to remove possible aggregates in a buffer containing 25 mM Tris, pH 8.0, 100 mM sucrose, 10 mM NaCl, and 0.2% (w/v) LDAO.

2.3. Cloning, expression and purification of CopB-N

To generate the expression vector for the N-terminal fragment of CopB (CopB-N), a stop codon was introduced into the pBAD-*Af*CopB vector at the position Arg303 to generate pBAD-*Af*CopB-N, which was introduced into *E. coli* LMG194 cells. The protein was expressed and the membrane was prepared in a manner similar to that used for the full-length protein. For purification, the membrane was solubilized in a loading buffer containing 10 mM Tris pH 7.3, 500 mM NaCl, 10% (v/v) glycerol, and 30 mM imidazole, supplemented with 1% FOS-Choline-12. After the removal of unsolubilized material, the detergent extract was applied to a Ni-NTA column pre-equilibrated with the loading buffer plus 0.05% FOS-choline-12. Following the washing step with the same buffer containing 0.05% FOS-Choline-12 and 50 mM imidazole, and CopB was eluted with the loading buffer plus 500 mM imidazole. The purified protein sample was further dialyzed against a

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buffer containing 10 mM Tris, pH 7.5, 150 mM NaCl, 5% (v/v) glycerol, 0.025% FOS-

Choline-12.

2.4. Blue-Native PAGE

Most BN-PAGE experiments were performed with the NativePAGE^{TM} Novex Bis-

Tris Gel System (Invitrogen, Carlsbad CA) according to the instructions. The most

frequently used native gel was the 4-16% gradient gel (8 × 8 cm, 1 mm thick and 10 slots);

some gels were poured in-house. Membrane protein samples were diluted to a final

concentration of approximately 1 mg/ml with their respective buffers (see figure legends

for details). To this solution, an additional detergent to be tested was added at a final

concentration of 0.4% (1.0% in the case of β-OG) and incubated for 10 minutes prior to

BN-PAGE. This sample was mixed with 4× NativePAGE^{TM} loading buffer (Invitrogen,

Carlsbad CA) at a volume ratio of 3:1. To each lane of a native gel, 3 to 5 μg of protein

were loaded. Anode buffer was made by diluting the 20× NativePAGE^{TM} running buffer

(Invitrogen, Carlsbad CA), and the cathode buffer by mixing the NativePAGE^{TM} running

buffer with Cathode Buffer additive (Coomassie Blue G-250 dye, Invitrogen, Carlsbad

CA) according to the instructions. For BN-PAGE with membrane proteins, the

concentration of the blue dye is 0.02% (w/v), which is 10-fold higher than that for soluble

proteins. The electrophoresis was performed in an ice bath and ran at 150 volts for one

hour followed by another hour or until the running front reaches the gel end at 250 volts.

The gel was stained using the Colloidal Blue Staining Kit (Invitrogen, Carlsbad CA).

2.5. Tricine-SDS-PAGE

The Schagger's Tricin-SDS-PAGE (Schagger, 2006) was followed with modifications.

Briefly, 3.75 ml 40% (w/v) acrylamide-bisacrylamide stock (37:1, Sigma) is mixed with 5

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ml 3× gel buffer (3 M Tris, 1M HCl, 0.3% SDS, pH 8.45) and 1.5 ml glycerol; water is

added to a final volume of 15 ml. 75 μl 10% (w/v) APS and 7.5 μl TEMED are added

immediately before casting. Pour the mixture into the gel cassette (8 × 8 cm, 1.5 mm thick

and 10 slots, use cassette from Invitrogen) to a height of 6 cm; wait until the

polymerization is complete before adding 4% sample gel (mix 1.2 ml acrylamide-

bisacrylamide stock and 3 ml gel buffer, and add water to 12 ml followed by addition of 90

μl APS and 9 μl TEMED) on top of the separating gel. To 15 μl of bovine bc_1 or $Rsbc_1$

(approximately 5 mg/ml and 2 mg/ml, respectively) sample, 5 μl tricine-SDS-PAGE

sample buffer (12% SDS (w/v), 6% mercaptoethanol (v/v), 30% glycerol (v/v), 0.05%

Coomassie Blue G250, 150 mM Tris pH 7.0) is added, and to each lane, 10 μl of the

mixture is applied. The anode buffer contains 0.1 M Tris-HCl, pH 8.9, and the cathode

buffer consists of 0.1M Tris-HCl, pH 8.25, 0.1M Tricine, and 0.1% SDS. Initial voltage for

electrophoresis is set to 30V and runs for 45 min, letting the sample completely runs into

the separating gel. Then the voltage is raised to 150V until the dye front reaches the gel

end.

2.6. Dynamic light scattering (DLS) measurement

Protein or detergent samples in a buffer containing 10 mM Tris-HCl, pH 7.5 and

150 mM NaCl were filtered through a 0.02 μm filter (Anodisk 13, Whatman) before

measurements. DLS data collections were carried out at 4°C using a DynaPro™ 90

instrument with a wavelength of 830 nm and at a scattering angle of 90° (Wyatt

Technology, Santa Barbara CA). A volume of 20 μl of each sample was used to fill a 1.5 ×

1.5-mm cuvette (Hellma, Plainview NY). Autocorrelations for 20 seconds were collected

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and the data were averaged over at least 10 repeats. The scattering data were then analyzed

with the DYNAMICS version 5.24.02 and plotted with DYNALS release 1.51.

2.7. Size exclusion chromatography experiments

Protein samples (250 μl) in their respective buffers at a concentration of 1 mg/ml

were injected into a Superdex 200 column (10/30). The chromatography was run at a flow

rate of 0.5 ml/min for 1.5 column volumes (34 ml total).

2.8. Crystallization screening

An initial crystallization screen of CopB-N was performed using a Mosquito

micropipette liquid dispense system (TTP LabTech, Royston UK) by hanging-drop vapor

diffusion method with 96 well format plates. Drops were setup by mixing 150 nl of protein

sample with 150 nl of reservoir solutions. Crystallization screening kits were purchased

commercially from Hampton Research, Molecular Dimension, and Sigma.

2.9. X-ray diffraction experiment

X-ray diffraction experiments were conducted at the SER-CAT beamline at the

Advanced Photon Sources (APS), Argonne National Lab (ANL) equipped with a MAR300

CCD detector. Since all CopB-N crystals were grown in mother liquor containing 30-32%

PEG400 (see Results and discussion), which acted as an effective cryo protectant, crystals

were frozen readily in liquid propane. The data was collected at 100 K. Crystal to detector

distances were set between 250 - 300 mm, and oscillation range was either 0.5° or 1°. Raw

data frames were indexed and intensities integrated using the DENZO program; integrated

intensities from each diffraction image of the same crystal were merged and scaled with

the SCALEPACK program. Both programs are part of the HKL2000 software package

(Otwinowski & Minor, 1997).

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3. Results and discussion

Structure determination of polytopic membrane proteins by X-ray diffraction presents a unique challenge in the field of contemporary structural biology. Although there is a general consensus on the main obstacles in the process, namely the difficulties in purifying large amounts of high quality membrane proteins, especially for eukaryotic proteins, and in obtaining diffraction quality crystals of these proteins, specific issues in each and every step in the process often lack sufficient treatment. One of the critical issues is the sample monodispersity, which has been shown to strongly correlate with the sample's propensity to crystallize for both soluble and membrane proteins. However, the question of how to obtain reliable information on the monodispersity or aggregation states of a membrane protein sample quickly and with little sample consumption lacks sufficient discussion. Here we demonstrate the utility of BN-PAGE in the assessment of monodispersity of membrane proteins and discuss pros and cons of its application to membrane protein samples.

3.1. Application of the BN-PAGE technique to membrane protein samples

The classic native-PAGE technique requires that proteins have a net charge under the given buffer conditions, and this excludes its application to proteins that are neutral. In contrast, the use of Coomassie Blue G-250 in Native-PAGE (BN-PAGE) negatively charges all proteins regardless of their charge and magnitude in native states. Furthermore, BN-PAGE can be used to estimate molecular weight, since the magnitude of the negative charge that results from attached dye molecules is roughly proportional to the size of the protein. More importantly, proteins or protein complexes charged with Coomassie Blue,

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unlike SDS, are not denatured or disrupted in complex associations, permitting the analysis of aggregation or oligomeric states.

The device used for SDS-PAGE can easily be adapted for BN-PAGE; all running solutions should be absent of SDS or any other detergents and the cathode buffer must contain Coomassie Blue dye for charging protein molecules. While BN-PAGE analysis is applicable to both soluble and membrane proteins, significant differences exist due to the presence of detergent micelles in the membrane protein application. The most important one is the presence of 10-fold more Coomassie Blue dye for the membrane protein application, which is 0.02% in our protocol. The reasons for the higher dye concentration are that most detergents used in membrane protein purifications are non-ionic, forming detergent micelles and PDCs that are capable of binding to dye molecules. Since PDCs and detergent micelles compete for dye molecules, the presence of a sufficient amount of dye becomes important during electrophoresis in order to cover the entire surface of a protein, keeping it fully charged and soluble. Insufficient amount of dye causes partial charging of protein molecule, leading to errors in molecular weight estimation. Worse, once a detergent micelle acquires dye molecules, it becomes charged and runs alongside the protein during electrophoresis, facilitating removal of more dye molecules from the protein and aggravating the problem. Secondly, unlike BN-PAGE analysis for soluble proteins, it is not necessary to add Coomassie Blue dye to membrane protein samples because sufficient amount is already present in the cathode buffer. Finally, since membrane proteins are prone to aggregate during electrophoresis even at room temperature, all our BN-PAGE experiments were performed in an ice bath, which reduced smearing significantly.

3.2. Correlation of monodispersity revealed by BN-PAGE   with   the   propensity   of membrane proteins to crystallize

To test whether BN-PAGE would be a good indicator of monodispersity for membrane proteins, we applied the method to two membrane protein samples: the cytochrome bc_1 (cyt bc_1 or bc_1) complexes from bovine heart mitochondria and from the anoxygenic, photosynthetic bacterium *R. sphaeroides* (Xia *et al.*, 1997, Esser *et al.*, 2008). The bovine bc_1 complex is the mid-segment of the cellular respiratory chain located in the mitochondrial inner membrane; the physiological form of the complex is dimeric with a molecular weight of 486 kDa and each monomer consists of 11 different subunits. Among the 11 different subunits, three are important for function: cyt *b*, cyt c_1 and the iron-sulfur protein (ISP) subunit. Six subunits are membrane bound, accounting for about 45% of the total mass, and the rest are soluble subunits on the periphery of the complex. The bovine bc_1 complex is well behaved in solution and was crystallized more than a decade ago (Yu *et al.*, 1996). Notably, all eleven subunits were present in the crystal structures (Kim *et al.*, 1998, Gao *et al.*, 2002, Esser *et al.*, 2004). The bc_1 complex from the photosynthetic bacterium *R. sphaeroides* (*Rsbc₁*) also exists as a dimer with a molecular weight of 235 kDa. In contrast to the bovine bc_1, *Rsbc₁* consists of only four subunits per monomer: three essential subunits plus an additional subunit (Subunit 4). In the crystal structure of *Rsbc₁*, only the three essential subunits were resolved, lacking subunit 4 (Esser *et al.*, 2008).

When tested using the BN-PAGE technique, the bovine cyt bc_1 complex was resolved as a sharp, single band corresponding to a molecular weight of ~598 kDa, consistent with the molecular weight of dimeric bc_1 (Fig. 1A). The single band in BN-PAGE not only indicates that the purified bovine bc_1 complex exists in a monodispersed

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state in solution, it is also in accordance with the successful crystallization of the complex

sample and reflects the true oligomeric state of the purified complex. The same sample

gives rise to at least ten bands on a Tricine-SDS-PAGE gel (Fig. 1A), showing the

composition of an intact bovine complex. $Rsbc_1$, on the other hand, was resolved as a sharp

major band corresponding to a molecular weight of approximately 310 kDa, and a minor

band with an estimated molecular weight of 32 kDa (Fig. 1B). This result corresponds well

with the successful crystallization and structure determination of the three-subunit $Rsbc_1$

complexes, lacking the subunit 4. Tricine-SDS-PAGE of the same sample produced four

well-separated subunits (Fig. 1B). In conclusion, the BN-PAGE provides a sensitive tool

for monitoring the monodispersity of membrane proteins in solution and additionally, there

appears to exist a correlation between the aggregation states detected by the BN-PAGE and

those revealed by crystal structures.

The apparent molecular weight of the bc_1 complexes estimated by BN-PAGE (Fig.

1) appears to be higher than expected for the membrane proteins tested. Since the rate of

migration of proteins in an electric field is a function of the charge-size ratio, all other

things being equal, the observed lower migration rates appear to suggest that (1) membrane

proteins have a significant number of bound detergent molecules, (2) the hydrophobic

surfaces may attract fewer dye molecules due to the presence of detergents, or (3) bound

dye molecules are stripped away by the presence of detergent micelles. The third

possibility could be eliminated by running a BN-PAGE experiment with varied detergent

concentrations. We found that, under our running conditions, the migration of $Rsbc_1$ bands

did not change as a function of systematically varied detergent concentrations (data not

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shown), suggesting that the overestimated molecular mass measured by BN-PAGE is likely due to attachment of detergent molecules to the surfaces of membrane proteins.

3.3 The correlation between oligomeric states shown by BN-PAGE and those found in protein crystals

To further verify the above observed correlation for bc_1 complexes between the aggregation states detected by the BN-PAGE and those seen in crystal structures, we analyzed two soluble protein complexes with known crystalline oligomeric states, namely, *E. coli* ClpA and human p97. Both ClpA and p97 are members of the broad family of AAA+ ATPases (<u>A</u>TPases <u>A</u>ssociated with various cellular <u>A</u>ctivities; (Neuwald *et al.*, 1999). The *E. coli* ClpA is an Hsp100/Clp protein unfoldase and an integral component of the ATP-dependent ClpAP protease (Gottesman & Maurizi, 1992), participating in post-translational protein quality control and regulation. The human AAA+ protein p97 is one of the most abundant proteins in cells, accounting for ~2% of cytoplasmic proteins; it participates in a number of cellular pathways including the ubiquitin-proteasome degradation pathway and endoplasmic reticulum associated degradations (Woodman, 2003). Both ClpA and p97 are type II AAA+ proteins, each subunit containing an N-terminal domain followed by two AAA ATPase domains (D1 and D2) in tandem. Electron microscopy has shown that ClpA subunits form a homo-hexamer in the presence of nucleotide, but the hexameric association is rather delicate, as demonstrated biochemically (Kessel *et al.*, 1995). In contrast, p97 subunits form stable hexamers in solution.

When these protein samples were analyzed by BN-PAGE, ClpA was resolved as a single band corresponding to an apparent molecular weight of 146 kDa, larger than a ClpA monomer calculated at 84 kDa but smaller than a dimer of 168 kDa (Fig. 2). Most likely,

ClpA exists as a monomer in solution. This result is consistent with the ClpA crystal structure determined as a monomer in the symmetry of $P6_5$ space group, in which the ClpA subunits arrange as a spiral (Guo *et al.*, 2002). Two different forms of p97 were also tested by BN-PAGE: the full-length p97 and the N-D1 (1-481) fragment (Fig. 2). Both the full-length protein and the N-D1 fragment were resolved as a single band in BN-PAGE, but they migrated much slower than ClpA, indicating a higher molecular weight species. The molecular weights estimated from the gel are 660 kDa and 440 kDa, respectively, for the full-length and the N-D1 fragment. These values correspond to p97 and N-D1 hexamers (582 kDa and 324 kDa, respectively), in full agreement with the hexameric states of their respective structures determined crystallographically (Zhang *et al.*, 2000, Huyton *et al.*, 2003, DeLaBarre & Brunger, 2003). Clearly, the oligomeric states measured by BN-PAGE analysis echo those found in crystal structures. Conceivably, BN-PAGE could be a useful tool to predict oligomeric states of proteins even prior to structure solutions.

3.4. BN-PAGE as a sensitive indicator of membrane protein aggregation

Bacterial copper transporters, CopBs, are members of the P_{1B}-type ATPase family and power the transport of metal ions across the membrane by ATP hydrolysis (Solioz & Odermatt, 1995). Their human orthologs, ATP7A and ATP7B, are essential copper transporters, whose mutations have been linked to a number of neurological disorders such as Menkes and Wilson diseases (Bull *et al.*, 1993, Vulpe *et al.*, 1993). Despite extensive efforts to determine the structures of soluble fragments of both human and bacterial transporters (Sazinsky, Mandal *et al.*, 2006, Sazinsky, Agarwal *et al.*, 2006, Achila *et al.*, 2006, Banci *et al.*, 2007, Banci *et al.*, 2006), no success has been reported on structures of copper transporters containing transmembrane helices (TMH). The thermophilic bacterium

A. fulgidus CopB (*Af*CopB) has 690 amino acid residues and eight predicted TMHs (Mandal *et al.*, 2002). The N-terminal region of *Af*CopB contains a soluble, histidine rich domain thought to have the ability to bind Cu^{2+} ions. The ATPase domain of *Af*CopB is located in a large insertion between TMH6 and TMH7. Since, as with many other membrane transporters, crystallization of this protein appears to be intractable, we chose to use it as a model system to test the effectiveness of the BN-PAGE technique as an indicator of membrane protein monodispersity.

The full-length *Af*CopB was over-expressed in *E. coli* and the expressed *Af*CopB was localized to the cytoplasmic membrane (Fig. 3A). Since the N-terminal portion of this protein contains a histidine rich and presumably a metal ion binding domain, full-length *Af*CopB was purified using one-step Ni-NTA affinity chromatography after solubilization with 1% DDM. In addition to the full-length *Af*CopB, Ni-NTA affinity chromatography co-purifies a fragment of *Af*CopB, named *Af*CopB-N (see next section), which can largely be removed by SEC (Fig. 3B). However, the SEC profile shows clearly at least two overlapping peaks for the purified full-length *Af*CopB in 0.2% LDAO (Fig. 3B), suggesting multiple aggregation states or polydispersity of this protein in solution. Moreover, the estimated molecular weight for *Af*CopB by SEC is in the range of 158 to 670 kDa, significantly larger than its calculated molecular weight of 75 kDa. This observation is consistent with difficulties in crystallizing full-length *Af*CopB.

When the purified full-length *Af*CopB (SDS-PAGE given in Fig. 4A) was tested by BN-PAGE, it gave rise to a large smeared band in the gel with an estimated molecular weight in the range of 451 to 1,335 kDa (Fig. 4A), which is consistent with the observation from SEC that the protein exists in multiple aggregation states in solution. A number of

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different detergents were used in purification and all gave rise to similar results (data not shown). Thus, both SEC and BN-PAGE obtained consistent results, indicating a polydispersed *Af*CopB preparation.

3.5. Application of BN-PAGE to AfCopB-N

As mentioned earlier, a small protein was co-purified with the full-length *Af*CopB and mass spectrometry provided evidence that the co-purified protein was an N-terminal fragment of *Af*CopB (*Af*CopB-N, data not shown). Although the C-terminal portion of *Af*CopB shows a strong homology to the P_2-type calcium pump (SERCA), whose structure is already known (Toyoshima *et al.*, 2000), the N-terminal part of *Af*CopB bears no similarity to any structure in PDB. The *Af*CopB-N consists of an N-terminal metal-binding domain, a four-helix TM domain (from TMH1 to TMH4), and the entire actuator domain.

To characterize *Af*CopB-N, we expressed and purified the *Af*CopB-N fragment in much the same way as for the full-length *Af*CopB (Fig. 3C). Unlike the full-length protein, membranes were solubilized in 1% FOS-Choline-12 and *Af*CopB-N was purified in one step with Ni-NTA affinity chromatography in the presence of 0.025% FOS-Choline-12. Although the eluent from the Ni-NTA column displayed a single peak in the SEC profile (Fig. 3D), suggesting a monodispersed protein preparation, it nonetheless showed a slight asymmetry skewed toward high molecular weight fractions. The apparent molecular weight estimated from the SEC experiment was in the range of 80 to 158 kDa, in comparison to a calculated molecular weight of 34 kDa for a monomeric *Af*CopB-N. Additionally, DLS experiments for the purified *Af*CopB-N in FOS-Choline-12 showed significant polydispersity with a C_P/R_H ratio of 40.8% (Table 1), which suggests that this

protein preparation is less likely to crystallize. Thus, the SEC and DLS experiments seem

to give contradictory results on the aggregation states of the purified *Af*CopB-N.

We tested the purified *Af*CopB-N sample (single band in SDS-PAGE, Fig. 4A) by

BN-PAGE in the same buffer as for the SEC experiment and found that the sample

consists of many different oligomeric species, ranging in molecular weight from 68 kDa to

982 kDa (Fig. 4A). Thus, the result from BN-PAGE confirmed the conclusion derived

from the DLS experiment but appeared to provide a better visualization or resolution to the

aggregation states of *Af*CopB-N in solution. Indeed, intensive efforts to crystallize the

*Af*CopB-N thus purified did not render a single promising condition.

3.6. Use of BN-PAGE to screen detergents for membrane protein crystallization

Because of the well-established correlation between the propensity of a protein to

crystallize and the monodispersity of the protein in solution, it is conceivable that BN-

PAGE could be used as an indicator of protein oligomeric state when searching for suitable

detergents to be included in membrane protein solutions. In the previous sections, we have

shown that BN-PAGE correlates rather well the oligomeric states of proteins with those in

crystals and with the propensity of proteins to crystallize. Furthermore, the amount of

protein needed for a BN-PAGE experiment is small compared to both the SEC and DLS

methods; this is particularly advantageous in screening a large number of detergents for

membrane proteins where purified proteins are often precious and detergents expensive.

To search for appropriate conditions for crystallizing *Af*CopB-N, we tested the

effects of different detergents on the oligomeric state of *Af*CopB-N in solution. Purified

protein (in 0.025% FOS-Choline-12) was diluted ten-fold before adding a second detergent

at a concentration of 0.4% (β-OG at 0.8%). The solution was incubated for 10 minutes and

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then loaded on a BN-PAGE gel (Fig. 4B). For most detergents tested, the *Af*CopB-N showed significant high molecular weight aggregations, except for CYMAL-7, in which the protein appeared to be in only two-distinct aggregation states with estimated molecular weights of 68 kDa and 146 kDa, corresponding to monomeric and dimeric PDC, respectively (Fig. 4B).

3.7. Crystallization condition for AfCopB-N in agreement with the finding from BN-PAGE

Based on the result from BN-PAGE, crystallization screens were setup robotically using purified *Af*CopB-N protein in CYMAL-7 and various commercial crystallization screen kits. Spindle shaped crystals were obtained under one condition, in which the equilibrium solution contained 0.1 M citric buffer, pH 5.6, 0.1 M Li_2SO_4, and 30% PEG400 (Fig. 5A), and the crystals diffracted X-rays to 12 Å resolution with synchrotron radiations (Fig. 5B). The diffraction pattern was successfully indexed to the orthorhombic crystal form with unit cell dimensions of *a*=79.2 Å, *b*=161.5 Å and *c*=175.6 Å. The Matthews' coefficient (V_M) based on a homo-tetramer in the crystallographic asymmetric unit is 3.9, which is rather typical for membrane protein crystals. The successful crystallization of *Af*CopB-N supports the notion that detergents play a very important role in defining aggregation states of membrane proteins in solution and critically influences crystal growth. The low diffraction limit may be due to the two distinct species in solution and optimization of the crystallization condition is currently underway.

Although BN-PAGE results indicated polydispersity of *Af*CopB-N purified in various detergents, crystallization trials with purified proteins in these detergents were nevertheless conducted extensively. However, all attempts were unsuccessful. The fact that

diffracting crystals were obtained with CYMAL-7 demonstrates the predictive power of BN-PAGE in guiding successful crystallization of membrane proteins.

3.8. Comparison of BN-PAGE with DLS and SEC measurements

DLS, which was designed to measure the shape and size of solutes in solution, has become a useful tool in the protein crystallization field to monitor aggregation states of proteins in solution. Protein samples that display sharp, symmetrical, and unimodal distributions (or monodispersed states) have been positively correlated with the propensity for crystallization, while polydispersed samples are rarely crystallized (D'Arcy, 1994, Rigaud *et al.*, 2000). DLS has the advantage of using small amounts of protein samples compared to the SEC method, and samples used have little modification and can be recovered easily. However, the usefulness of DLS to guide membrane protein crystallization has not been extensively studied, in part because of the interference coming from detergent micelles.

In our experiments with cyt bc_1 complexes from bovine mitochondria and *R. sphaeroides*, DLS results did not correlate well with crystallization propensities for these two membrane protein complexes. As shown in Table 1, the polydispersity index C_P/R_H is 78.4% for the bovine bc_1 at 1 mg/ml concentration and is 68.3% for the *Rsbc$_1$* at the same concentration, both of which are too large to suggest successful crystallization trials (Borgstahl, 2007). Additionally, the molecular weights of the complexes were also overestimated. Interestingly, the count rates for the measurements were very high, but the sum-of-squared errors (SOS) in the two experiments were also big. At higher protein concentrations, the DLS results (C_P/R_H) indicated more polydispersed solutions for these membrane proteins (data not show). This result is in apparent contradiction to our

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experience with crystallizations of these two complexes, both of which were crystallized at

concentrations of 11 and 7 mg/ml, respectively, and the discrepancy could be due to a

rather different behavior of PDCs detected by DLS in solution from that of soluble

proteins, calling into question the validity of using DLS as a measure for crystallization

propensity of large membrane proteins or membrane protein complexes. The large

hydrophobic surface area of membrane proteins attracts large numbers of detergent

molecules, forming a surface layer that is more fluid than normal soluble proteins. This

fluid layer is conceivably influencing the behavior of membrane proteins in solution seen

by DLS. Large membrane protein complexes such as cyt bc_1 covered with a thick layer of

detergent molecules would behave very differently from similarly sized soluble proteins.

Smaller membrane proteins, such as *Af*CopB-N, should be less affected by an extra layer

of detergent.

Purified *Af*CopB-N was mixed with various detergents and then subjected to DLS

measurements; measurements for detergent alone were also performed (Table 1). All

detergents are well behaved in solution, consistent with reported micellar sizes and

dimensions, provided that sufficient number of detergent micelles is present. However, the

DLS results for *Af*CopB-N solution are non-discriminatory at best. While the FOS-

Choline-12 and DDM show very large polydispersities at 40.8% and 38.6%, respectively,

the polydispersity indices for β-OG, LDAO, $C_{12}E_8$ and CYMAL-7 (22%-26%) are in the

range often recommended for crystallization trials. Unlike BN-PAGE, which clearly shows

CYMAL-7 as the best detergent for crystallization trials, DLS is incapable of making a

distinction. Furthermore, unlike working with soluble proteins, results from DLS often

depend on the membrane protein concentrations. However, we observed that the average

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molecular weights estimated from DLS for proteins in various detergents are remarkably consistent with the aggregation patterns from BN-PAGE (Figure 4B), except that BN-PAGE provides a greater level of details on the distribution of various aggregation species. For example, in the presence of β-OG, the estimated average molecular weight is 466 kDa for *Af*CopB-N, and BN-PAGE shows nearly all proteins are forming aggregates (Fig. 4B, lane 6). Similarly, DLS estimates a molecular weight of 240 kDa for DDM, and BN-PAGE shows a distribution of both low and high molecular weight species of *Af*CopB-N in the presence of DDM (Fig. 4B, lane 2).

Size exclusion chromatography gives a single, sharp peak for both bovine mitochondrial bc_1 complex and $Rsbc_1$ (data not shown), consistent with crystallization propensity for both proteins. However, although monodispersity was predicted for the four-subunit $Rsbc_1$ by SEC, the final crystallized form lacks the subunit 4. BN-PAGE, on the other hand, showed the separation of the bacterial bc_1 complex into a dimeric three-subunit core and the fourth subunit during electrophoresis. It is conceivable that while the SEC is milder in treating membrane protein complexes and maintains their subunit integrity, BN-PAGE does exert stronger forces, breaking apart subunits that are only very weakly associated, a process possibly akin to crystallization. SEC is apparently able to maintain very weak, sometimes non-physiological, interactions between subunits or molecules. Therefore, the forces exerted upon membrane proteins during BN-PAGE could be used to our advantage in predicting the right forms of the protein to crystallize.

4. Summary

BN-PAGE is a promising method in evaluating membrane protein samples for crystallization. It features high sensitivity, simplicity, and speed in obtaining results.

Furthermore, it requires a very small amount of sample, which is important when working with low-expressing membrane proteins. The results from BN-PAGE are more informative on the sample aggregation states in solution than other techniques such as DLS and SEC. Also, BN-PAGE is particularly useful for efficient detergent selection for membrane protein crystallization.

5. Acknowledgements

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Figure legends

Figure 1 BN-PAGE analysis of membrane proteins with known crystal structures (A) BN-PAGE and Tricine-SDS-PAGE of the 11-subunit bovine heart mitochondrial cytochrome bc_1 complex. Two PAGE gels are shown; the left two lanes are results from a BN-PAGE run and are as labeled; the right lane shows the result from Tricine-SDS-PAGE (Schagger, 2006) of the same sample, which gives rise to at least 10 bands for the intact bovine bc_1 complex. Purified bovine bc_1 in a buffer (50 mM Tris-HCl, pH 8.0 supplemented with 0.66 M sucrose) was diluted to a final concentration of 1 mg/ml using the same buffer before a BN-PAGE run. There was no needed for additional detergents in the dilution because the purified bovine bc_1 contained sufficient amount of potassium deoxycholate. The arrow indicates the position of the bovine bc_1 complex in BN-PAGE. (B) BN-PAGE and Tricine-SDS-PAGE of the 4-subunit bacterial cytochrome bc_1 complex from *R. sphaeroides*. The *Rsbc₁* sample in a buffer (50 mM Tris-HCl, pH 8.0, 200 mM NaCl, 200 mM histidine, 0.5% β-OG) at a concentration of 1 mg/ml was used for BN-PAGE. Two bands were shown by the BN-PAGE as indicated by arrows; the top arrow indicates the position of the three-subunit *Rsbc₁* complex dimer and the lower arrow indicates the position of the dissociated subunit 4. Four subunits are shown for the same sample by the Tricine-SDS-PAGE as indicated.

Figure 2 BN-PAGE analysis of soluble protein complexes Three AAA+ proteins were analyzed by the BN-PAGE. The *E. coli* ClpA runs at 146 kDa as a monomer, human full-length p97 runs at 660 kDa, forming a hexamer, and the p97 N-D1 fragment runs at 440 kDa, also a hexamer.

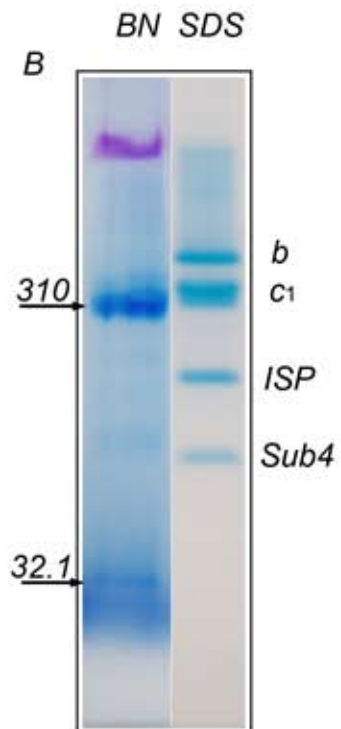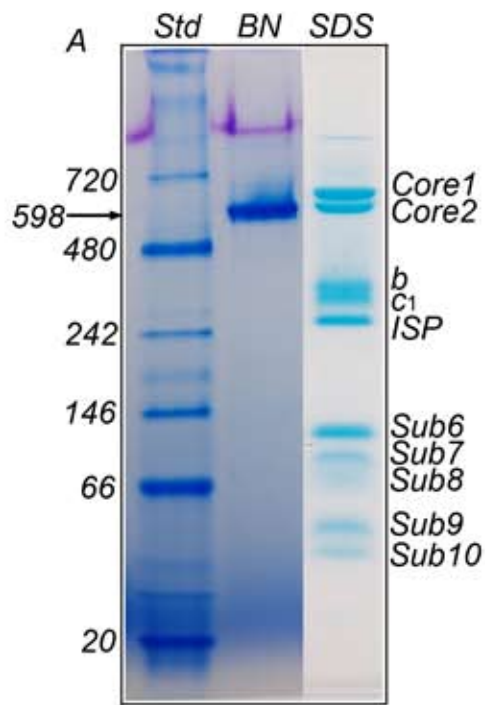**Figure 3 Expression, purification and characterization of the full-length *Af*CopB and its N-terminal fragment *Af*CopB-N** (A) SDS-PAGE gel following the purification procedure of the full-length *Af*CopB. Protein samples were run on a 4-12% Bis-Tris gel in a MOPS buffer. The lane labeled with WC is for the whole cell lysate, M is the crude membrane, Ni is the eluent from Ni-NTA column, and SEC is the purified *Af*CopB after size exclusion chromatography. The two major bands after Ni-NTA chromatography are the full-length *Af*CopB and a co-purified N-terminal fragment of *Af*CopB named *Af*CopB-N. (B) Elution profile of a full-length *Af*CopB sample eluted from SEC. The blue line represents the profile for the protein sample from a Superdex 200 column, showing two peaks. The first one is from the full-length *Af*CopB and the second from the co-purified *Af*CopB-N fragment. The pink line is from commercial molecular weight standards. (C) SDS-PAGE gel following the purification of *Af*CopB-N. Protein sample was run on a 12 % Bis-Tris gel in MOPS buffer. The lane labeled with WC is for the whole cell lysate, M is crude membrane, Ni is eluent from Ni-NTA column, and SEC is the purified *Af*CopB302 after SEC. (D) The SEC profile of *Af*CopB-N after a Superdex 200 column.

Figure 4 BN-PAGE analysis of full-length *Af*CopB and *Af*CopB-N (A) BN-PAGE and SDS-PAGE of the full-length and N-terminal fragment *Af*CopB. Approximately 20 μg of the proteins in a buffer containing 25 mM Tris-HCl, pH 7.5, 100 mM NaCl, and 0.025% FOS-Choline-12 were applied to each lane for BN-PAGE. SDS-PAGE shows the purified *Af*CopB-N and full-length *Af*CopB with corresponding molecular weights of 33.8 and 75.4 kDa, respectively, whereas BN-PAGE clearly indicates polydispersity of these two proteins in 0.025% FOS-Choline-12. (B) BN-PAGE for the *Af*CopB-N fragment in various detergents. Protein sample in 25 mM Tris-HCl, pH 7.5, 100 mM NaCl, and 0.025% FOS-

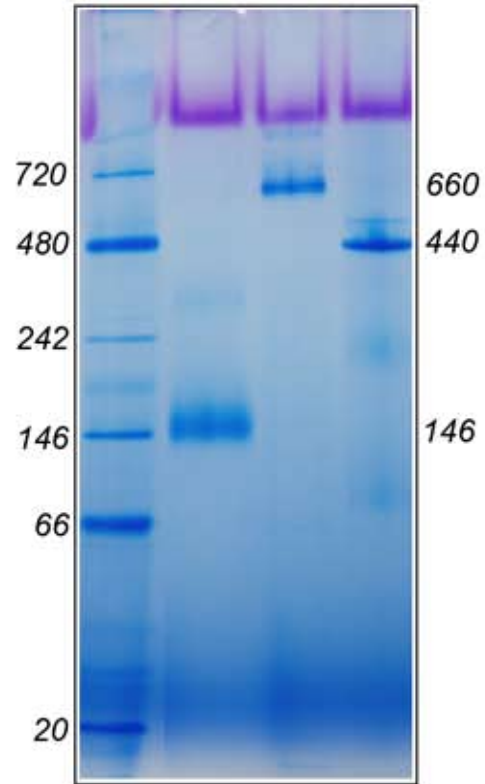--

Choline-12 was diluted to 1 mg/ml and each detergent was added to a final concentration

of 0.4% (w/v) (1% for β-OG). Lanes 1 FOS-Choline-12, lane 2 DDM, lane 3 $C_{12}E_8$, lane 4

CYMAL-7, lane 5 LDAO, and 6 β-OG.

Figure 5 Crystals and diffraction image from an *Af*CopB-N crystal (A) *Af*CfaB-N

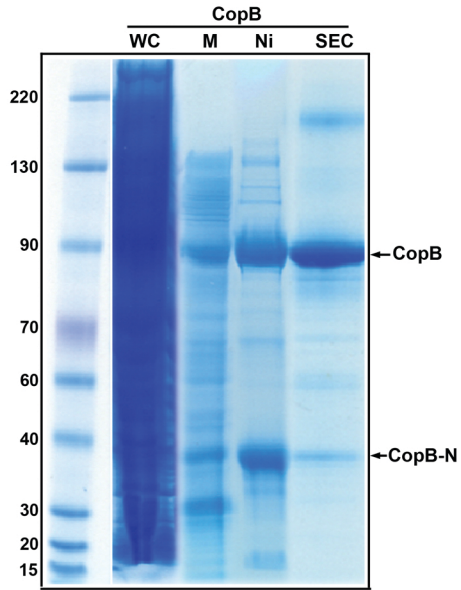fragment crystals obtained. (B) A diffraction image from an *Af*CfaB-N crystal.

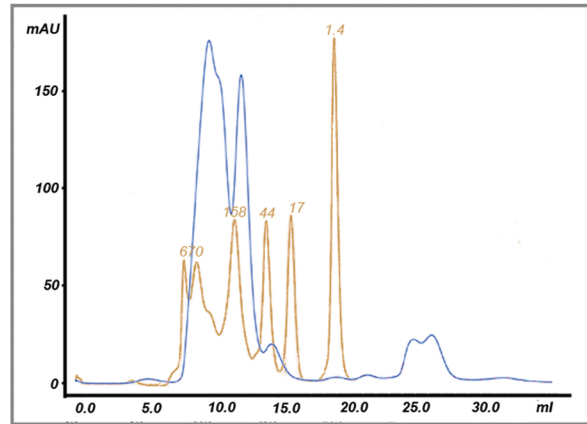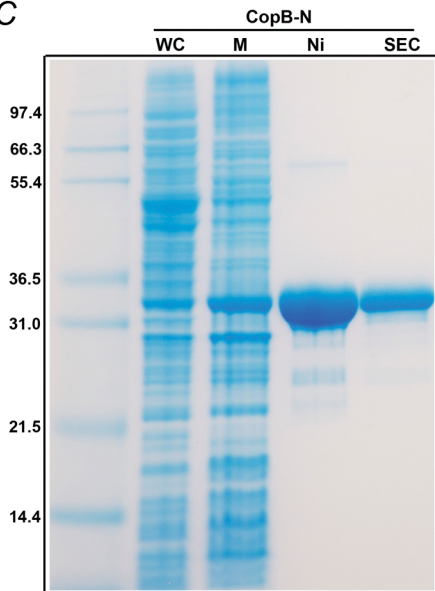**Table 1 DLS measurement of detergent alone and PDC solutions.**

A

Std BN SDS

720
598 →
480

Core1
Core2

b
c₁
ISP

242

146

Sub6
Sub7
Sub8

66

Sub9
Sub10

20

B

BN SDS

310 →

b
c₁

ISP

Sub4

32.1 →

A

CopB
WC M Ni SEC

220
130
90 ← CopB
70
60
40 ← CopB-N
30
20
15

B

mAU
150
100
50
0

670 158 44 17 1.4

0.0 5.0 10.0 15.0 20.0 25.0 30.0 ml

C

CopB-N
WC M Ni SEC

97.4
66.3
55.4
36.5
31.0
21.5
14.4

D

mAU
150
100
50
0

670 158 44 17 1.4

0.0 5.0 10.0 15.0 20.0 25.0 30.0 ml

A

SDS   BN
302   FL   302   FL

1,335
982
451

75.4

146

68

33.8

B

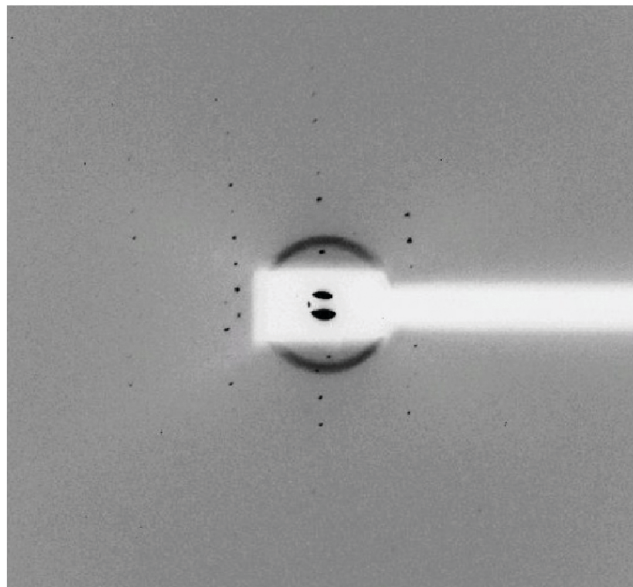1   2   3   4   5   6
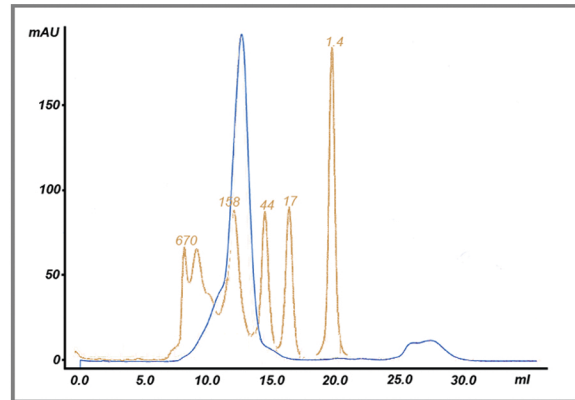
A

B

Table 1 DLS measurement of detergent alone and PDC solutions[1].

Preparation	Concentration prot mg/ml, det %	CMC (%)	Count rate (kcps)	Scattering amplitude	R_H^2 (nm)	Est. MW^3 (kDa)	C_P^4 (nm)	C_P/R_H^5 (%)	Baseline error	SOS error[6]
DDM[7] – bovine bc_1	1, 0.1	-	5,165	0.421	12.5	1,340	9.8	78.4	1.000	58.9
SMC[7] - $Rsbc_1$	1, 0.1	-	4,849	0.424	18.6	3,480	12.7	68.3	1.001	50.6
β-OG	1.25 %	0.68[8]	124	0.838	2.7	33	0.5	15.2	1.000	6.1
β-OG – CopB-N	0.7, 0.4	-	373	0.847	8.1	466	1.9	23.4	1.000	6.9
LDAO	0.4	0.0032[8]	108	0.818	2.6	28	0.4	13.7	1.000	4.7
LDAO – CopB-N	0.7, 0.4	-	229	0.862	4.0	85	0.9	22.4	1.000	6.2
$C_{12}E_8$	0.4 %	0.0048[9]	51	0.713	3.3	53	0.9	26.3	1.000	18.9
$C_{12}E_8$ – CopB-N	0.7, 0.4	-	447	0.873	7.8	417	2.0	26.0	1.000	5.7
CYMAL-7	0.4	0.0099[8]	214	0.875	3.6	64	0.6	15.5	1.000	2.6
CYMAL-7 – CopB-N	0.7, 0.4	-	490	0.871	5.0	147	1.2	23.7	1.000	5.8
DDM	0.4	0.0087[10]	186	0.857	3.0	43	0.4	12.4	1.000	2.6
DDM – CopB-N	0.7, 0.4	-	555	0.831	6.2	240	2.4	38.6	1.000	17.9
FOS-Choline-12	0.4	0.047[10]	87	0.800	2.5	28	0.6	22.1	1.000	7.0
FOS-Choline-12 – CopB-N	0.7, 0.4	-	149	0.854	3.6	66	1.5	40.8	1.000	28.7

[1] All data acquisitions were collected for a period of 20 seconds and averaged over 10 measurements.
[2] R_H is the mean hydrodynamics radius defined as weighted average of the number of bins comprising the peak.
[3] Estimated molecular weight by DLS.
[4] C_P is the polydispersity measuring the width of the peak.
[5] C_P/R_H is % of polydispersity.
[6] Sum of square error.
[7] These detergents were used at a concentration of 0.1 %.
[8] CMC were reported with detergents dissolved in 100 mM NaCl solution.
[9] CMC were reported with detergents dissolved in 10 mM TES buffer, pH 7.5, containing 50 mM NaCl and 0.1 mM CaCl₂.
[10] CMC were reported with detergents dissolved in water.