In the cauldron of cell-free synthesis of membrane proteins: playing with new surfactants

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Cell-free protein synthesis is a well-known technique for the roles it has played in deciphering the genetic code and in the beginnings of signal sequence studies. Since then, many efforts have been made to optimise this technique and, recently, to adapt it to membrane protein production with yields compatible with structural investigations. The versatility of the method allows membrane proteins to be obtained directly stabilised in surfactant micelles or inserted in a lipidic environment (proteoliposome, bicelle, and nanodisc) at the end of synthesis. Among the surfactants used, non-detergent ones such as fluorinated surfactants proved to be a good alternative in terms of colloidal stability and preservation of the integrity of membrane proteins, as shown for *Escherichia coli* homo-pentameric channel, MscL (Park *et al.*, *Biochem. J.*, 403: 183–187). Here we report cell-free expression of *Escherichia coli* leader peptidase (a transmembrane protease), *Halobacterium salinarium* bacteriorhodopsin (a transmembrane protein binding a hydrophobic cofactor) and *E. coli* MscL in the presence of non-detergent surfactants, amphipols and fluorinated surfactants in comparison to their expression in classical detergents. The results confirm the potentialities of fluorinated surfactants and, although pointing to limitations in using the first generations amphipols, results are discussed in the light of membrane protein refolding, especially in the case of bacteriorhodopsin. Preliminary experiments using new generations of amphipols supports choices made in developing new molecules.

**Introduction**

Over-expression of membrane proteins (MPs) *in vivo* is notoriously difficult because, unlike soluble proteins, MPs will not fold properly unless targeted to a membrane: large amounts of MPs can either be toxic for the cell or clog the translocation machinery. An alternative strategy is to synthesise the protein in a cell-free system, in the absence of a biological membrane but in the presence of either detergent micelles or liposomes. This strategy has proved to be promising because cell-free synthesis (CFS) of MPs in their functional form has been reported for a large panel of both α-helical bundles and β-barrels, among which are included ion channels [1–3], several G-protein coupled receptors [4–6], and bacteriorhodopsin (BR) [7–10]. The versatility and the open nature of this technique have boosted structural [2,3] and functional [11–13] studies of MPs. The advantage of this approach is to obtain directly solubilised MPs, but it does not abolish a major problem.
encountered in MP biochemistry: time-dependent inactivation of MPs in detergent solution. The requirement to keep MPs stable and functional in solution has led to the development of ‘protein-friendly’ detergents [14–17] and alternative non-detergent surfactants [18,19].

**CFS of MPs in the presence of detergent**

Detergents are used in CFS both to limit soluble protein aggregation and to solubilise the expressed MPs [20,21]. The major difference using CFS compared to in vivo expression is that the detergent molecules are present during protein synthesis. As has been shown from early reports using CFS, not all detergents are compatible with transcription and/or translation. High critical micellar concentration (CMC) detergents, such as octylglucoside (OG), inhibit the expression machinery of cell extracts at concentrations hardly exceeding their CMC. However, many others can be used at concentrations of several times their CMC [5,20,21]. A second important point is the solubility of the produced MP, which will depend on the amount of detergent micelles introduced into the synthesis medium. Most detergents compatible with CFS have a tendency to inhibit protein production with increasing concentration. CFS of MPs in detergent is thus a compromise between a detergent concentration high enough to solubilise the synthesised proteins, but low enough to allow protein synthesis. Mild detergents such as Brij-derivatives seem to be quite well adapted to CFS. These detergents are very mild, as they cannot solubilise biological membranes. This ‘mildness’ could explain why the window of compatible concentrations that can be used for CFS is particularly wide compared to that of other detergents [22].

**Fluorinated surfactants and amphipols**

Some of the ideas that led to the conception of hopefully milder surfactants for the biochemistry of MPs relied on (i) reducing the miscibility of the surfactant and lipids, and (ii) increasing the stiffness of the hydrophobic domain of the molecule. Indeed, hypotheses that can explain the destabilising effect of detergents are their ability to solubilise in the micellar phase stabilising hydrophobic cofactors (e.g. lipids) and their very flexible hydrophobic tail that could destabilise the α-helical bundle by direct intrusion between helices. These requirements can be met by fluorinated surfactants (FS). The chemical structure of FS is similar to that of classical detergents, but their hydrophobic tails contains fluorine atoms. These FS are both hydrophobic and lipophobic [23 and refs. therein]. However, after solubilisation using a classical detergent, they can substitute for the latter to keep MPs watersoluble [24]. To improve interactions with the hydrogenated transmembrane surface of MPs, a hydrogenated tip can be grafted at the end of the fluorinated tail, yielding ‘hemifluorinated’ surfactants (HFS). Our working (H)F-S molecules are (H)F-TAC, bearing a small Tris polymer as a head group [23]. Previous work has shown that three MPs – BR, the transmembrane domain of OmpA and the cytochrome b$_6$f complex – remain soluble when transferred in (H)F-TAC, and in the case of BR and the b$_6$f, are stabilised when stored in (H)F-TAC, as compared to storage in equivalent concentrations of detergent [24,25].

Another approach to limit MP instability in detergents consists in trying to minimise the micellar phase. To this end, a new class of amphiphatic polymers suitable for membrane protein biochemistry was engineered. The first Amphipols (APols) were designed in 1994 [26] and one of them, A8-35, has become the most studied ‘working molecule’ (e.g. [27,28]). APols are composed of a hydrophilic polymeric chain onto which hydrophobic alkyl chains are randomly grafted [26]. Thus, these molecules have such a high affinity for the surface of the protein (multipoint attachment) that even very low amounts of free surfactant allow the protein to remain soluble, the protein-APol association being quasi-irreversible. A membrane protein in such a medium retains its associated lipids, cofactors, and/or subunits and is, therefore, strongly stabilised. Furthermore, the diminished protein–protein interaction and the reduced dynamics of conformational transitions in the helix bundle, may limit aggregation and unfolding phenomena [16]. New APols such as zwiternionic [29], sulfonated (SApol, Dahmane et al., in preparation) or non-ionic [30,31] amphipols have been synthesised to overcome some limitations of A8-35 (precipitation at pH < 7 and in the presence of divalent cations).

Both FS and amphipols have been tested for many applications [18,19]. Here, we test the usefulness of these molecules for CFS using the (H)F-TAC series of (H)FSs, and A8-35 and SAPols as members of the APols family.

**Materials and methods**

**Production and biochemical analysis of recombinant proteins**

*E. coli* Leader peptidase (Lep), *E. coli* MscL, and H. salinarum bacterioopsin (bO) genes were amplified by PCR from chromosomal DNA and subcloned into the pLVEK-2.4b and -2.3 plasmids (Roche). Syntheses were performed with 0.5 µg of plasmid in 50 µl of RTS100 HY (Roche) for 6–9 h incubation at 600 rpm and at 23–30°C. Syntheses of BR were done in the presence or absence of 50–70 µM all-trans-retinal (from a 10 mM stock solution prepared in 100% ethanol). Different surfactants (F-, HF-TAC, F-, HF-Lac, DDM, and Triton X-100) were tested at concentrations ranging from 2 to 5 mM (0.2–0.5% for Triton X-100), and Apos A8-35 and SAPols at concentrations of 0.5–4 mg/ml; the addition of 1 g/l sonicated egg phosphatidylcholin liposomes (from a 30 g/L stock prepared in 10 mM HEPES-KOH pH 7.4, 100 mM KCl) was also tested. After the reaction, samples were diluted twice with 20 mM Tris pH 8 and centrifuged at 16,000 × g for 10 min at 4°C to separate aggregated from soluble protein. Pellet and supernatant were analysed on SDS-Urea 12–18% polyacrylamide gels and detected by immunoblotting using an anti-(His)$_6$-tag antibody.

For preparative experiments of full-length form of leader peptidase, 15 µg of plasmid was incubated for 20 h in 1 ml of the Roche RTS 500 HY lysate. The reaction system and the feeding chamber were supplemented with 0.2% Triton. *In vitro* produced Lep protein was purified in one step using a Ni-NTA column. The extract was incubated 1 h at 4°C with 1 ml of Ni-NTA agarose beads (Qiagen) equilibrated in phosphate saline buffer (PBS) (50 mM Na$_2$HPO$_4$, pH 7.5, 300 mM NaCl, and 0.2% Triton X-100) containing 10 mM imidazole. The column was packed and washed with PBS, 20 mM imidazole and bound protein was eluted with PBS, 250 mM imidazole. After purification, fractions were analyzed on 15% polyacrylamide gels, and concentrations were determined using the bicinchoninic acid method (Pierce Chemical).
When detergents are added, solubilising the membrane, the cleavage site becomes accessible to the catalytic domain, and Lep undergoes intermolecular cleavage [39] (Fig. 1b). After purification, samples were analysed by Synchrotron Radiation Circular Dichroism. Secondary structure percentages in the three protein species are as expected from predictions (Wien and Park, unpublished results).

The BR affair

Another protein amenable to study the feasibility of CFS is BR, the major component of purple membrane [40], which converts light into electrochemical energy. BR consists of a polypeptide folded in seven membrane-spanning $\alpha$-helices (bacterioopsin) covalently bound to a retinal molecule [41]. The correct fold of the protein and binding of its cofactor is witnessed by its purple colour, which is lost when the protein unfolds (free retinal is yellow). It is thus simple to evaluate visually the presence of correctly folded BR after CFS.

The bo gene was synthesised in the absence or presence of a large excess of all-trans-retinal and 2 mM dodecylmaltoside (DDM) or 2–5 mM (H)F-TAC with a (His)$_6$-tag in N-ter (a C-ter His-tag led to less protein expression). In the presence of DDM, the protein production was higher without retinal, but bo was aggregated. When retinal was added, the yield was reduced, but at least part of the protein was correctly folded into BR, as shown by the pink colour of the tube (Fig. 2), and in this case, most of the protein was soluble (not shown). The yield of aggregation and correctly folded BR, as determined by the intensity of the colour of the tube, was however subject to variation from one experiment to the next.

When syntheses were carried out in the presence of (H)F-TAC, both without and with retinal, protein production yields were

![FIGURE 1](image1.png)

**FIGURE 1**
(a) Schematic representation of the transmembrane arrangement of leader peptidase and the SecYEG complex. The catalytic site of the peptidase lies in the periplasm. The scissors indicate the position of the self-cleavage site. (b) In vitro synthesis of full-length leader peptidase. 1: before purification; 2: intermolecularly cleaved full-length form; 3: cleaved form, after purification.

**Results**

**CFS in the presence of FS**

**Leader peptidase**

We tested the synthesis of different domains of a transmembrane protease, the bacterial Leader peptidase (Lep), both in a classical detergent and in FS. Proteins meant to be exported through the bacterial cytoplasmic membrane are generally synthesised as precursors containing a cleavable N-terminal signal sequence which is recognised and cleaved by an inner membrane protease, Lep, which acts jointly with the general secretory machinery. *E. coli* Lep is a 37-kDa MP comprising of two putative transmembrane helices and a periplasmic catalytic domain (Fig. 1a) [32,33]. The choice of this protein was driven by the observation that the structure of the Lep transmembrane domain (tLep) remains unknown [34,35]. It is clear that interfering with this essential protein is fatal for bacterial survival [36] and it thus represents an interesting target for the design of novel antibacterial drugs.

The full-length or different truncated lep genes were fused to a (His)$_6$-tag at the C- and N-terminus, respectively. Three protein lengths were generated corresponding to the tLep (Met1-Pro84), the same domain with part of the linker to the periplasmic domain (tLeplg, Met1-Gly90) and the full-length peptidase. CFS was performed using F-TAC (2–5 mM) or Triton X-100 (0.2%), and the indicated surfactant at the indicated concentrations. Top: the reaction tube after CFS. The pink colour of the tube (Fig. 2), and in this case, most of the protein was soluble (not shown). The yield of aggregation and correctly folded BR, as determined by the intensity of the colour of the tube, was however subject to variation from one experiment to the next.

When syntheses were carried out in the presence of (H)F-TAC, both without and with retinal, protein production yields were
similar to the DDM conditions with retinal, as determined by immunoblot analysis (Fig. 2). However, the yellow colour (or very faint pink) of the tube pointed to a very low yield of native folding of BR. UV–vis spectra of the sample could not be done at this point, due to the scattering of the synthesis medium. The protein synthesised was however colloidally stable, because after 2 weeks storage at 4°C it was still soluble, whereas the protein in DDM was partially aggregated (Fig. 2). (Note that to perform large-scale synthesis that would allow protein purification and thereby a direct and unambiguous estimation of the folding yield, a 1 ml synthesis was performed in 5 mM F-TAC, unfortunately resulting in aggregated protein.) Retinal addition after the synthesis did not improve folding, and all the samples retained the yellow colour of free retinal. Addition of exogenous lipids (1 g/l egg phosphatidylycholine) did not improve folding yields. Synthesis was also performed in the presence of FS with different head groups, the (H)F-Lac family [42], with similar results (not shown).

**CFS in the presence of APols**

CFS of MscL, Lep and BR was achieved in the presence of different concentrations of APols (0.5–4 mg/ml) to establish the best ratio between synthesised proteins and surfactants. However, as shown in Fig. 3, both A8-35 and SAPol seem to inhibit CFS of MPs. To investigate if this inhibition was specific to MPs, we tested the expression of the soluble Green Fluorescent Protein, in the same condition (Fig. 3c). No detectable inhibition occurred, suggesting that inhibition is specific to MPs.

The inhibition was variable for MscL and tLep, but we could not determine the adequate conditions to give reproducible results (Fig. 3b). To trace a rationale for this inhibitory behaviour of APols, we changed some parameters during the synthesis, using a homemade S30 lysate derived from *E. coli* Rosetta (DE3), following the protocol described by Zubay [43], and modified according to Kim *et al.* [44]. PEG-8000 was omitted during the S30 preparation and MP expression was done without or with 2% PEG-8000. We tested also the effect 0.5 mM EDTA before running expression tests, to prevent APol precipitation in the presence of Ca2+. None of these conditions gave any improvement in the production of the different MPs.

**Discussion**

**CFS in the presence of FS**

(H)F-TAC preserve activity and prevent aggregation of membrane proteins such as BR, the cytochrome *b*₅₆₃ or tOmpA [24,25], the yeast ATP-synthase [45], and two membrane receptors (Ptc and Smo) [46]. Moreover, a role as molecular chaperones for pH-dependent insertion of diphtheria toxin in lipid bilayers has been reported [47,48].

The mechanosensitive channel MscL from *E. coli* [49] was the first MP synthesised in the presence of (H)F-TAC. It had activities similar to the ones of MscL synthesised either *in vitro* in Triton X-100 or *in vivo* [50]. We demonstrated that FSs have the prerequisite of being tolerated by the cell-free system even at concentrations exceeding their CMC several folds. Here, we further tested CFS in the presence of FSs of two MPs, leader peptidase and BR, the latter will be discussed below. All three tested forms of Lep were soluble, confirming the ability of F-TAC to accommodate an MP co-translationally. We could not demonstrate the correct folding of the protein, but the protein produced in Triton X-100 shows catalytic activity and its transmembrane domain is organised into α-helices. Furthermore, we have shown that the compatibility for CFS could be extended to other type of FSs such as (H)F-Lac (this work), or (H)F-Diglu [51].

**CFS of BR**

BR is a protein widely used as a model to test different kinds of application for MPs. In particular, it has been used in the 1990s to validate CFS as a tool to label specifically given aminoacids and study the photocycle of refolded BR by Fourier transform InfraRed spectroscopy. The protein, over-expressed without detergent or lipids, was subsequently refolded into *H. salinarum* lipids in the presence of retinal [7,52]. More recently, Kalmbach *et al.* [8] and Shimono *et al.* [9] synthesised photoactive BR in the presence of small liposomes or a variety of lipid/detergent mixtures, respectively, to allow the correct co-translational membrane insertion and folding of the protein. Lastly, a very elegant study has been performed showing that photoactive BR, co-expressed in CFS with an apolipoprotein and in the presence of both lipids and retinal, could be reconstituted into nanolipoprotein particles (nanodisc) [10].

Here, we show that BR could be synthesised without added lipids and in the presence of solubilising amounts of DDM and retinal, and that the resulting product was correctly folded BR, as shown both by the fact that the protein was soluble and by the pink colour of the reaction medium. In itself, this is an interesting result as BR, while very stable in the purple membrane, is much less stable when solubilised in detergent micelles [25]. In particular, we can note that the synthesis temperature is rather high (25–30°C), which sometimes results in the formation of aggregation. In this paper, we verified that the synthesis could be done at lower temperatures, but this would require further investigations.
which is against optimal stability conditions for most MPs solubilised in detergent. Indeed, in our hands, synthesis at lower temperatures (23°C) resulted in higher synthesis yield. When synthesised with DDM but in the absence of retinal, however, bO was not collooidally stable and had a great tendency to precipitate. The addition of retinal after the synthesis did not restore correct folding. This is somewhat surprising, for refolding of BR has been widely studied and documented [53,54]. It suggests that the co-translational folding state trapped in DDM micelles in the absence of retinal is not a folding intermediate that can lead to active BR and that the folding of MPs needs to follow a precise path to lead to a functional protein.

The synthesis of BR in FS was somewhat disappointing, as no significant native folding could be evidenced. This is all the more surprising, as refolding from SDS-denatured purple membrane could be achieved using (H)F-TAC, with yields ranging from 30% to 95% depending on the surfactant concentration (Lebaupain and Breyton, unpublished results). When performed on delipidated bO, however, refolding yields were much lower. Furthermore, a close analysis of refolded BR from SDS-solubilised purple membrane showed that most probably patches of membrane were reconstituted, pointing to the importance of the lipids in the refolding process. Lipids could thus be important for the correct co-translational folding of BR. CFS in DDM could benefit from the lipids present in the CFS lysate; however (H)FS micelles being very poor solvents for lipids, it is not the best system in which to test this hypothesis. Indeed, the addition of phosphatidylcholine to the CFS mixture did not change folding yields. The lack of solubility of the retinal in the fluororinated micelles could explain poor refolding, but we have shown that retinal is solubilised by (H)F-TAC micelles [55].

The reason for the lack of correct folding of BR in CFS in the presence of FSs could be that, during translation, the polypeptide is trapped in a conformation that does not allow a correct rearrangement of the helix bundle around the retinal molecule. The difference between refolding from SDS-denatured protein and co-translational folding could be the kinetics of the transition between unstructured and structured conformation. By analytical ultracentrifugation, we have determined that the denaturation products of BR transferred from OTG to F-TAC have a sedimentation coefficient smaller than that of the monomer in F-TAC (Lebaupain et al., manuscript in preparation). This reflects either a smaller amount of bound surfactant, or more probably, a less compact structure. The structure of bO stabilised in F-TAC micelles during translation in CFS could resemble this expanded structure. Moreover, BR does not turn out to be the best system to test the ability of FSs to allow a correct folding of the protein, as when transferred into F-TAC, it becomes blue and is rather unstable (Lebaupain et al., manuscript in preparation). However, in contrast to CFS in DDM, bO solubilised in (H)F-TAC is remarkably stable from the colloidal point of view.

**CFS in the presence of APols**

Non-detergent amphipathic polymers such as the anionic A8-35 and the zwitterionic sulfonated SAPols are among the most characterised of the APols family. CFS of three model MPs in the presence of these polymers was found to be inhibited to a variable extent. We were unable to pinpoint any mechanism explaining this negative effect, which could account for the non-reproducibility of the results. However, we believe that the inhibition observed is not at the transcription level but more probably at the translational one. Indeed, in the presence of APols, CFS is inhibited for three different MPs, but not for a soluble protein (GFP), which is not supposed to interact with APols. For this reason one can exclude all general mechanisms which would block protein synthesis, for example, Mg²⁺ concentration, rapid depletion of energy sources or accumulation of translation by-products. It seems necessary then to invoke specificity to MPs. One may argue that APols, which have a high affinity for transmembrane domains, would be able to interact with the newly generated polypeptide, thus congesting the synthesis of the entire protein. All the proteins tested in this work are indeed highly hydrophobic and thus tightly bound by APols. Some antibiotics are able to inhibit protein synthesis by blocking the exit route for the nascent peptide [56–58]. One of these is erythromycin, which inhibits elongation of the nascent peptide after two to five residues. The deletion of Met82-Lys83-Arg84 in E. coli L22, a ribosomal protein found on the cytoplasmic side of the 50S ribosomal subunit, confers erythromycin resistance to the cells [59]. Moreover Lys83 and Arg84 are part of a large positive stretch, possibly close to the exit tunnel for the nascent protein [58]. It is difficult at this stage to determine precisely the cause and effect relations, but it seems plausible that the charges conveyed by A8-35 and SAPols may be responsible for a specific interaction with the positive amino acids on the surface of the ribosome. APols bound to the neosynthesised peptides would be able to interact sterically with the positives aminoacids of the L22 protein, thus mimicking erythromycin inhibition. This hypothesis can be reinforced by the evidence that, when employing a new category of non-ionic APols [60], we are able to synthesise *in vitro* a stable form of BR (Bazzacco et al., manuscript in preparation) and several other membrane proteins (Billon-Denis and Zito, manuscript in preparation). In this case, the electrostatic interactions between APols and ribosome are abolished due to the absence of charges in the non-ionics APol.

**Conclusion**

APols yielded disappointing results, because both A8-35 and SAPols were found to inhibit, totally or partially, the transcription/translation machinery of MPs. Nevertheless, we have proven the feasibility of CFS in the presence of neutral APols which, furthermore, seems to boost the synthesis of the tested MPs (Billon-Denis et al., manuscript in preparation). There is no doubt that ‘classical’ APols cannot be engaged in a constructive ménage with MP during their CFS; nevertheless, they constituted the basis for the development of other molecules, which are very promising indeed for the over-expression of MP in CFS.

We have shown that FSs are compatible with CFS and that they are fully competent to stabilise MPs. Previously, we have demonstrated that MscL, in the presence of (H)F-TAC concentrations above the CMC, is synthesised and, once the protein purified, inserts rapidly into preformed liposomes, without the latter being solubilised by the surfactants [50]. We confirm that CFS in the presence of (H)F-TAC leads to the synthesis of other MPs, such as bO and Lep, and that the synthesised proteins remain soluble over a long period of time.
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