Fluorinated and hemifluorinated surfactants as alternatives to detergents for membrane protein cell-free synthesis

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Hemifluorinated and fluorinated surfactants are lipophobic and, as such, non-detergent. Although they do not solubilize biological membranes, they can, after conventional solubilization, substitute for detergents to keep membrane proteins soluble, which generally improves their stability [Breyton, Chabaud, Chaudier, Pucci and Popot (2004) FEBS Lett. **564**, 312–318]. In the present study, we show that (hemi)fluorinated surfactants can be used for *in vitro* synthesis of membrane proteins: they do not interfere with protein synthesis, and they provide a suitable environment for MscL, a pentameric mechanosensitive channel, to fold and oligomerize to its native functional state. Following synthesis, both types of surfactants can be used to deliver MscL directly to pre-formed lipid vesicles. The electrophysiological activity of MscL synthesized *in vitro* in the presence of either hemi- or per-fluorinated surfactant is similar to that of the protein expressed *in vivo*.

Key words: detergent, fluorinated surfactant, hemifluorinated surfactant, membrane delivery, membrane protein, MscL.

INTRODUCTION

Non-ionic surfactants with fluorocarbon hydrophobic tails [FSs (fluorinated surfactants)] (Figure 1) are both highly tensioactive and lipophobic: because they do not interact favourably with the alkyl chains of natural lipids, their partition coefficient into biological membranes is low, which renders them non-cytolytic and inefficient at extracting MPs (membrane proteins) (see, e.g., [1–4]). However, after solubilization using a classical detergent, they can substitute for the latter to keep MPs water-soluble [5]. To improve interactions with the hydrogenated transmembrane surface of MPs, a hydrogenated tip can be grafted at the end of the fluorinated tail, yielding HFSs (hemifluorinated surfactants) [6]. Previous work has shown that two model MPs, bacteriorhodopsin and the cytochrome b_6f complex, are stabilized by transfer to an HFS, HF-TAC {$C_2H_5C_6F_{12}C_2H_4$-*S*-poly[tris(hydroxymethyl)-aminomethane]} (Figure 1), as compared with storage in equivalent concentrations of detergent [7]. This is likely to be due to the fact that (H)FSs are less dissociating than classical detergents. The non-cytolytic character of HFSs has been exploited to reinsert into pre-formed lipid vesicles the water-insoluble low-pH form of the pore-forming fragment of diphtheria toxin without destabilizing the target vesicles [8].

Overexpressing MPs *in vivo* is notoriously difficult because, unlike soluble proteins, MPs will not fold properly unless targeted to a membrane. If insertion into the host membrane is a limiting factor, an alternative strategy is to synthesize the protein in a cell-free system, in the absence of membrane but in the presence of surfactant. Cell-free synthesis of MPs under their functional form has recently been reported for EmrE [9,10], MscL [11], a bacterial light-harvesting complex [12], several G-protein-coupled receptors [13,14] and Tsx, an MP of the β-barrel type [14]. Most MPs are unstable in detergent solutions, however, which indicates that their native fold does not, in this environment, lie at their free energy minimum. It is therefore of great interest to examine whether milder surfactants such as (H)FSs are compatible with cell-free synthesis and folding. As a model MP, we have used the mechanosensitive channel MscL from *Escherichia coli*, a pentameric protein whose protomer (14 kDa) comprises two transmembrane helices [15]. We have shown previously that MscL can be produced *in vitro* in the presence of various detergents [11]. After purification and reconstitution, the functionality of the protein can be assessed at the single-molecule level by patch–clamp measurements. In the present study, we have established that (H)FSs are compatible with *in vitro* synthesis, folding and oligomerization of MscL. In addition, we have shown that they allow direct and rapid incorporation of functional MscL into pre-formed lipid bilayers.

EXPERIMENTAL

Organic synthesis

Two forms of F-TAC, bearing either a C_6F_{13}- (C_6F-TAC {$C_6F_{13}C_2H_4$-*S*-poly[tris(hydroxymethyl)aminomethane]}) or a C_8F_{17}- (C_8F-TAC {$C_8F_{17}C_2H_4$-*S*-poly[tris(hydroxymethyl)aminomethane]}) fluorinated chain, were synthesized by radical polymerization in the presence of fluoroalkyl mercaptan as a transfer reagent [1]. The synthesis of HF-TAC followed the same chemical pathway, using a hemifluorinated mercaptan as transfer reagent [6]. Stock solutions (100 mM in water) were kept at 4 °C. Chemical structures, molecular masses and CMCs (critical micellar concentrations) are given in Figure 1.

In vitro protein synthesis

Cell-free expression was carried out using the Roche system [16], in which an *E. coli* lysate provides the machinery to drive coupled

Abbreviations used: CMC, critical micellar concentration; FS, fluorinated surfactant; HFS, hemifluorinated surfactant; HF-TAC, $C_2H_5C_6F_{12}C_2H_4$-*S*-poly[tris(hydroxymethyl)-aminomethane]; C_6F-TAC, $C_6F_{13}C_2H_4$-*S*-poly[tris(hydroxymethyl)-aminomethane]; C_8F-TAC, $C_8F_{17}C_2H_4$-*S*-poly[tris(hydroxymethyl)aminomethane]; MP, membrane protein; Ni-NTA, Ni^{2+}-nitrilotriacetate.

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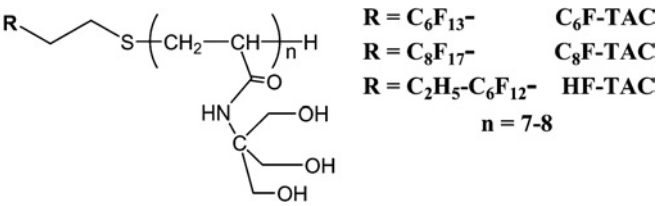

Figure 1 Structures of HF-TAC, C_6F-TAC and C_8F-TAC

Chemical structures of HF-TAC, ($n \approx 8$, molecular mass $\approx$ 1790 Da, CMC = 0.45 mM), C_6F-TAC ($n \approx 7$, molecular mass $\approx$ 1605 Da, CMC = 0.3 mM) and C_8F-TAC ($n \approx 7$, molecular mass $\approx$ 1705 Da, CMC = 0.03 mM).

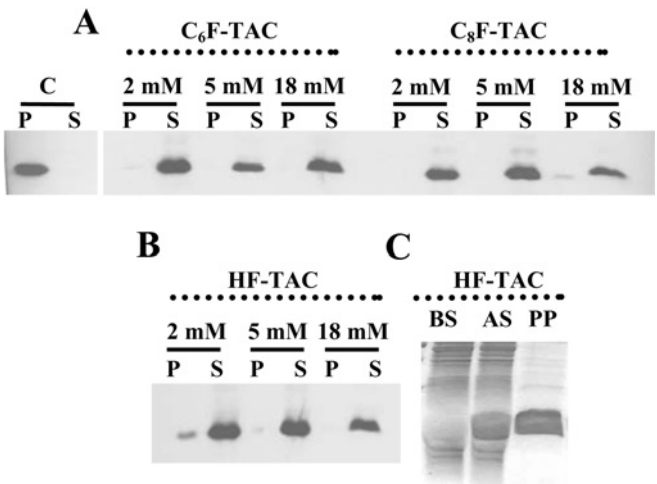

Figure 2 *In vitro* synthesis and purification of histidine-tagged MscL

(**A** and **B**) Small-scale synthesis was carried out in the absence of surfactants (C, control) and in the presence of C_6F-TAC, C_8F-TAC or HF-TAC at the concentrations indicated. At the end of the synthesis, samples were centrifuged at 16000 ***g*** for 10 min and the pellet (P) and supernatant (S) were analysed by 8 M urea SDS/15 % PAGE. MscL was detected by immunoblotting using an anti-(His_6 tag) serum. (**C**) One-step purification of radiolabelled MscL synthesized *in vitro* in the presence of 2 mM HF-TAC. BS, total extract before synthesis; AS, total extract after synthesis; PP, purified protein after purification on a Ni-NTA column in the presence of 2 mM HF-TAC. Samples were analysed by SDS/15 % PAGE and stained with Coomassie Blue.

transcription and translation in the presence of a DNA template. For large-scale production, a semi-permeable membrane allows for a continuous supply of substrates and the removal of inhibitory by-products, thus extending the duration of expression and the protein synthesis yield [17].

For a small-scale test (50 μl), 10 μg/ml plasmid carrying the *MscL* coding region [11] was incubated for 6 h in Roche RTS 100 HY (High Yield) lysate with HF-TAC, C_6F-TAC or C_8F-TAC at the concentrations indicated in Figure 2(A).

When the RTS 500 HY lysate (1 ml) was used for preparative experiments, 15 μg/ml plasmid was incubated for 20 h in Roche RTS 500 HY lysate. The reaction system was supplemented with either 2 mM HF-TAC or 4 mM F-TAC. Surfactants were added at the same concentration in the feeding chamber. Radiolabelling of MscL was achieved by addition of 0.4 and 2.2 MBq (2.8 TBq/mmol) of L-[*methyl*-^{3}H]methionine (Amersham Biosciences) in the reaction and feeding chamber respectively.

All syntheses were carried out according to the manufacturer's protocols.

Biochemical analysis and protein purification

At the end of the small-scale tests, 10 μl samples were diluted with the same volume of 5 mM Tris/HCl buffer, pH 8.0, then were centrifuged at 16000 ***g*** for 10 min at room temperature (25 °C) to check for protein solubility. For Western blotting, proteins were separated by 8 M urea SDS/15 % PAGE. The tagged protein was detected by immunoblotting, using an antibody against the His_6 tag (Sigma).

Following large-scale synthesis, *in vitro* produced MscL was purified as described in [11], in the presence of 2 mM (H)FSs. The purified protein samples were separated by SDS/15 % PAGE. Concentrations were determined using the bicinchoninic acid method (Pierce Chemicals). Purified samples were kept at 4 °C until reconstitution into liposomes.

Integration of MscL into liposomes

Purified radiolabelled MscL was added to a suspension of sonicated liposomes (1 mg of asolectin from soybean, type IV-S; Sigma) in 10 mM Hepes/KOH, pH 7.4, and 100 mM KCl (liposome suspension buffer), to achieve a lipid/protein ratio of 40:1 (w/w). The suspension of liposomes contained (H)FSs at the same concentration as the MscL solution. Either immediately or after 30 min of incubation at room temperature, the mixture was centrifuged at 300000 ***g*** for 20 min and the radioactivity associated with the pellet and the supernatant was counted. Control experiments were performed in the same way but in the absence of liposomes.

For flotation experiments, liposomes were incubated with MscL in HF-TAC. The mixture was adjusted to 1.5 M sucrose (final volume 400 μl), overlaid with 330 μl of 0.3 M sucrose in suspension buffer and topped with 100 μl of sucrose-free buffer. Following centrifugation at 90000 rev./min for 150 min using a TLA-100.2 rotor of a TL100 centrifuge (Beckman), five fractions (100 μl, 350 μl and three of $\sim$120 μl) were collected from the top, the last fraction being used to resuspend any eventual pellet, and the radioactivity present in each fraction was counted.

Formation of giant proteoliposomes

Proteoliposomes were prepared as described in the previous section, except that the suspension was diluted in 3 ml of liposome suspension buffer before ultracentrifugation. The pellet was washed in 3 ml of the same buffer, and then resuspended in 20 μl of 10 mM Hepes/KOH, pH 7.4. To obtain giant proteoliposomes amenable to patch–clamp measurements, this suspension was subjected to a dehydration/rehydration cycle as described previously [18].

Patch–clamp measurements

Single-channel activity was recorded using standard patch–clamp methods [19]. Patch electrodes were pulled from Pyrex capillaries (Corning code 7740) using a P-2000 laser pipette-puller (Sutter Instruments); they were not fire-polished before use. Micropipettes were filled with a buffer similar to that of the patch–clamp chamber, supplemented with 5 mM $MgCl_2$ and 2 mM $CaCl_2$. Negative pressure (suction) was applied to the patch pipette by a syringe and monitored with a piezo-electric pressure transducer (Bioblock Scientific). Unitary currents were recorded using a Biologic RK-300 patch–clamp amplifier with a 10 GΩ feedback resistance. Records were subsequently filtered at 1 kHz ($-$3 dB point) through a four-pole Bessel low-pass filter, digitized off-line at a rate of 2 kHz, and analysed on a personal computer using Pclamp (Axon). Data were plotted using Sigmaplot software (Jandel).

RESULTS

Cell-free synthesis of MscL in HFSs and FSs

Previous experiments have shown that it is possible to express MscL in a cell-free system in the presence of various detergents, in the absence of membranes [11]. In the present study, we performed *in vitro* synthesis of MscL in the presence of (H)FSs [7,20,21]. Small-scale syntheses were carried out in the presence of C_6F-TAC, C_8F-TAC or HF-TAC at 2, 5 or 18 mM, which corresponds respectively to ~6, ~15 and ~60 times the CMC of C_6F-TAC, ~60, ~150 and ~600 times that of C_8F-TAC, and ~2, ~10 and ~40 times that of HF-TAC (Figures 2A and 2B). For the two fluorinated surfactants, we additionally performed syntheses at 50 mM FS, i.e. ~150 times (C_6F-TAC) and ~1500 times (C_8F-TAC) their CMC (results not shown). All conditions were found to be compatible with *in vitro* synthesis. In all cases, the protein produced was soluble (Figures 2A and 2B). In contrast, when 1 mM HF-TAC was used, part of the protein was found in the pellet after centrifugation, indicating that this concentration of surfactant was not sufficient to maintain the protein in a soluble form (results not shown).

To obtain large amounts of purified protein, we scaled up the synthesis using the RTS 500 HY lysate. Large-scale synthesis of MscL was carried out in the presence of 2 mM HF-TAC (Figure 2C), 4 mM C_6F-TAC or 4 mM C_8F-TAC. The protein was radiolabelled using L-[*methyl*-^{3}H]methionine in order to facilitate monitoring of MscL incorporation into lipid vesicles. Syntheses were performed for 20 h at 30 °C and the protein was purified on a Ni-NTA (Ni^{2+}-nitrilotriacetate) column (Figure 2C). The yield of all three large-scale syntheses was ~1 mg of pure protein per ml of lysate, a value comparable with that obtained in the presence of 0.2 % (v/v) Triton X-100 [11]. The oligomeric state of MscL synthesized in the presence of (H)FSs was examined as described in [11]. The protein was found to form native-like oligomers (results not shown).

Insertion of MscL into liposomes

Insertion of MscL into pre-formed liposomes was performed in the presence of 1 mM HF-TAC, 3 mM C_6F-TAC or 3 mM C_8F-TAC. In keeping with previous observations [8], light-diffusion measurements at 430 nm indicate that liposomes are not solubilized under these conditions, even though all three surfactants are present above their CMC. Purified radiolabelled MscL, synthesized in the presence of either HF-TAC or C_6F-TAC, was added to a 10 mM Hepes/KOH, pH 7.4, and 100 mM KCl buffer containing asolectin liposomes, and either surfactant at the above concentration. Aliquots of the suspension were centrifuged either immediately or 30 min after addition of the protein, and the amount of [^{3}H]methionine present in the pellet and supernatant was determined. Most of it (~95 % for HF-TAC, ~90 % for C_6F-TAC) was found in the liposome pellet of the aliquot taken immediately after addition of the protein. The distribution observed after 30 min of incubation was not significantly different. Incorporation of MscL into liposomes therefore is faster than the time (20 min) required for centrifugation. All of the protein remained in the supernatant when the centrifugation was performed under similar conditions, but in the absence of liposomes, showing the ability of all tested surfactants to maintain the protein in solution at the concentrations used.

To rule out the possibility that, during the insertion experiment, part of the radiolabelled proteins aggregated and subsequently pelleted with the liposome fraction, we performed flotation experiments with liposomes incubated with MscL in HF-TAC (see the Experimental section). Liposomes floated to the top of the 1.5 M sucrose fraction. Fractions were collected and counted for radioactivity. In two separate experiments, 90 % and 85 % of the labelled MscL were recovered with the 0.3 M sucrose fraction (results not shown). The radioactivity present in the last fraction, including any eventual pellet, was less than 4 %.

MscL synthesized *in vitro* in the presence of HFS or FS forms active channels once integrated into giant lipid vesicles

For functional assays, MscL was integrated into asolectin vesicles at a lipid/protein ratio of 40:1 (w/w) and the suspension was washed free of surfactants by two rounds of ultracentrifugation. Giant proteoliposomes were generated by vesicle fusion, induced by a cycle of dehydration/rehydration. The resulting structures were assayed using the patch–clamp method. After seal formation in the micropipette, suction was applied to the membrane patch to induce opening of mechanosensitive channels, at a membrane voltage of +10 mV. Mechanosensitive channels similar to the native ones were detected in all three preparations, opening under the applied pressure and closing upon its release (Figure 3A).

The mechanosensitive activity was studied in detail so as to determine whether the use of (H)FSs is compatible with functional *in vitro* expression of MscL. The average conductance of the channels was found to be 1.52 ± 0.06 nS (HF-TAC), 1.44 ± 0.06 nS (C_6F-TAC) or 1.55 ± 0.05 nS (C_8F-TAC), the number of active channels per patch was 6.0 ± 1.4, 4.3 ± 1.3 and 5.2 ± 1.2 respectively (Table 1, N_{obs}). These values are close to those observed for the same protein expressed *in vivo* and analysed under identical buffer and voltage conditions (1.5 nS of unitary conductance and 9.3 ± 5.5 channels per patch, $n = 26$; [11]). For each surfactant, up to 20 cumulated openings were observed in a single membrane patch. MscL channel is activated by membrane tension (T) rather than pressure (p). The channel dependence on pressure, $P_o(p)$, was therefore converted into dependence on tension, $P_o(T)$, according to a method used previously by Sukharev et al. [22]. Seven $P_o(p)$ curves were obtained for HF-TAC, five for C_6F-TAC and five for C_8F-TAC. For each (H)FS, $P_o(T)$ data sets were either fitted individually (Table 1), or cumulated (Figure 3B), with a fixed half-maximal tension of 11.8 dyn/cm. Average values of N_{max}, calculated from individual $P_o(T)$ curve fittings, are 7.1 ± 2.4 ($n = 7$), 10.0 ± 4.4 ($n = 5$) and 8.0 ± 1.3 ($n = 5$) channels per patch for channels synthesized in HF-TAC, C_6F-TAC and C_8F-TAC respectively. Their respective sensitivity to tension ($1/\beta$) was 0.63 ± 0.13, 0.53 ± 0.20 and 0.68 ± 0.06 dyn/cm (see Table 1), which is similar to that of native MscL channel (0.63 dyn/cm; [22]).

DISCUSSION

Perfluorocarbons are strongly hydrophobic without being lipophilic. As a result, compounds carrying a polar headgroup attached to a fluorinated chain, while they are very strong surfactants, are not detergents, owing to their poor miscibility with lipids (see, e.g., [2,3]). Presumably because they do not compete effectively with the protein–protein and protein–lipid interactions that stabilize the native fold of MPs, FSs and HFSs can keep MPs water-soluble under particularly mild conditions [5,7].

In the present study, we show that FSs and HFSs (i) are compatible with *in vitro* protein synthesis, and (ii) provide an environment that allows efficient solubilization, folding and assembly of a pentameric MscL. In parallel experiments, we have observed that, in addition to MscL, other MPs can also be synthesized *in vitro* in the presence of FSs or HFSs (K.-H. Park, unpublished work). At variance with detergents, (H)FSs do not interfere with protein synthesis up to 50 times their CMC, suggesting that

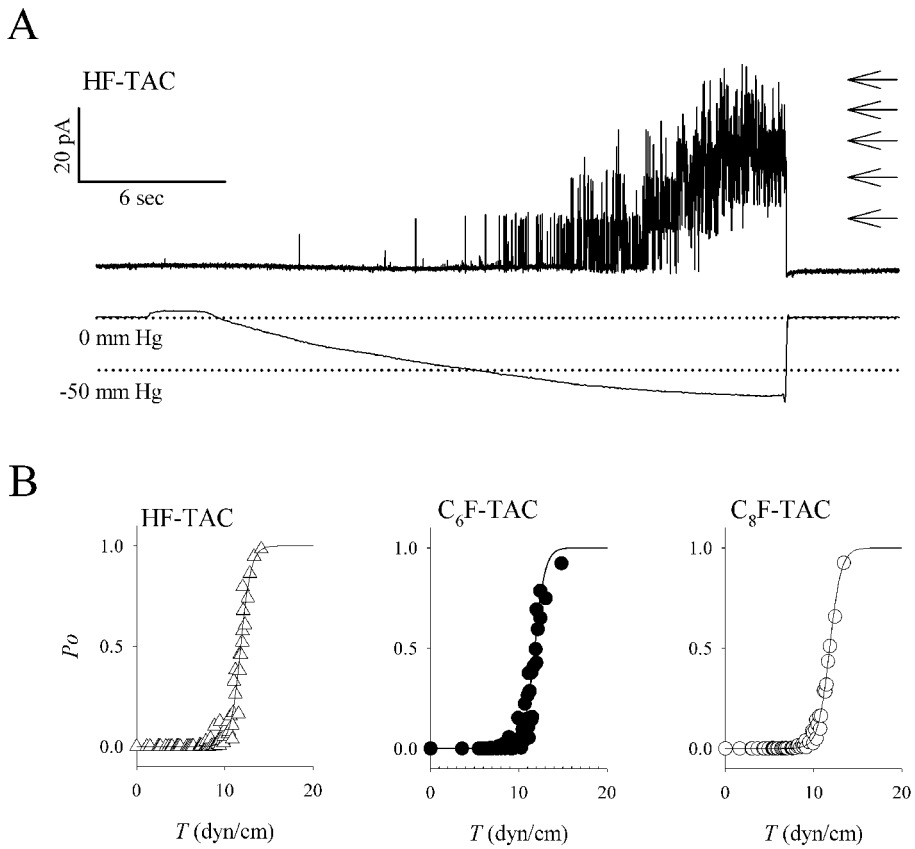

Figure 3 Functional properties of *in vitro* synthesized MscL

Patch–clamp experiments were performed on giant proteoliposomes in the inside-out excised configuration. (**A**) The upper trace shows the current and the lower trace the pressure applied to a membrane patch containing MscL synthesized *in vitro* in the presence of HF-TAC. Time and current scales are indicated by small bars; arrows indicate the cumulative fully opened levels of channels. (**B**) Sensitivity to tension of MscL synthesized *in vitro*. Channel activity was recorded at different levels of suction. The open probability was calculated for each level, on a 20 s recording segment, and plotted against the applied pressure. Data were first fitted with a Boltzman distribution of the form $P_o = [1 + \exp \alpha \cdot (p_{1/2} - p)]^{-1}$, where P_o is the open probability, p is the applied pressure, $p_{1/2}$ is the pressure at which P_o is 0.5, and $1/\alpha$ is the sensitivity to pressure. Each $P_o(p)$ curve was converted into a $P_o(T)$ curve by rescaling to a midpoint $T_{1/2}$ of 11.81 dyn/cm, and fitted to the equation $P_o = [1 + \exp \beta \cdot (T_{1/2} - T)]^{-1}$, where $1/\beta$ is the sensitivity to tension and T is the tension. Left to right, $P_o(T)$ curves were fitted by a single-fitting curve from seven, five and five recordings for HF-TAC (△), for C_6F-TAC (●) and for C_8F-TAC (○). The corresponding sensitivity factor $1/\beta$ is 0.59, 0.59 and 0.65 dyn/cm for HF-TAC, C_6F-TAC and C_8F-TAC respectively.

Table 1 Properties of reconstituted MscL after synthesis in the presence of each surfactant

The number of active channels per membrane patch, N_{obs}, and the slope factor or sensitivity to tension, $1/\beta$, are presented as means ± S.E.M. The values of $1/\beta$ were calculated from individual fitting curves, as indicated in the legend to Figure 3. For data regarding MscL synthesized *in vivo*, see [11].

Surfactant	N_{obs}	$1/\beta$ (dyn/cm)
HF-TAC	6.0 ± 1.4 ($n = 24$)	0.63 ± 0.13 ($n = 7$)
C_6F-TAC	4.3 ± 1.3 ($n = 20$)	0.53 ± 0.20 ($n = 5$)
C_8F-TAC	5.2 ± 1.2 ($n = 10$)	0.68 ± 0.06 ($n = 5$)

these surfactants interact weakly if at all with the transcription–translation machineries. Following integration into lipid vesicles, MscL synthesized *in vitro* in the presence of (H)FSs presented similar properties as native MscL, in terms of mechanosensitivity and number of channels formed per patch: this implies correct folding and oligomerization, in keeping with biochemical observations. (H)FSs therefore appear as a very interesting alternative for the cell-free production of those MPs that will not achieve their native fold when synthesized in the presence of classical detergents.

Another interesting result of this study, which is relevant to membrane biochemistry and cell biology applications of (H)FSs, is the demonstration that MscL, in the presence of (H)FS concentrations above the CMC, can insert rapidly into pre-formed liposomes, without the latter being solubilized by the surfactants. This behaviour, which is in keeping with previous observations with the pore-forming fragment of diphtheria toxin [8], is consistent with the hydrophobic regions of MPs having a higher affinity for the lipids than for (H)FSs. Insertion without lysis of the target membrane is difficult to control using classical detergents. In this case, indeed, if the concentration of free detergent falls below the CMC, the layer of MP-associated detergent disassembles, which leads to protein aggregation, whereas detergent concentrations above the CMC, which keep MPs soluble, are lytic. F-TAC and HF-TAC can be applied, above their CMC, to cultures of mouse embryonic stem cells or fibroblasts without killing the cells (F. Lebaupain, K. Mesbah and J. Barra, unpublished work). Using (H)FSs, it should therefore be possible to deliver MPs directly not only to artificial lipid bilayers, but also to the plasma membrane of living cells.

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