**TITLE:** **The yin and yang of solubilization and stabilization for wild-type and full-length membrane protein.**

**AUTHORS**:

Haut du formulaire

David HARDY1, 2, Elodie DESUZINGES MANDON1, Alice ROTHNIE2 & Anass JAWHARI1¶

1 CALIXAR, 60 avenue Rockefeller 69008, Lyon, France

2 Life & Health Sciences, Aston University, Birmingham B4 7ET, U.K

**¶Corresponding author:** [ajawhari@calixar.com](mailto:ajawhari@calixar.com)

Bas du formulaire

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**Keywords:** Membrane proteins, detergent, surfactant, solubilization, stabilization.

**Running title:** solubilization and stabilization of membrane proteins.

Membrane proteins (MP) are stable in their native lipid environment. To enable structural and functional investigations, MP need to be extracted from the membrane. This is a critical step that represents the main obstacle for MP biochemistry and structural biology. General guidelines and rules for membrane protein solubilization remain difficult to establish. This review aims to provide the reader with a comprehensive overview of the general concepts of MP solubilization and stabilization as well as recent advances in detergents innovation. Understanding how solubilization and stabilization are intimately linked is key to facilitate MP isolation toward fundamental structural and functional research as well as drug discovery applications. How to manage the tour de force of destabilizing the lipid bilayer and stabilizing MP at the same time is the holy grail of successful isolation and investigation of such a delicate and fascinating class of proteins.

**Highlights**

* What parameters control stabilized membrane protein production?
* Study of membrane protein structure/function requires their solubilization from the membrane. What are the detergent solubilization solutions and alternatives to detergents?
* Solubilization tends to be de-stabilizing for the protein. What are the methods that allow to screen for good solubilization and stabilization conditions?
* Tools for stabilizing solubilized wild-type, full-length membrane proteins are discussed. How to evaluate the protein quality?
* How to stabilize already solubilized membrane proteins?
* Endogenous vs recombinant membrane proteins expression of is discussed

**Introduction**

Membrane proteins (MP) represent 20-30% of human proteins. They are crucial for cellular physiology as they are directly involved in a large spectrum of cellular processes including cell adhesion, cell-cell communication, signal transduction and transport. This may explain why they represent 70% of therapeutic targets [1]. Many of the difficulties associated with MP structure, function and drug discovery have stemmed from the need to solubilize them from the membrane bilayer with detergents. Thus, it is essential to develop tools for membrane protein solubilization and stabilization to unlock structure function details as well as drug discovery.

**What parameters control stabilized membrane proteins production?**

There is not only one parameter to consider for successful MP production. MP production work flow can be improved, at the expression, solubilization, purification and formulation steps **(Figure 1)**. A tremendous effort has been made with MP thermostabilization expression approaches using truncation, multiple alanine scan mutagenesis and protein fusion (such as T4 lysozyme or BRIL). Co-expression of membrane protein partners may also help improve stability. It was recently reported that co-expression of mini-protein G helped to stabilize a GPCR [2]. Protein engineering strategies were applied to different GPCRs such as β1 adrenergic or adenosine A2A receptors [3, 4] resulting in different atomic structures [5-9]. Although these approaches have proven very successful for generating crystals for structure determination, the **modification of the protein sequence may have a significant impact on the protein conformation and can therefore provide misleading information to drug discovery.** For example, a study has demonstrated structural deviations of a fused receptor in the crystal due to the protein fusion [10]. Therefore, in this review we will focus on approaches for studying non-mutated full-length proteins which we term wild-type protein. The expression system used may also have an impact on the quality of MP. For some MP, posttranslational modifications can be important for function. Gene optimization as well addition of a variety of tags/ fusion at different location may also help improve expression and stability of membrane proteins. Over-expression can also sometimes generate misfolded proteins and a good compromise is to be established. Co-expression with MP partner and/or sometimes in presence of specific ligands may affect expression yield or stability features. Therefore, the choice of the expression system needs to take into account all the cited parameters. To assess the influence of expression conditions on protein stability in solution you could use fluorescence size exclusion chromatography, utilizing either fluorescence proteins such as GFP fused to the protein of interest or fluorescent multivalent NitriloTriacetic Acid dye that interact with His-tagged proteins [11]. In addition to stabilization at the expression level, the addition of high-affinity ligands, lipids or lipid-like molecules during membrane preparation, solubilization and/ or purification can provide conformational or oligomeric stabilization [12-14]. Moreover, the use of antibodies, nanobodies and fusion proteins as chaperones can significantly improve the stability of different classes of membrane proteins [15-17].

**Solubilizing membrane proteins with detergents**

Arguably the most important parameter is to search for the best solubilization conditions that help maintain the structural and functional integrities. Solubilization and stabilization are intimately linked and balancing solubilization efficiency with protein stability can be extremely challenging. To date the majority of studies have utilized detergents for MP solubilization. Detergents are amphiphilic molecules made of hydrophobic and hydrophilic moieties that display monomeric and micellar organization in solution depending on the detergent concentration (**Figure 2**). Detergents are commonly defined according to their charge, anionic (eg. sodium dodecyl sulfate; sodium deoxycholate), cationic (eg. cetyltrimethylammonium bromide), zwitterionic (eg. CHAPS for 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; LDAO for lauryldimethylamine-N-oxide or fos-choline) or non-ionic (eg. triton X-100 or DDM for n-dodecyl-b-maltoside) and the nature of hydrophilic (Carboxylate for example) and hydrophobic groups (lipid like structure such as aliphatic chain or alicyclic structures). Differences in detergent features directly influence their Critical Micelle Concentration (CMC), aggregation state, solubility in water, protein solubilization efficiency, protein stabilization ability and pH sensitivity. CMC is specific to each detergent and is defined as the concentration below which only detergent monomers exist in solution and above which detergent micelles start to form. Often biochemists assume that a concentration of 1% (w/v) of detergents is sufficient for comparison of solubilization efficiencies but this is not always true since detergents have very different CMC. Therefore, it is more relevant to rank detergent solubilization efficiency using concentrations related to the CMC. For example, a concentration of 10x CMC is commonly used to compare detergents for their solubilization. There is no direct correlation between solubilization efficiency and stability of membrane proteins [18, 19]. The physical-chemical proprieties of detergents (headgroups and tail) which are variable from one detergent to another are critical for solubilization and stabilization efficiencies.

To date, no detergent has been reported which tackles membrane protein solubilization and stabilization universally. Indeed, a very successful detergent for one protein can turn out to be very inefficient for others. This is very often related to the sensitivity of each protein to the critical extraction step. Therefore, the classical definition of harsh, mild and soft detergent needs to be revaluated taking into account the specificity of each MP. In addition to classically used detergents such as DDM, LDAO, fos-choline or CHAPS which are routinely used for MP solubilization and purification with certain successes and limitations, new families of amphiphilic reagents have emerged recently. Novel calixarene based detergents with CMCs ranging from 0.05 to 1.5 mM, have been successfully applied to solubilize, purify and stabilize membrane proteins belonging to a range of different families including one trans-membrane (TM) macrophage receptor (IFITM2), 12 TM transporters, 7TM GPCRs, multiple TM ion channels [20-24]. These novel detergents were initially designed to structure the membrane domains through hydrophobic interactions and a network of salt bridges with the basic residues found at the cytosol-membrane interface of membrane proteins, in addition to π-stacking interactions between the calixarene platform and aromatic residues [25]. This new class of calixarene detergents and others recently designed bearing glycoside or cholesterol-like groups have been used to stabilize membrane proteins. **Figure 3** shows their chemical structures alongside other promising detergents for structural biology such as glucose neopentyl glycols (GNGs), maltose neopentyl glycol (MNGs) and facial amphiphiles [26-32].

**Solubilization & stabilization screening methods?**

The need to find the optimal solubilization condition for each protein is an important pre-requisite for MP isolation in solution. The choice of the best detergent to use can seem overwhelming. Often selection of the reagent used is based primarily on previous successes with other MPs. For membrane protein crystallography the historically most successful detergents have been previously discussed and evaluated [33-35](data is also readily available via Stephen White’s website (http://blanco.biomol.uci.edu/)). However, a systematic detergents screening including a wide range of detergents with different physical-chemistry features is the best approach to tackle solubilization as one of the major obstacles for MP production. Several methodologies have been described to monitor solubilization efficiency including size exclusion chromatography or FSEC [36] ultracentrifugation dispersity sedimentation assay [37], differential filtration assay [38] and more recently *BMSS (Biotinylated Membranes Solubilization & Separation)* that uses streptavidin binding of solubilized biotinylated membranes to screen solubilization conditions in 96 well plate format [20, 24]. *BMSS* was applied to a GPCR expressed in two different expression systems (insect cells and yeast) and demonstrated very similar tendencies for solubilization conditions regardless of the cell type/ lipid composition [20]. This suggests that solubilization relies more on MP physical chemical and structural proprieties and less on the properties of the membrane it is embedded in. Further studies are still required though to nail down the determinant of MP solubilization.

In addition to screening for solubilization efficiency, a careful screening of stability is absolutely required for a successful MP production. Thermostability assays can help rank stabilization conditions. Different methods have been reported for this purpose such as microscale fluorescent thermal stability assay [39] as well as western blot based thermostability assay [40]. It is also possible during the purification to identify the best stabilizing conditions. In fact, stabilizing reagents can also be added after solubilization steps to improve protein stability as illustrated in **Figure 4**. Detergents tend to strip away all the lipids from a membrane protein, many of which have been shown to be important for protein function. The use of particular lipids or cholesterol homologs can have a positive impact on MP stability and functionality [41, 42]. This is why cholesteryl hemisuccinate and similarly calixarene cholesteryl derivative were designed **(Figure 3**). Depending on the purification scheme, MP can be more or less stable and functional. A careful evaluation of the protein’s behavior in solution, stability and functionality needs to be carried out, similarly to the solubilization step.

**Alternatives to detergents?**

Apart from typical detergents, Lipid Like Peptides (LLPs) have detergent like properties and have been recently proposed as alternatives to solubilization of integral membrane proteins [43]. Polymer based solubilizing agents have also recently emerged. Styrene-maleic acid (SMA) co-polymer was described to efficiently solubilize MPs forming of discs of lipid bilayer, containing MP and encapsulated by the polymer, termed SMALPs [44-49] (SMA lipid particles) (**Figure 5)**. Solubilizing and stabilizing effects rely on the chemical nature of the solubilizing reagent and the intrinsic stability of a particular MP in solution. MP production roadmap consists of solubilization followed by purification steps resulting in protein detergent complexes, or SMALPs or equivalent depending on the used solubilization agent (**Figure 5**). Purification of MP is often carried out by affinity chromatography (His, Flag, HA, Myc, Strep or other tags), ion exchange chromatography and gel filtration. Following purification detergent solubilized MPs are often reconstituted into lipid bilayer environments or exchanged into amphipols to improve stability. Amphipols and nanodiscs are amphipatic polymers and polypeptides (also peptide [50]) that wrap around MP creating a water-soluble complex. Although Amphipols/ Nonionic amphipols (NAPols), hemifluorinated agents and nanodiscs often show efficient stabilization of MPs they lack efficient initial extraction capacity [51-56]. Thus, their use for MP reconstitution/ stabilization requires prior use of detergent for the initial solubilization stage. Recently a new platform for MPs was reported [57]. This amphiphilic block comprised of copolymer, membrane and two MSPs (Membrane Scaffold proteins). To avoid any solubilization trials, a recent strategy called *SIMPLEx* (Solubilization of Integral Membrane Proteins with high Level of Expression) was described[58]. The method consists of fusing MP with truncated apolipoprotein A-1 to promote its solubilization. As an alternative scaffold to Apolipoprotein A-1, saposin-lipoprotein nanoparticle system (Salipro) have also been described [59, 60]. Aside from the various forms of nanodiscs and polymers, another important reconstitution format is the proteoliposome. It is often obtained after dialysis or the use of other buffer exchange methods to slowly remove detergent in the presence of lipids. Like nanodisc and amphipol, detergent is still required for solubilization and purification if proteoliposomes are to be obtained from purified protein. The orientation of protein within the liposome is difficult to control and is often close to 50% inside out. The choice of using MP in detergent micelles, in nanodisc, amphipol or proteoliposome is often based on stabilization effect for each formulation condition and their relevance for the final application. Indeed, structural biology (NMR, crystallography, cryo-EM), ligand screening (Biacore and NMR) and antibody discovery have all different preferential formulation requirement.

**What is the perfect tool?**

Detergents offer a great opportunity to solubilize and stabilize MP, but in some cases, they can interfere with the absorbance at OD 280nm and optical spectroscopy. Some detergents are difficult to dialyze/ exchange or mask the purification tag or the antibody epitope. SMA offer a great advantage over nanodisc to allow solubilization and purification similarly to detergent. However, in some cases, it may interfere with the accessibility of the protein tag to the affinity purification column. In **Figure 6**, we show a side by side solubilization and purification study of two MPs (MP-A and MP-B) conducted either in classical detergent (DDM and FC12 for MP-A and MP-B, respectively) or using SMA. While for MP-A efficient purification and better purity was obtained with SMA in comparison to DDM condition; result of MP-B purification show inefficient purification. In fact, SMA allows good solubilization but in this case, it interferes with the binding to the His-tag column. The same situation can also be observed with detergent depending on the micelle and protein tag locations. Thus protein purification needs to be optimized with SMA as with detergent. The most commonly used SMA is SMA2000 (with a 2:1 styrene to maleic acid ratio). This was recently shown to be the most efficient SMA for membrane solubilization [61]. Despite this, due to interactions of SMA with divalent cations, it can be challenging to investigate MP functionalities such as ATPase activity. Other polymers such as Diisobutylene/Maleic Acid Copolymer (DIBMA) were also reported to have the advantage of not interfering with optical spectroscopy in the far‐UV range, and not precipitating divalent cations [62] in contrary to most detergents and SMA, respectively. The nanodisc and amphipol format are particularly attractive options for studying membrane proteins, especially for ligand-receptor interactions, cryo-EM and antibody discovery. However, they both require finding good detergent conditions for solubilization and purification. Therefore, MP can benefit from this format only if it was properly extracted from the membrane. To this end good solubilization conditions need to be obtained.

**How to evaluate the quality of purified membrane proteins?**

Suitable detergent has to guarantee efficient solubilization and the conservation of structural and functional integrities of MPs. This remains an important challenge that has hampered MP structure function investigation and drug discovery. The effect of detergent on protein structure and/ or function can be variable from one protein to another. Detergents can introduce structural deviations from the initial 3D structure of the protein. They can also break interactions within MP complexes or with co-factors/ partners. The first parameter to analyze after solubilization and purification of MP is the aggregation status. This can be easily assessed by size exclusion chromatography (SEC) where aggregates can be easily distinguished by their elution at the column void volume. Native PAGE can also be used to evaluate the existence of aggregates, oligomers or monomers of MP, therefore giving insight into the positive or negative effect of the chosen detergent/ surfactant for structural integrity conservation of MP. Due to the existence of different oligomeric states and different protein/ detergent complexes (PDC), it is not always a straightforward method even if in general the presence of aggregates can be indicated by signal in the wells of the gel. Other methods such as dynamic light scattering (DLS) as well as SEC-MALS are more informative to study oligomeric assemblies in solution, but they require pure and homogenous protein and a good idea about detergent micelles size. A second and very important parameter to assess is MP functionality. This depends on the specific dedicated function of each MP. Purified protein such as GPCR can be analyzed by radioligand/ fluorescent binding/ NMR binding or after immobilization on SPR (surface plasmon resonance) chip. This provides indirect evidence about functionality but does not prove a direct functional state as real GPCR function is not ligand binding but rather cell signaling. Protein G activation assays have been developed to bridge the gap between direct and indirect functional assays of GPCRs. This is even more complex for orphan GPCRs for which no ligand binding can be performed. For some ion channels where the functional oligomeric state is known, a simple investigation of the oligomeric state can already give good indication about the functional status. This was the case for the tetrameric proton selective ion channel M2 from influenza [21]. A current voltage measurement of purified protein inserted into a lipid bilayer in the presence or absence of specific inhibitor could be applied for further functional validation. This is very often a limiting factor for all transporters and ion channels for which functionality can only be assessed when the protein is inserted into the membrane. This requires proteo-liposome or other bilayer system reconstitution and adds more complexity and variability to the functional validation. This is true for ABC transporters as measuring transport, not just ATPase activity, is the best way to test its functionality. Because some aggregated MPs can still be functional, it is important to consider MP homogeneity and good behavior in solution as one of the best quality control criteria to be complemented by functional assays if possible.

**Can already solubilized protein still be stabilized?**

Solubilization can be the best step to stabilize membrane proteins. But even after this step, it is still possible to impact positively on MP stability by addition of suitable detergent/ surfactant. **Figure 4** illustrates how some reagents can improve MP thermostability and help maintain functionality over time. Using previously described western blot based thermostability assay [40], we show that addition of stabilizing CALX-reagent 1 and 2 did improve thermostability of MP-A by +9 and +16°C, respectively (**Figure 4**). This demonstrates that thermostability can already be significantly optimized by providing the right stabilizing chemical environment without having to apply mutagenesis or other heavy engineering methodologies.

**Endogenous vs recombinant?**

The same tools used for recombinant MP can also apply to endogenous MP. **Figure 7** shows a side by side study on solubilization and purification/ enrichment of MP expressed recombinantly (in HEK cells) versus endogenously (cancer cells). The main difference is the purity of the final sample. Indeed, in recombinant systems it is always possible to add affinity tags to the MP which facilitate purification and allow high purity. Alternatively, successive solubilization trials consisting of first solubilizing most of the protein contaminants before extracting the MP of interest can be applied to endogenous MP enrichment. Ion exchange chromatography, immuno-purification and/or gel filtration can also be used instead of affinity purification to improve purity/ enrichment without using any tags.

**Perspectives**

The future of MP biochemistry and structural biology is bright given the tremendous progress made in new innovative solubilizing/ stabilizing reagents and the development of systematic methodologies for MPs solubilization and purification. More innovation in this field require the combination of expertise on chemistry, biochemistry, structural biology, molecular simulation and modelling that will provide valuable tools to understand the determinants of MP solubilization and stabilization. Experimental and rational approaches are to be engaged in parallel to tackle this critical step of extracting MPs from their native environment and provide an adapted strategy for stabilization by helping rationally design new generation of customized stabilizing reagents. A special focus on designing novel tailored reagents that interact gently with MP, for example by tuning their polar head to be able to clamp membrane domains using salt bridges. Additional improvements could focus on the micelle size reduction and the detergent/ protein interaction dynamics. Such innovations are key to the success of future structural/ functional studies as well as drug discovery.

**FIGURES LEGENDS**

**Figure 1:** Parameters to consider for the optimization of membrane protein production including expression, solubilization, purification and formulation. PTM, SEC, Tm, PAGE, MP stand for post-translational modifications, size exclusion chromatography, melting temperature, polyacrylamide gel electrophoresis and membrane protein, respectively.

**Figure 2**: Equilibrium between detergent monomers and micelles depending on the detergent concentration. Critical micelle concentration (CMC) is defined as the concentration of detergents above which micelles are spontaneously formed.

**Figure 3:** Structure of different novel stabilizing detergents. **A**- Structure of different families of calixarene based detergents. CALX-1, CALX-2 and CALX3 correspond to 5,11,17-tris[(carboxy)methyl]-25-monooctyloxy-26,27,28-trihydroxycalix[4]arene; 5,11,17-tris(2-[4-(β-D-glucopyranosyl)-1H-1,2,3-triazol-1-yl]ethyl),-25- monoheptoxy-26,27,28-trimethoxycalix[4]arene; and 5,11,17-tris[(carboxy)methyl]-25-monomethoxytriazolo-(3α-cholesteryl)-26,27,28-trihydroxycalix[4]arene, respectively. **B**- Structure of GNG (Glucose Neopentyl Glycols), MNG (Maltose Neopentyl Glycol) and TFA (Tandem Facial Amphiphile).

**Figure 4:** Western blot based thermostability assay[40] and corresponding Tm curves of membrane protein transporter (MP-A) obtained using either DDM alone or in presence of calixarenes stabilizing reagents (CALX-R1 and CALX-R2) showing the positive impact on MP-A stability after heating from 25 to 80°C. **~~B~~**~~- Representation of the activity of 7 transmembrane protein MP-D (% activity) over time (days) after solubilization and purification on octyl glucoside (OG) and addition either Calixarene based reagent (CALX), Lauryl Maltose Neopentyl Glycol (LMNG), Amphipol (A835) or Facial Amphiphile 3 (FA-3). ­­~~

**Figure 5:** Roadmap of membrane protein production describing all steps and tools for solubilization, purification and reconstitution. Solubilization can be carried out using detergent or Styrene-maleic acid (SMA) resulting in protein/ lipid/ detergent complex and SMA lipid particles (SMALP), respectively. Proteins can be purified in detergent or within SMALPs. Finally, detergent purified protein can be reconstituted in proteoliposome, nanodisc/ peptidic or amphipol/ protein complexes.

**Figure 6**: Solubilization and purification of two membrane proteins MP-B and MP-C using either conventional detergent (DDM and FC12 for MP-A and MP-B, respectively) or SMA (Styrene-maleic acid co-polymer). Purification was carried out using His-tag affinity chromatography (Cobalt resin) and fractions were analyzed by Coomassie stained SDS-PAGE. T, P, S, FT, W and E correspond to Total, Pellet, Soluble, Flow through, Wash, Elution fractions, respectively.

**Figure 7:** Expression solubilization and enrichment/ purification of native MP target from endogenous (Cancer cells) and recombinant expression (HEK).SDS-PAGE stain free gel showing partially pure/ enriched native MP obtained after differential solubilization from cancer cells (lane 1, indicated by arrowhead) and pure wild-type protein obtained after solubilization and affinity purification from HEK cells (lane 2, indicated by arrowhead).

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