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A diglucosylated fluorinated surfactant to handle integral membrane proteins in aqueous solution

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ABSTRACT

The use of fluorinated surfactants as efficient tools for handling membrane proteins in aqueous media was demonstrated many years ago. The work reported herein deals with the synthesis of a new sugar-based surfactant bearing two glucose moieties and labelled F_6-DigluM. The synthesis of F_6-DigluM is based on a one-pot reduction/alkylation of a fluorinated thioacetate onto an acrylamido-type polar head precursor, using $NaBH_4$ in refluxing methanol. Its physical–chemical properties in aqueous solution were studied by surface tension measurement, dynamic light scattering and analytical ultracentrifugation. F_6-DigluM exhibits a critical micellar concentration of ~0.4 mM and self-assembles into small and well-defined aggregates, very likely to globular micelles. Finally, the homogeneity and the stability of complexes of bacteriorhodopsin and F_6-DigluM over time were observed, demonstrating that F_6-DigluM is a suitable tool for biochemical applications.

1. Introduction

Membrane proteins (MPs), which represent 20–30% of the proteins encoded by the genome, perform a wide range of essential and vital cellular functions and have therefore a substantial physiological and biomedical importance. Thus solving their structure to understand the molecular details of their mode of action constitutes a major issue in basic life sciences as well as public health care. MPs need to be extracted from their original membrane to be purified and studied. Such a procedure is carried out using surface active molecules, called detergents, which are able to solubilise biological membranes [1,2]. In other words, detergents need to efficiently compete with lipid–lipid and lipid–protein interactions in order to disrupt membranes and to disperse its components under the form of detergent-solubilised particles. Unfortunately, whereas most of MPs are reasonably stable in their natural environment, they tend to become unstable once transferred to detergent solutions. Indeed, the dissociating character of detergents is difficult to control and frequently provokes an irreversible inactivation of the protein [3–5]. This denaturation appears to originate from either the interference of the flexible hydrophobic tail of the detergent into the structure of the protein itself or the solubilisation of the lipids and stabilizing hydrophobic co-factors of the protein in the detergent micelles. Therefore, it is of increasing interest to generate amphiphilic tools able to replace detergents. A variety of innovative tools have been developed to go beyond the threshold of stability that can be achieved using classical detergents [6]. Among them, one can cite surfactants with stiffer hydrophobic tails as well as milder amphiphilic molecules such as amphipathic peptides [7], tripod amphiphiles [8], whose hydrophobic moiety is made up of two short hydrocarbon segments and one cyclic moiety, amphiphilic polymers (APols) [9–11] and fluorinated surfactants (FSs) [12–14].

FSs have the same general structure as classical detergents, i.e. a hydrophilic head group and a hydrophobic tail, but the latter, rather than being a fully hydrogenated aliphatic chain, comprises fluorine atoms. They were designed based on the fact that alkanes and perfluorinated alkanes, while both hydrophobic, are poorly miscible [15–17]. Therefore, surfactants with fluorinated alkyl chains are expected to be less aggressive towards membrane proteins than detergents are, basically because of these two

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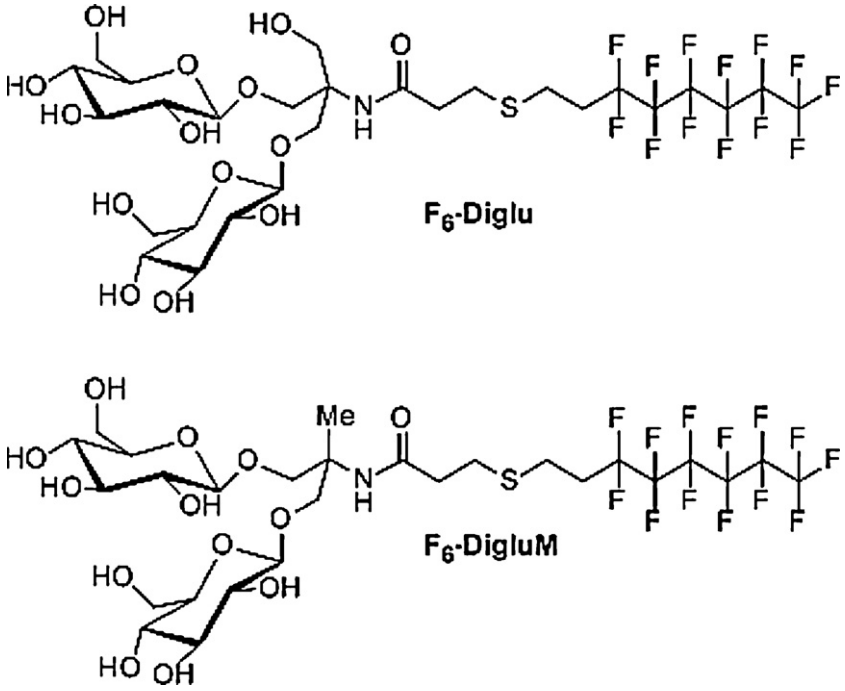


Fig. 1. Chemical structures of F_6-Diglu and F_6-DigluM.

features: (i) their micellar phase is a poor solvent for lipids and other stabilizing hydrophobic cofactors; (ii) their hydrophobic moieties, being bulkier and more rigid than their hydrogenated counterparts and having little affinity for the hydrogenated surfaces of transmembrane protein segments, compete less efficiently with protein/protein interactions and intrude less easily into the protein structure itself. For these reasons, fluorinated surfactants do not partition well into biological membranes, have little cytolytic effect [18], and were considered as potential tools for handling MPs in aqueous solution. The interest of using fluorinated surfactants was demonstrated with surfactants bearing a polymeric hydrophilic head derived from Tris-(hydroxymethyl)aminomethane [14,15,17,19] (THAM), F-TAC. We confirmed that fluorinated surfactants could not solubilise a biological membrane, but very fragile membrane proteins (the cytochrome b_6f or bacteriorhodopsin (BR), for instance) purified in detergent and transferred in F-TAC solutions, remained soluble in their native form and enzymatically active [12–14]. FSs have also been successfully used in biochemistry for in vitro synthesis of MPs [20] as well as for their insertion into pre-existing lipid bilayers [21]. To improve their interactions with the transmembrane surface of MPs, a hydrogenated tip was grafted at the end of the fluorinated tail, yielding a novel series of surfactants, the so-called 'hemifluorinated' surfactants (HF-TAC) [13,22]. However, the synthesis of the polar head of HF-TAC involves radical polymerisation, and leads therefore to inevitable polydispersity and batch-to-batch variations. We thus investigated the potentialities of hemifluorinated surfactants bearing chemically defined polar heads such as aminoxide groups [23], lactose [24], maltose [25], or a polyglucosylated moiety [26]. In the latter series, the F_6-Diglu derivative, where "F_6" corresponds to the six fluorinated carbons within the hydrophobic tail and "Diglu" for the two glucose grafted moieties (Fig. 1) was found to self-assemble into small and homogeneous well-defined spherical micelles (5–6 nm), as it is required in the biochemistry of membrane proteins.

F_6-Diglu formed stable and homogeneous complexes in presence of two MPs (bacteriorhodopsin and cytochrome b_6f) [27]. This has proved that diglucosylated polar head fluorinated surfactants constitute a very promising class of surfactants that could be used as an alternative to classical detergents. Although the preparation of F_6-Diglu is considered uncomplicated and can be achieved in a satisfactory overall yield, the preparation of its diglucosylated polar head requires multi-step syntheses which could be limiting for large scale synthesis. This led us to search for a new compound that enables us to easily graft two glucose moieties and improves thereby the experimental procedure and overall yield. In this aim, our choice went to the use of the 2-amino-2-methylpropane-1,3-diol commercially available. The work reported herein deals with the synthesis of a new diglucosylated fluorinated surfactant, called F_6-DigluM, which can be easily prepared in only five steps from 2-amino-2-methylpropane-1,3-diol and 1*H*,1*H*,2*H*,2*H*-tridecafluoro-1-iodooctane as commercially available starting materials. Its physical–chemical properties in aqueous solution were studied by surface tension measurement, dynamic light scattering and sedimentation velocity. The hydrophobicity of F_6-DigluM was measured by reversed-phase chromatography and the homogeneity and stability of complexes of bacteriorodopsin, a very fragile MP, in F_6-DigluM over time were studied.

2. Results and discussion

2.1. Synthesis

The retrosynthetic approach for the elaboration of F_6-DigluM consists of three key steps (Fig. 2): (1) synthesis of an acrylamido-type diglucosylated polar head derived from 2-amino-2-methyl-1,3-propandiol, (2) synthesis of a fluorinated thiol from the commercially available 1*H*,1*H*,2*H*,2*H*-tridecafluoro-1-iodooctane, and finally (3) formation of the thioether bond leading to F_6-DigluM.

2.1.1. Synthesis of the polar head group

The preparation of the glucosylated derivative is shown in Scheme 1. Condensation of commercially available acrylic acid and 2-amino-2-methyl-1,3-propandiol using EEDQ [28] in ethanol

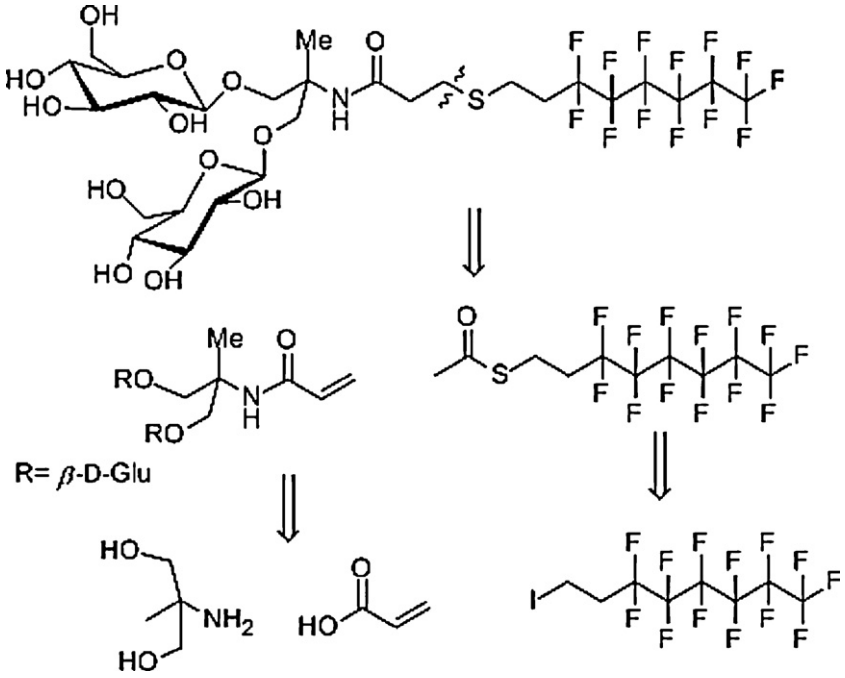


Fig. 2. Retrosynthetic pathway for the fluorinated surfactant F_6-DigluM.

under reflux led to compound **1** in satisfactory yield (56%). Diglucosylation of compound **1** following Helferich's method was next performed under ultrasound activation [29,30] as previously reported [26,31]. It is worth to mention that ultrasound activation is a versatile activation method used for many chemical reactions including carbohydrate synthesis [32]. Thereby, under ultrasound activation, the glucosylation of compound **1** using an excess of acetobromoglucose and mercury cyanide was almost immediate and afforded compound **2** in satisfactory yield (67%). Finally, removal of the acetyl protective groups was carried out under the Zemplén [33] conditions and led to compound **3** in 91% yield.

2.1.2. Synthesis of the fluorinated thiol

The synthesis of the fluorocarbon chain bearing a thiol group consists of the conversion of the commercially available 1*H*,1*H*,2*H*,2*H*-tridecafluoro-1-iodooctane into its corresponding thioacetate **4** (Scheme 1). The introduction of a thioacetate group is usually done by reaction of potassium acetate [34] with a monoiodide compound. Since thiols are known for their vulnerability towards oxygen (formation of stable and unreacting sulfoxide even at low concentration of O_2), the presence of the acetyl as protecting group for the thiol was also targeted. Therefore, monoiodide compound was reacted with potassium thioacetate (1.5 equiv.) in anhydrous DMF and led to compound **4** (75%) following a similar strategy as that previously reported [26].

2.1.3. Thioether bond formation

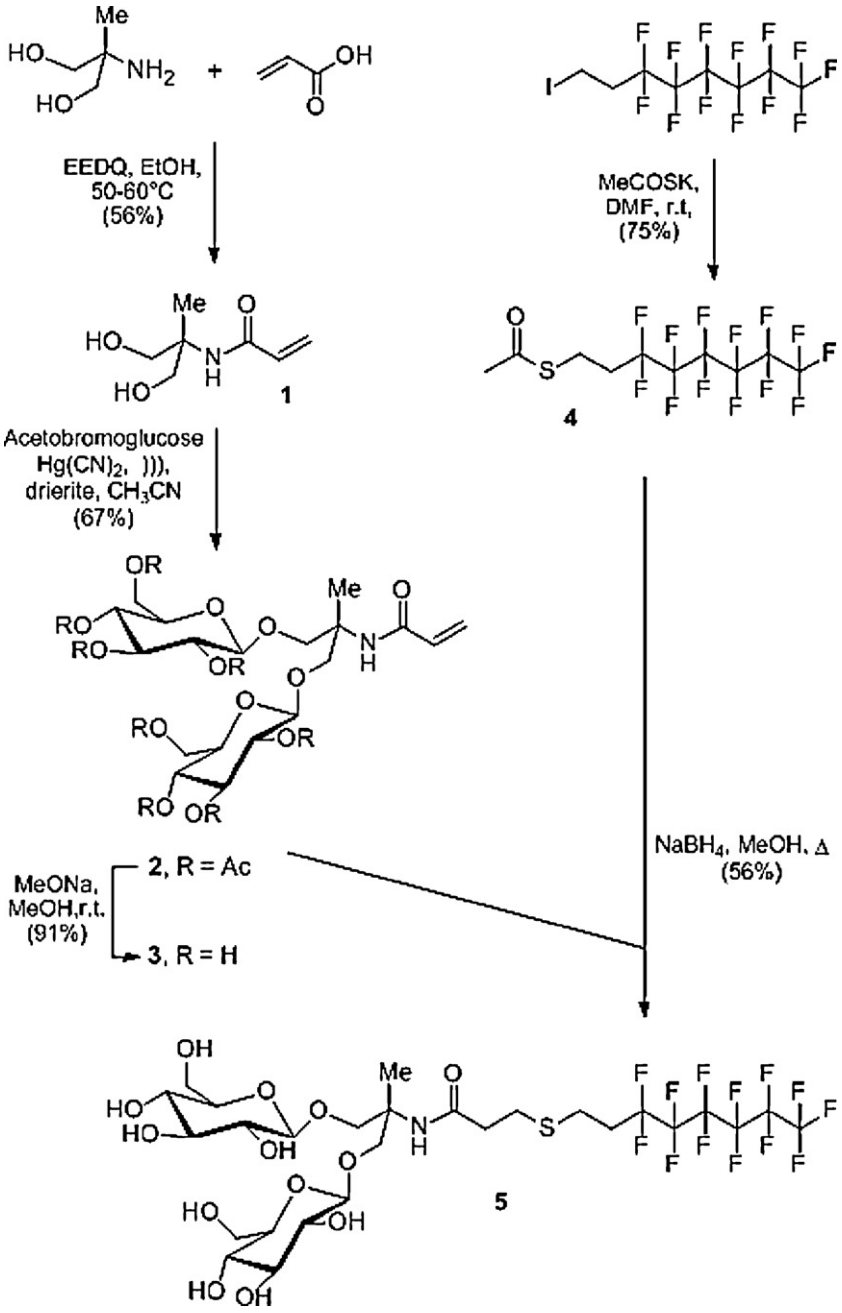
Taking into consideration the Michael acceptor character of the acrylamido group, the connection of the fluorinated thiol to the glucosylated derivative, following a Michael addition, represents the final step in the synthesis of F_6-DigluM. This was achieved under a very mild one-pot reduction/alkylation method using $NaBH_4$ in refluxed methanol (Scheme 1) in which the transformation of the 1*H*,1*H*,2*H*,2*H*-perfluorooctylthioacetate into its thiol was completed *in situ*. The ability of the $NaBH_4$/methanol couple [26], to afford a thioether directly from thioacetate compound, in a very good yield, was pointed out in a previous work [26]. Indeed, performing Michael reaction generally requires the presence of a base, which could be, as stated above, harsh with fluorinated compounds. The methanol in the presence of the reducing agent $NaBH_4$ is capable of transesterification of the thioacetate group, yielding consequently the corresponding thiol *in situ*, which is then converted to its thiolate due to the excess of $NaBH_4$. Following this method, thioacetate **4** was reacted with compound **3** in the presence of 1.5 equiv. of $NaBH_4$ in refluxed methanol, and afforded the final compound **5** in a satisfactory yield (56%). Compound **5** was purified by Sephadex LH-20 size exclusion chromatography and by C18 reversed-phase HPLC and then lyophilized to give the pure surfactant F_6-DigluM as a white powder. F_6-DigluM was fully characterized by ^1H, ^{13}C, ^{19}F, DEPT sequences and HMQC NMR experiments as well as high-resolution mass spectrometry, allowing the observation of characteristic adducts. Finally, the purity of the sample was checked by RP-HPLC and was higher than 98%.

2.2. Physical–chemical characterization

In order to study the physical–chemical properties of F_6-DigluM, three different parameters were considered: (1) its surface tension activity which was determined by the Wilhelmy plate technique, (2) its overall hydrophobic character ($\log k'_W$) which was measured by reversed-phase HPLC, and finally (3) the size of its supra-molecular assemblies in aqueous media; this was studied by dynamic light scattering (DLS) and analytical ultracentrifugation (AUC).

2.2.1. Hydrophobic character ($\log k'_W$)

This parameter is closely related to the molecule water/octanol partition coefficient and has been used to reflect the hydrophobic character of molecules [26,35,36]. It can be easily obtained from reversed-phase HPLC and the value for F_6-DigluM was found to be 5.1 (Table 1). As shown in Table 1, F_6-Diglu and F_6-DigluM exhibit similar $\log k'_W$ values. However, F_6-DigluM is slightly more hydrophobic than its former analogue due to the presence of a methyl group instead of a hydroxymethyl group in the polar head of the former surfactant; evidence for this comes



Scheme 1. Synthesis of the fluorinated surfactant F_6-DigluM.

from the Hansch [37] partition constant water/octanol, with π values of 0.52 and −1.30 for CH_3 and CH_2OH group, respectively. Moreover, our present results suggest, in agreement with our previous work, that the volume and the nature of the polar group have only a relatively limited impact on the $\log k'_W$ parameter [26,35].

2.2.2. Surface tension activity

The surface tension activity data, measured by the Wilhelmy plate method, are summarized in Table 1 and the curve of F_6-DigluM is presented in Fig. 3. From this technique, different parameters can be determined: (a) the critical micellar concentration (CMC), (b) the limit surface tension (γ_{CMC}), (c) the minimum area occupied by the surfactant (A_{min}) and its corresponding surface excess concentration (Γ_{max}) at the air/water interface once this latter is saturated and finally, (d) C_{20}, the minimum concentration needed to reduce the surface tension of the air/water interface by 20 mN/m.

The CMC of a surfactant is obtained from the intersection of the two straight lines for the linear concentration-dependent section and for the baseline of the limit surface tension (γ_{CMC}). Fluorinated surfactants, in contrast to their hydrocarbon analogues, are known for their high surface activity, thereby, for a same hydrophilic head and a same tail length, they exhibit lower CMC values than their hydrogenated derivatives [38]. Another typical feature for fluorinated surfactants is that they adsorb in a compact fashion at the air/water interface. Therefore, their limit surface tension at the CMC drops to low values ~20–30 mN/m, while the surface tension at the air/pure water interface is 72 mN/m [38].

The CMC value of F_6-DigluM was found to be 0.383 mM from surface tension measurements, which is in the same range of that of F_6-Diglu (0.246 mM). This demonstrates that the substitution of

Table 1
Physical–chemical data for F_6-DigluM and F_6-Diglu.

Surfactant	F_6-DigluM	F_6-Diglu
$\log k'_W$ (HPLC)	5.1	4.8[c]
CMC (mM) (ST/AUC)	0.383 ± 0.034/0.48	0.246 ± 0.002[d]; (0.233)[c]/0.68 ± 0.1[c]
γ_{CMC} (mN/m) (ST)	27.7 ± 0.1	28.7 ± 0.1[*,d]; (28.3)[c]
A_{min} (Å^2) (ST)	74.7 ± 8.5*	93.1 ± 7.8[d]; (97.5)[c]
$\Gamma_{max} \times 10^{-12}$ (mol/mm^2) (ST)	2.24 ± 0.25*	1.78 ± 0.14[d]; (1.79)[c]
$C_{20} \times 10^{-3}$ (mM) (ST)	4.48 ± 2.35*	1.34 ± 0.43[d]; (1.54)[c]
CMC/C_{20} (ST)	96.1 ± 52.2*	196.5 ± 62.0[d]; (151.8)[c]
D_H (nm) (DLS/AUC)	5.5/≥5.4[a]; 6.0[b]	5.8[c]/≥5.2[c,a]; 7.0[c,b]
Vol (%) (DLS)	100.0	99.8[c]
HHW (nm) (DLS)	2.0	1.3[c]
M (kDA) (AUC)	≥47[a]; 44[b]	≥44[c,a]; 60[c,b]
N_{agg} (AUC)	≥54[a]; 51[b]	≥50[c,a]; 68[c,b]

Measurements were done by reversed-phase HPLC (HPLC), surface tension (ST), analytical ultracentrifugation (AUC) and dynamic light scattering (DLS). ST data result from the average of three experiments unless specified by "*" indicating the average of only two experiments. A_{min} and Γ_{max} were estimated from the slope of the surface tension curve using the Gibbs equation; C_{20} values were obtained by extrapolation of the surface tensions versus log C to 52 mN/m. Values from AUC for the hydrodynamic diameter (D_H), molar mass (M) and aggregation numbers (N_{agg}) – estimated with a precision of about 10%.

[a] Minimum values estimated from the coefficient of sedimentation, considering a frictional ratio of 1.2.

[b] Estimates from the analysis in terms of sedimentation and diffusion coefficients. DLS measurements were done at 25 °C and at 1.7 mM and 4 mM for F_6-DigluM and F_6-Diglu, respectively. The values reported are the average of 10 runs. D_H is given for the main peak, as the volume particle size distribution (Vol) and the width of the peak at half-height (HHW), an indication of the degree of polydispersity of the aggregates.

[c] Data from Abla et al. [26].

[d] The values were obtained by averaging data from this work and those from the previous work by Abla et al. [26].

the hydroxymethyl group to a methyl group does not strongly affect the CMC of the surfactant in agreement with the very similar $\log k'_W$ values.

Regarding the limit surface tension value, F_6-DigluM exhibited a value of 27.7 mN/m, which is very close to that of F_6-Diglu (28.7 mN/m) suggesting similar properties of adsorption at the air/water interface. However, despite the fact that both F_6-Diglu and F_6-DigluM possess very close surface tension values, their respective C_{20} values were found slightly different. The C_{20} parameter is referred to as the efficiency of a surfactant at the air/water interface and gives an indication on its ability to adsorb at this interface, the lower the value of C_{20}, the more efficiently the adsorption at the interface [38]. F_6-DigluM exhibited a C_{20} value of 4.48×10^{-3} mM that is ~3 times higher than that of the F_6-Diglu (1.34×10^{-3} mM), although they have the same fluorinated tail. This suggests that F_6-DigluM molecules are less packed at the air/water interface at 52 mN/m. This is in line with the lower A_{min} value of F_6-DigluM compared to that of F_6-Diglu (74.7 Å^2 and 93.1 Å^2, respectively). The difference between A_{min} values of the two surfactants could result from the presence of the non-polar methyl group in the DigluM polar head, which in turn could minimize the hydration of the polar head and thus induce a decrease of its area at the air/water interface while destabilizing at the same time the formation of hydrogen bonds between polar heads. This would result in an increase of the gap between two molecules decreasing therefore the efficiency of the surfactant at the air/water interface.

Finally, we determined the CMC/C_{20} ratio, which measures the tendency of a surfactant to adsorb at the air/water interface relative to its tendency to form micelles. Table 1 shows that the CMC/C_{20} ratio decreases when replacing the hydroxymethyl group of F_6-Diglu by a methyl group (F_6-DigluM). This is in line with our previous findings on the polyglucosylated series, where we demonstrated that for a given chain an increase of the volume of the polar head led to an increase of the CMC/C_{20} ratio, with for instance the following order within the F_6 series: F_6-Triglu > F_6-Diglu > F_6-Monoglu. Confirmation of this observation is made here with F_6-Diglu and F_6-DigluM exhibiting CMC/C_{20} ratio of 196.5 and 96.1, respectively. This suggests that for branched polar heads, such as those of the polyglucosylated series, the volume of the polar head is the most important parameter in the CMC/C_{20} ratio. Indeed, while F_6-DigluM is slightly less hydrophilic than F_6-Diglu, its A_{min} was found smaller than that of F_6-Diglu, leading to a lower CMC/C_{20} value.

2.2.3. Size and shape of the micelles

Analytical ultracentrifugation allows investigating the structural homogeneity of macromolecules in solution as well as

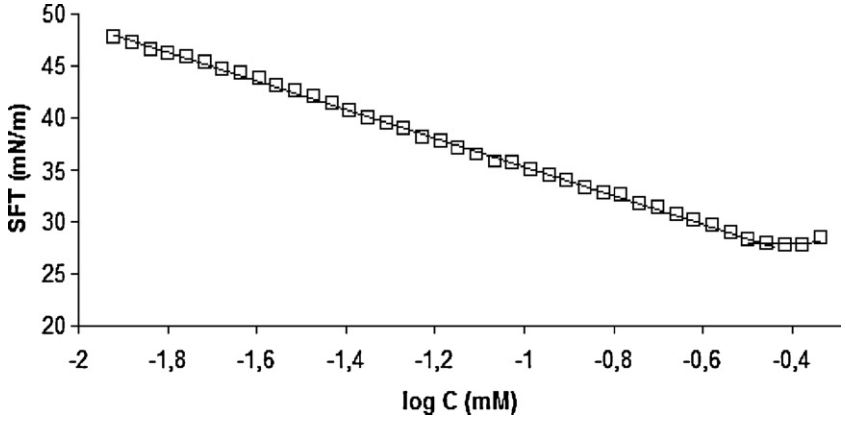


Fig. 3. Surface tension versus log C plot for F_6-DigluM.

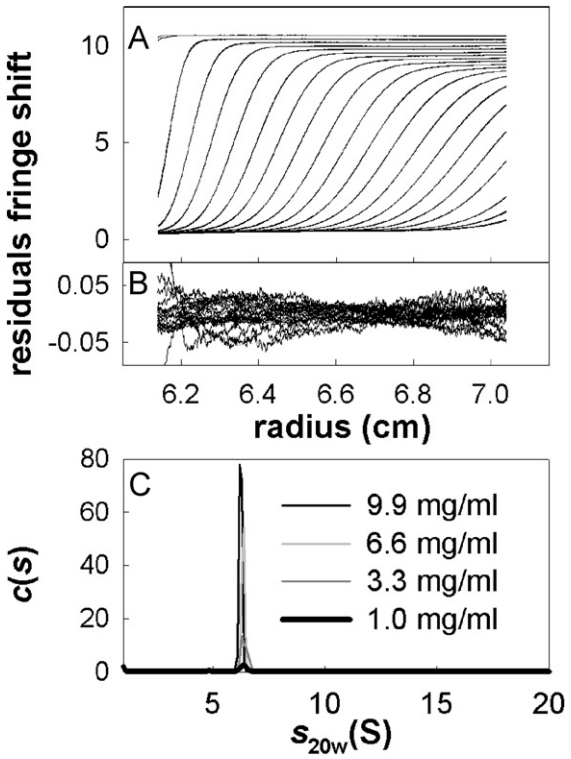


Fig. 4. Sedimentation velocity of F_6-DigluM in H_2O at 42,000 rpm and 20 °C. (A) Superimposition of experimental and modelled from the $c(s)$ analysis (Schuck, 2000) profiles corrected for all systematic noises for F_6-DigluM in H_2O at 6.6 mg/mL in 3 mm optical path length double sector centrepieces. Selection includes the first twenty profiles obtained every ≈7 min during 4 h. (B) Superimposition of the differences between the experimental and fitted curves. (C) Superimposition of the distributions of sedimentation coefficients obtained in H_2O at the indicated concentrations.

characterizing the mass and size of the assemblies they form in solution [39,40]. The degree of homogeneity of the preparation can be easily defined, and components of a mixture can be distinguished according to their sedimentation coefficient, s. The analysis in terms of distributions of sedimentation coefficients – the $c(s)$ analysis – shows that F_6-DigluM in H_2O solution in the concentration range of 1–9.9 mg/mL is very homogeneous (Fig. 4) with one essential contribution corresponding to the micelles. A similar behaviour was also observed in D_2O (data not shown). Since the number of fringe shifts of the boundary is proportional to the micelle concentration, we calculated from its linear dependency with F_6-DigluM concentration, a CMC of 0.42 mg/mL (0.48 mM) and a refractive index increment $\partial n/\partial c$ = 0.091 mL/g. The value of the CMC obtained by this technique is in rather good agreement with the value obtained by surface tension measurement (0.383 mM). The sedimentation coefficient s is very slightly decreasing when increasing the concentration, by 0.2 S at 10 mg/mL, this shift being related to the expected non-ideality from excluded volume effects. At infinite dilution the sedimentation coefficient was found to be s_0 = 6.4 ± 0.1 S in H_2O (s_0 = 4.5 ± 0.1 S in D_2O). The minimum values of molar mass (M), aggregation number (N_{agg}) and hydrodynamic diameter (D_H) calculated using Svedberg's equation and considering a globular compact shape with a frictional ratio f/f_{min} = 1.2, gave values of 47 kDa, 54 molecules, and 5.4 nm, respectively from the sedimentation coefficient at infinite dilution. We analysed the data for F_6-DigluM at 0.99 mg/mL in H_2O in terms of two non-interacting species, the monomer and the micelle. This procedure gave independent estimates for the micelle of 44 kDa for the molar mass, 51 molecules for the aggregation number, and 5.0 for the hydrodynamic diameter, confirming therefore, the compactness and globular shape of the micelle. The values are reported in Table 1.

The dynamic light scattering technique was also employed to determine the size distribution profile of F_6-DigluM self-assemblies in aqueous solution and Table 1 shows the hydrodynamic diameters observed at a relatively high concentration of 1.7 mM (∼5 times its CMC). F_6-DigluM formed small and well-defined monodisperse particles with apparent hydrodynamic diameter 5.5 nm, which is in perfect agreement with the sedimentation velocity data and suggests the formation of spherical micelles as it was previously observed for F_6-Diglu [26,27].

2.3. Biochemical applications

The ability of F_6-DigluM to keep MP water-soluble was investigated using bacteriorhodopsin (BR), an archaebacterial protein folded into a bundle of seven transmembrane α-helices with small extramembrane loops [41]. A molecule of retinal is covalently but loosely bound to the protein, whose visible absorption spectrum is a sensitive and convenient reporter of whether it is in its native state or not. Purple membrane was solubilised using octylthioglucoside (OTG), and surfactant exchange was performed on a sucrose gradient, as described in Breyton et al. [12].

Fig. 5 shows the migration of BR in 10–30% sucrose gradients. This procedure enables surfactant exchange, while the position of the protein/surfactant band yields an estimate of the sedimentation coefficient of the particles (which is related to their mass, size, shape and density) and its width an indication of their mono- or polydispersity. In OTG (not shown, see Ref. [27]) or H_{10}-Diglu, BR migrates as a sharp band, in the upper part of the gradient. When transferred to F_6-DigluM 2, 5 or 6 mM, it migrates deeper into the gradient (Fig. 5 and not shown). A similar behaviour was previously observed with other fluorinated surfactants [12,24,25,27], and can be explained by the high density conferred to the surfactants by the fluorine atoms. However, the bands are as narrow as in the detergent gradient, showing that the protein/

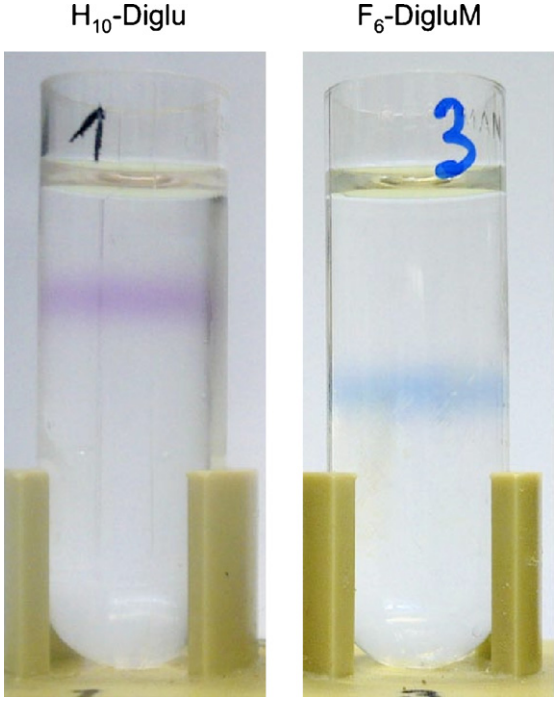


Fig. 5. Migration of bacteriorhodopsin in 10–30% sucrose gradients in the presence of either 6 mM H_{10}-Diglu or F_6-DigluM. Gradients were centrifuged 4 h at 200,000 × g in the TLS55 rotor of the TL100 centrifuge (Beckmann).

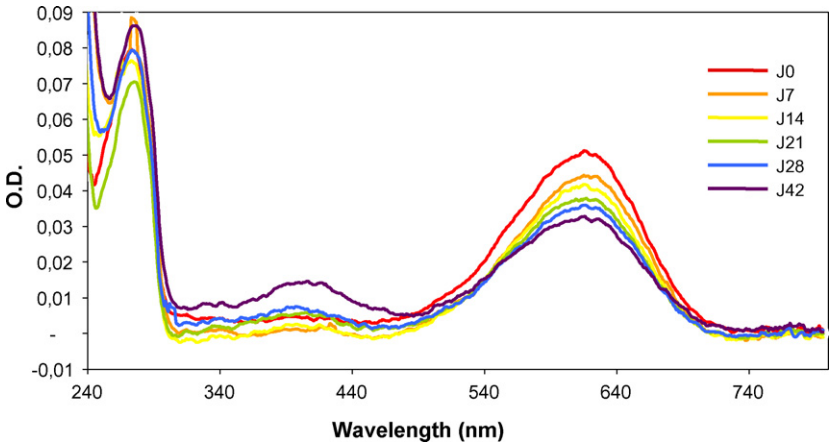


Fig. 6. Spectra of BR incubated in 5 mM F_6-DigluM, immediately after 4 h of centrifugation in sucrose gradients (red trace), or after incubation of the band collected from the gradients at 4 °C in the dark for the indicated time (day 0 to day 42). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

surfactant complex is homogeneous and monodisperse, as expected from the fact that the surfactant's micelles are small and homogeneous in size [27].

The integrity of BR in F_6-DigluM was investigated by spectroscopic measurements. BR was found to be in its native state, and its stability over time (Fig. 6) was higher than that in OTG, as previously observed with F-TAC [12], F-Lac [24], F-Malt [25], and F-Diglu [27]. Indeed, whereas after 14 days incubation at 4 °C in the dark the OTG sample has lost 40% of native protein [12], the F_6-DigluM sample has retained 80% of native protein (Fig. 6). The blue colour ($\lambda_{max} \sim 615$ nm) of the protein in F_6-DigluM, which appears to be a general feature of monomeric BR in surfactants with perfluorinated tails (Lebaupain et al., manuscript in preparation), indicates that the cofactor is still bound to the protein by a protonated Schiff base, but that its environment has changed. After 42 days incubation in 5 mM F_6-DigluM, we note that a proportion of the protein has shifted to a form with a $\lambda_{max} \sim 400$ nm. This latter form does not correspond to denatured protein having released retinal, as the maximum of absorption of free retinal is 380 nm. The 400 nm form rather corresponds to an unfolding intermediate [42]. When incubated in 2 mM F_6-DigluM over 27 days, BR's spectrum showed a shift from $\lambda_{max} \sim 615$ nm to 575 nm (not shown), witnessing oligomerisation of the protein during incubation (Lebaupain et al., manuscript in preparation). The spectra did not show, on this time scale, any diffusion that could indicate aggregation.

3. Conclusions

We reported herein the synthesis of a novel fluorinated surfactant bearing a diglucosylated polar head. The synthesis of F_6-DigluM is based on a one-pot reduction/alkylation reaction under mild conditions to form a thioether bond directly from a fluorinated thioacetate using an excess of $NaBH_4$ in refluxing methanol. The physical–chemical properties of F_6-DigluM in aqueous media were studied by surface tension measurements, dynamic light scattering, and sedimentation velocity as well as its behaviour upon reversed-phase chromatography and these parameters were compared to those of its former analogue F_6-Diglu. These studies showed a very similar behaviour for both surfactants in aqueous solution. We showed that F_6-DigluM, like its former analogue F_6-Diglu, forms small and monodisperse globular micelles, a requirement for biochemistry purposes. This was further confirmed by the homogeneity and stability of bacteriorodopsin solubilised in F_6-DigluM, demonstrating that F_6-DigluM is a suitable novel tool for biochemical applications.

4. Experimental

4.1. Methods

4.1.1. Surface tension measurements

The surface activity of F_6-DigluM in solution at the air/water interface was determined by the Wilhelmy plate technique according to our general procedure [26]. Briefly, the surfactant solution was prepared 12 h prior to the measurements using Milli-Q water (resistivity of 18.2 MΩ cm, surface tension of 72.0 mN/m at 20 °C) and the surface activity was determined by the dilution technique at a constant volume. An estimate of the area per molecule, A_{min}, at the interface is also given, as derived from the surface excess calculated using the Gibbs adsorption isotherm, $\Gamma_{max} = -(1/RT)\ (d\gamma/d(\ln C))$ where Γ_{max} is the surface excess (moles per unit area), R is the universal gas constant, T is the absolute temperature, γ is the surface tension, and C is the surfactant concentration. The A_{min} can be calculated as $A_{min} = 1/(N_A\Gamma_{max})$, where N_A is the Avogadro number. The C_{20} value was obtained by extrapolation of the slope of the surface tension curve to 52 mN/m.

4.1.2. Determination of $\log k'_W$ values

F_6-DigluM was dissolved in MeOH at 1 g/L and injected onto a reversed-phase column (C18, 5 μm granulometry, 250 mm × 4.6 mm) at room temperature. F_6-DigluM was eluted with various MeOH/H_2O mixtures (from 7:3 to 9:1 v/v) and a linear regression analysis ($r^2 > 0.999$) was performed on these three points. The measurements were performed at a flow rate of 0.8 mL min^{-1} and detected at 205 nm. The value of $\log k'$ was calculated as $\log k' = \log((t - t_0)/t_0)$, where t is the retention time of the surfactant and t_0 is the elution time of MeOH, which is not retained on the column. $\log k'_W$ values were obtained by extrapolation of the linear regression to 0% MeOH.

4.1.3. Dynamic light scattering

The hydrodynamic particle size distribution and polydispersity of F_6-DigluM solution at 1.7 mM was determined by using a He–Ne laser (λ = 633 nm, 4.0 mW) according to our general procedure [26]. A stock aqueous solution was made and stored at room temperature overnight and on the day of the experiment the solutions were passed through a 0.45 μm filter. The size of the

particles was measured 1 h after filtration and dilution at the target concentration.

4.1.4. Analytical ultracentrifugation (AUC) experiments

F_6-DigluM was investigated at 9.9, 6.6, 3.3 and 0.99 mg/mL in H_2O, and 10.3, 6.9, and 3.4 mg/mL in D_2O, according to the procedures described elsewhere [27,43,44] using the programs developed by Schuck [45] for the analysis. The value of the partial specific volume, 0.585 mL/g, is calculated from the chemical composition of the molecules [46].

4.2. Synthesis

Mercury cyanide $Hg(CN)_2$ was dried overnight on P_2O_5 under vacuum. All the solvents were of reagent grade, distilled and dried according to standard procedures prior to use. The progress of the reactions was monitored by thin layer chromatography (TLC, silica plates) and the compounds were detected either by exposure to ultraviolet light (254 nm) or by spraying with a 5% sulphuric acid solution in ethanol and/or with 2% ninhydrin solution in ethanol following heating at ~150 °C to detect glucose and amine containing group, respectively. Ultrasonication was performed with a sonicator equipped with a 13 mm diameter titanium probe. Flash chromatography purifications were carried out on silica gel (40–63 μm granulometry). Size exclusion chromatography purifications were carried out on Sephadex LH20 resin. High Pressure Liquid Chromatography (HPLC) purifications were performed on a reverse phase column (C18, 5 μm granulometry, 21.4 mm × 250 mm) at a flow rate of 16 mL min^{-1} with detection at 205 nm. Water was deionized with a Milli-Q water purification system. Melting points have not been corrected. The ^1H, ^{13}C, ^{19}F and DEPT NMR sequences were performed at 250, 62.86 and 235 MHz for ^1H, ^{13}C and ^{19}F experiments, respectively. Chemical shifts are given in ppm relative to the solvent residual peak as a heteronuclear reference for ^1H and ^{13}C. Abbreviations used for signal patterns are: s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet; dd, doublet of doublet; dt, doublet of triplet.

4.2.1. Synthesis of N-(1,3-dihydroxy-2-methylpropan-2-yl)acrylamide (1)

3 g (41.6 mmol, 1 equiv.) of acrylic acid, 4.81 g (45.7 mmol, 1.1 equiv.) of 2-amino-2-methylpropane-1,3-diol, and 12.34 g (49.9 mmol, 1.2 equiv.) of EEDQ were dissolved in 75 mL of EtOH and heated up to 60 °C. After 24 h of being stirred at 60 °C, the reaction mixture was cooled down and a spatula of IRC 50 resin was added. The reaction mixture was filtered and the solvent was evaporated under reduced pressure. The resulting crude compound was recrystallized from EtOH/Et_2O to give 3.7 g (23.2 mmol, 56%) of compound **1** as a white solid. R_f = 0.42 (EtOAc/MeOH 95:5 v/v). Mp = 60.2–61.3 °C. ^1H NMR (DMSO-d_6) δ 7.35 (NH, s, 1H), 6.15 (CH═, dd, J = 10.1 and 16.9 Hz, 1H), 5.86 (CH_2═, dd, J = 2.3 and 16.9 Hz, 1H), 5.35 (CH_2═, dd, J = 2.3 and 10.0 Hz, 1H), 4.67 (OH, t, J = 5.8 Hz, 3H), 3.35–3.30 (CH_2O, m, 4H), 0.98 (CH_3, s, 3H). ^{13}C NMR (DMSO-d_6) δ 170.2, 133.0, 128.7, 64.2, 59.0, 19.2.

4.2.2. Synthesis of N-Tris[di(2′,3′,4′,6′-tetra-O-acetyl-β-D-glucopyranosyl)oxymethyl]methyl but-3-enamide (2)

3.0 g (18.96 mmol, 1 equiv.) of compound **1**, 25.89 g (62.99 mmol, 6 equiv.) of 2,3,4,6 tetra-*O*-acetyl-α-D-glucopyranosyl bromide and 15.91 g (62.97 mmol, 6 equiv.) of $HgCN_2$ were dissolved in CH_3CN in the presence of drierite and then the solution was sonicated for 30 min. The reaction mixture was filtered over a pad of Celite and the solvent was removed under reduced pressure. The crude residue was dissolved in EtOAc and the organic layer was successively washed with $NaHCO_3$, KI (10%) and saturated $Na_2S_2O_3$ solutions, then dried over Na_2SO_4 and finally evaporated under reduced pressure. The resulting crude compound was purified by flash chromatography (EtOAc/cyclohexane 7:3 v/v) and by size exclusion chromatography (MeOH/CH_2Cl_2 1:1 v/v) to give 10.35 g (12.62 mmol, 67%) of compound **2** as a white solid. R_f = 0.29 (EtOAc/cyclohexane 7:3 v/v). Mp = 70.3–71.4 °C. $[\alpha]_D^{25}$ = −27.3° (c, 1, MeOH). ^1H NMR ($CDCl_3$) δ 6.28–6.03 (NH, CH_2═, m, 3H), 5.62–5.58 (CH═, m, 1H), 5.26–5.01 (H_2, H_3, H_4, m, 6H), 4.51 (H_1, d, J = 7.8 Hz, 1H), 4.50 (H_1, d, J = 7.9 Hz, 1H), 4.28–3.71 (H_5, H_6, CH_2O, m, 10H), 2.15–2.02 (CH_3CO, m, 24H) 1.41 (CH_3, s, 3H). ^{13}C NMR ($CDCl_3$) δ 170.7, −165.3, 131.1, 126.5, 101.2, 101.1, 72.7, 72.6, 72.4, 72.1, 71.9, 71.8, 71.3, 68.2, 61.7, 56.2, 20.9–20.6, 18.3. HRMS (ESI+) calculated for $C_{35}H_{50}NO_{21}$ ($[M+H]^+$): 820.2870, found: 820.2869.

4.2.3. Synthesis of N-Tris[di(β-D-glucopyranosyl)oxymethyl]methyl but-3-enamide (3)

1.2 g (1.46 mmol) of compound **2** was dissolved in methanol in the presence of a catalytic amount of MeONa and stirred overnight at room temperature. Two spatulas of IRC 50 resin were added, and the solution was filtered then concentrated under reduced pressure to afford 0.64 g of compound **3** (1.33 mmol, 91%) as a white foam, which was used without further purification.

4.2.4. Synthesis of 1H,1H,2H,2H-perfluorooctylthioacetate (4)

2.0 g (4.23 mmol, 1 equiv.) of 1*H*,1*H*,2*H*,2*H*-tridecafluoro-1-iodooctane were dissolved in 10 mL of anhydrous DMF under argon atmosphere. 0.58 g (5.08 mmol, 1.2 equiv.) of potassium thioacetate was added and the mixture was stirred for 1 h at room temperature. Then, 30 mL of water were added and the aqueous layer was extracted with EtOAc. The organic layer was washed with distilled water, dried over Na_2SO_4, and then concentrated under reduced pressure. The resulting crude compound was purified by flash chromatography (cyclohexane/CH_2Cl_2 9:1 v/v) to give 1.35 g (3.21 mmol, 75%) of 1*H*,1*H*,2*H*,2*H*-perfluorooctylthioacetate **4** as a translucent oil. R_f = 0.62 (cyclohexane/CH_2Cl_2 9:1 v/v). ^1H NMR ($CDCl_3$) δ 3.11 (t, J = 7.9 Hz, 2H, CH_2S), 2.48–2.34 (m, 2H, CF_2CH_2), 2.38 (s, 3H, CH_3CO). ^{13}C NMR ($CDCl_3$) δ 194.6, 31.6, 30.1, 20.1. ^{19}F NMR ($CDCl_3$) δ −81.4 (3F), −115.0 (2F), −122.2 (2F), −123.2 (2F), −123.8 (2F), −126.4 (2F).

4.2.5. Synthesis of N-1,1-di[(O-β-D-glucopyranosyl)oxymethyl]ethyl-4-thia-7,7,8,8,9,9,10,10,11,11,12,12,12-tridecafluorododecanamide (5)

1.46 g (3.86 mmol, 1 equiv.) of 1*H*,1*H*,2*H*,2*H*-perfluorooctylthioacetate **4** and 0.22 g (5.79 mmol, 1.5 equiv.) of $NaBH_4$ were dissolved in 10 mL of MeOH under argon atmosphere and the mixture was heated up to reflux of MeOH. After 15 min of being stirred in refluxing MeOH, a solution of 2.8 g (5.79 mmol, 1.5 equiv.) of compound **2** in 15 mL of MeOH was added, and the reaction was stirred for 3 h. The reaction mixture was cooled at room temperature and then concentrated under reduced pressure. The resulting crude compound was purified by size-exclusion chromatography (MeOH) and by RP-HPLC (H_2O/CH_3CN 60:40 v/v, U.V. detection at λ = 215 nm) to give after lyophilization 1.87 g (2.17 mmol, 56%) of compound **5** as a white powder. R_f = 0.6 (EtOAc/MeOH/H_2O 7:2:1 v/v/v). Mp = 153.7. ^1H NMR (CD_3OD) δ 4.34 (H_1, d, J = 7.7 Hz, 1H), 4.33 (H_1, d, J = 7.7 Hz, 1H), 4.15–3.67 (H_6, $H_{6'}$, CH_2O, m, 8H), 3.39–3.22 (H_2, H_3, H_4, H_5, m, 8H), 2.87–2.81 (CH_2SCH_2, m, 4H), 2.58–2.44 (CH_2CF_2, m, 2H), 2.52 (CH_2CO, t, J = 6.9 Hz, 2H), 1.42 (CH_3, s, 3H). ^{13}C NMR (CD_3OD) δ 173.2, 103.4, 103.3, 76.6, 73.6, 70.2, 67.7, 61.3, 61.9, 36.2, 31.5 (t, J = 20.4 Hz), 27.1, 22.0, 16.2. ^{19}F NMR (CD_3OD) δ −82.4 (3F), −115.3 (2F), −122.9 (2F), −123.9 (2F), −124.3 (2F), −127.3 (2F). HRMS (ESI+) calcd for $C_{27}H_{38}F_{13}NO_{13}S$ ($[M+H]^+$): 864.1929, found 864.1929.

4.3. Biochemistry

4.3.1. Protein purification

Purple membrane was purified from the overproducing strain S9 of *Halobacterium salinarum* as described in Oesterhelt et al. [47], and stored at −80 °C. BR was solubilised with 100 mM octylthioglucoside (OTG) at a membrane concentration of 2 g/L in 20 mM sodium phosphate buffer, pH 6.8. After 40 h incubation under constant stirring in the dark at 4 °C, the sample was centrifuged 10 min at 247.000 × *g*, diluted to reach a final OTG concentration of 15 mM and used extemporaneously.

4.3.2. Surfactant exchange

Purified BR in OTG was supplemented with 1 mM of F_6-DigluM and incubated 15 min prior to being loaded onto a 10–30% (w/w) sucrose gradient containing 20 mM sodium phosphate buffer, pH 6.8 and 2, 5 or 6 mM F_6-DigluM. Control gradients were performed in the same buffer with 15 mM OTG or 2 and 6 mM H_{10}-DigluM. Gradients were centrifuged for 4–5 h at 55,000 rpm (200,000 × *g*) in the TLS 55 rotor of a TL100 ultracentrifuge (Beckman). The bands containing the coloured protein were collected with a syringe and kept on ice in the dark for spectral analysis.

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