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Non-ionic hybrid detergents for protein delipidation

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ABSTRACT

Non-ionic detergents are important tools for the investigation of interactions between membrane proteins and lipid membranes. Recent studies led to the question as to whether the ability to capture protein-lipid interactions depends on the properties of detergents or their concentration in purification buffers. To address this question, we present the synthesis of an asymmetric, hybrid detergent that combines the head groups of detergents with opposing delipidating properties. We discuss detergent properties and protein purification outcomes to reveal whether the properties of detergent micelles or the detergent concentration in purification buffers drive membrane protein delipidation. We anticipate that our findings will enable the development of rationally design detergents for future applications in membrane protein research.

1. Introduction

Approximal 30% of all proteins are embedded in lipid membranes. They act in response to their environment and fulfil functions that are vital for every organism. Technologies that help to improve our understanding about membrane protein structures and functions are important across all disciplines relevant to life sciences, including chemistry, biology, and drug discovery. For structural and functional studies, membrane proteins are traditionally solubilized from lipid membranes with detergents, which contain a hydrophilic head and a lipophilic tail (Fig. 1) [1]. Detergents form water-soluble proteomicelles by shielding hydrophobic protein surfaces from water. Proteomicelles can be purified by chromatographic techniques and enriched to high concentration and purity. In this way, detergents enable the structural analysis of membrane proteins by different biophysical techniques and enable drug discovery research [2]. Ideal detergents maintain native protein structures throughout extraction from membranes and purification with chromatographic techniques. Furthermore, they enable the analysis of intact proteins with biophysical techniques, such as crystallography, spectroscopy, and mass spectrometry. In praxis, every detergent has its own pros and cons for protein purification and analysis. Given the diversity of the detergentome, which is the entity of all detergents, best detergents are selected empirically [3]. In this regard, detergents are also routinely exchanged to ensure that the protein is maintained in a stable environment and the detergent is compatible with the required application.

In the past years, we have seen an ever-growing number of new detergents entering membrane protein-related literature [4]. In addition, the number of alternative membrane mimetics is growing, as indicated by recent publications related to saposin-lipoprotein systems [5], amphiphilic polymers [6], peptidiscs [7], or nanodiscs [8]. While the number of different detergents and alternative membrane mimetics continues to grow, screening tools are emerging that allow parallelizing the testing of an increasing number of different membrane mimetics and conditions [9,10]. However, membrane proteins, in particular those that are medically relevant, such as G protein-coupled receptors or solute carriers, can be difficult to produce on large scales [2]. We noticed that this can motivate to include only the most established detergents in screenings, which causes dead ends for projects for which the most established detergents do not work.

To address this challenge, we established an interdisciplinary research collaboration with the aim to rationally design detergents for individual applications in membrane protein research. Recently, we found that modular oligoglycerol detergents (OGDs) can be used to decrease the time required to synthesize diversified detergent libraries [1]. In combination with miniaturized purification protocols and native mass spectrometry (nMS), first design rules were identified with which the structure of OGDs can be optimized for individual applications [1,11–13]. Lipid binding and drug binding to membrane proteins can affect one another, with implications for biological function [14].

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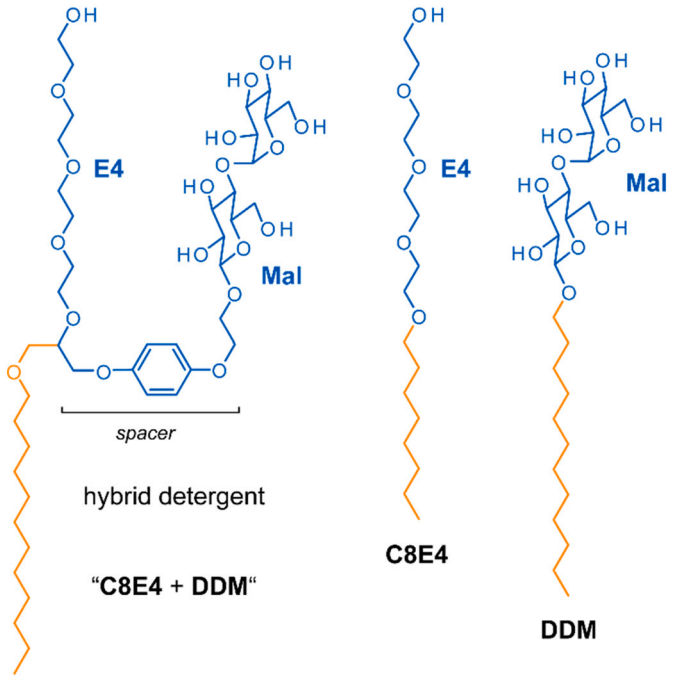


Fig. 1. Describing the molecular structure of detergents. The asymmetric hybrid detergent combines the maltose head group of DDM (Mal) and the tetraethylene gycol head of C8E4 (E4). Changing the relative size of head and tail affects the utility of detergents for membrane protein purification and delipidation.

Therefore, efforts have recently been focused on understanding how the preservation of protein-lipid interactions during purification can be controlled by changing the structure of detergents. While comparing different detergent classes, Reading et al. found that delipidating properties are linked to the properties of the detergent head group [15]. When investigating OGDs, we found that delipidation is also linked to the properties of the detergent micelle surrounding the protein in solution [1]. These findings seem to contradict the long-standing hypothesis that the preservation of protein-lipid interactions throughout purification depend on the detergent concentration in purification buffers [16].

To rationalize the delipidating properties of detergents, we here designed a hybrid detergent by combining the head groups of the mildly delipidating detergent n-dodecyl-β-D-maltoside (DDM) and strongly delipidating tetraethylene glycol monooctyl ether (C8E4) (Fig. 1). We investigate how detergent properties change when their head groups get combined and whether the properties of detergent micelles or the detergent concentration in purification buffers determine delipidation outcomes.

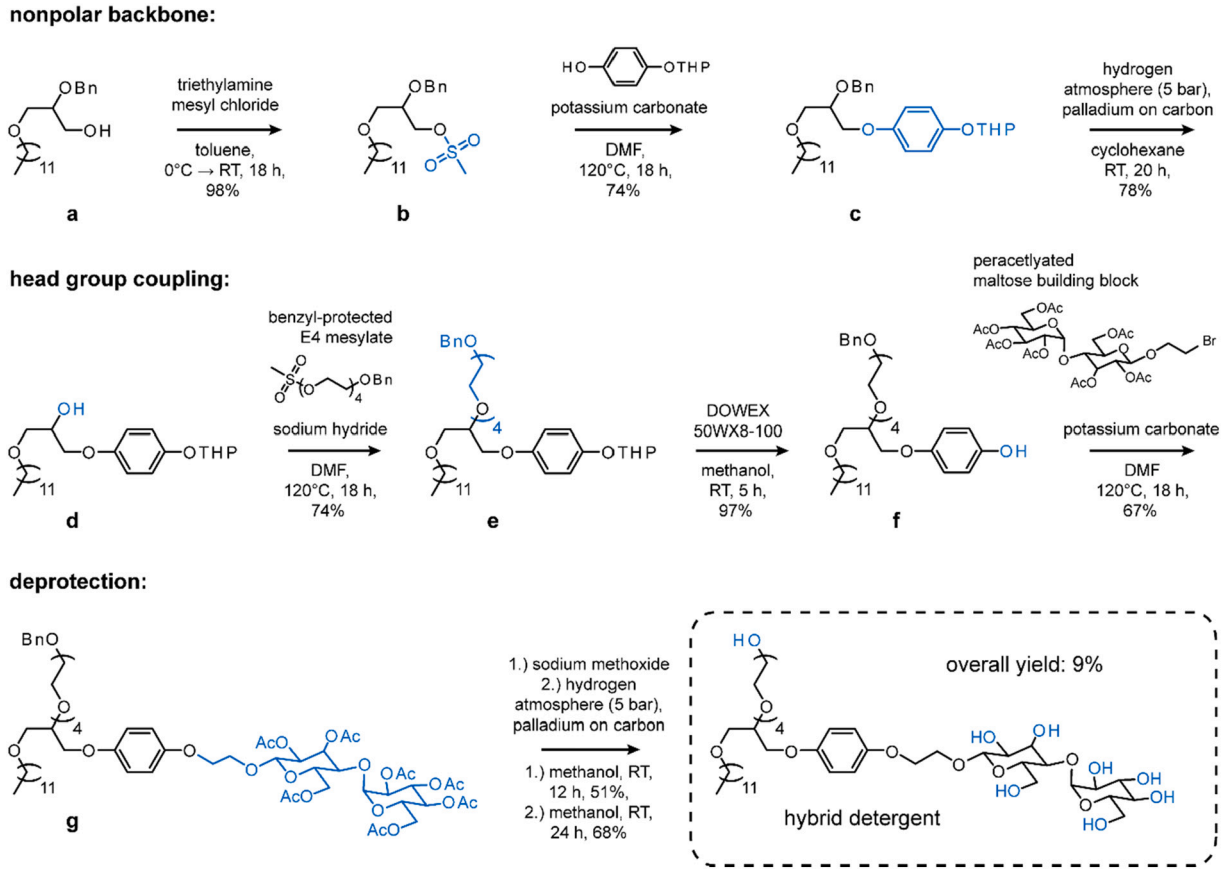
2. Results and discussion

To understand how detergent properties change when their head groups are covalently combined in one structure, we designed the hybrid detergent (Fig. 1). The maltose head of DDM (Mal) and the tetraethylene glycol head of C8E4 (E4) were merged into the hybrid detergent by using an asymmetric gemini detergent design. Unlike head-tail detergents, gemini detergents consist of a lipophilic tail and two head groups, which are bridged by a spacer. The synthesis of the hybrid detergent was achieved from monoalkylated 2-benzyloxy-1,3-propanediol (**a**) in eight steps with an overall yield of 9% (Scheme 1) (For details see ESI†). Alternative strategies, such as amide or triazole coupling could lead to higher synthesis yields. However, the individual detergents DDM and C8E4 do not contain any of these functional groups, *e.g.* amide or triazole, and were therefore not included in the hybrid detergent.

To accomplish the synthesis of the hybrid detergent, the hydrophobic backbone was first synthesized (Scheme 1). To do so, the starting material **a** was mesylated to obtain compound **b**. To finalize the hydrophobic backbone, compound **b** was further reacted with monoprotected 1,4-dihydroxybenzol under basic conditions to obtain compound **c**. Next, the individual head groups were attached (Scheme 1). For this purpose, we designed compound **c** to have a benzyl- and a tetrahydropyranyl-protected hydroxyl group. Each protecting group can be cleaved independently to enable selective coupling of individual head groups. The benzyl ether of compound **c** was removed using hydrogen gas and palladium on carbon (Pd/C) to obtain compound **d** (Scheme 1). The ability to preserve tetrahydropyranyl ethers under these conditions depends on the solvent system [17]. Commercial Pd/C catalysts usually contain $PdCl_2$. In the presence of alcohols or water, hydrochloric acid is formed, which subsequently catalyzes the deprotection tetrahydropyranyl ethers. Therefore, complete deprotection of benzyl and tetrahydropyranyl ethers was obtained in methanol (Fig. S1). However, in line with previous reports [17], an almost selective benzyl deprotection occurred in non-proteolytic solvents that have a low water content, such as tetrahydrofurane and cyclohexane. The tetrahydropyranyl ether of the desired product **d** was better retained in cyclohexane, which contained less water (Fig. S1). The ability to selectively remove the benzyl ether in compound **c** was the key to the preparation of the hybrid detergent because it enabled the selective coupling of benzyl-protected E4 mesylate with compound **d** under basic conditions (Scheme 1). Subsequent removal of the tetrahydropyranyl ether in compound **e** under acidic conditions and coupling of the compound **f** with a peracetylated maltose building block led to the obtainment of the detergent precursor **g** (Scheme 1). Subsequent deprotection of the acetate groups under Zemplén conditions and removal of the benzyl protecting group with hydrogen gas and Pd/C led to the obtainment of the hybrid detergent (Scheme 1).

The minimum information required for testing the utility of detergents for membrane protein purification is the critical aggregation concentration (cac) [12]. To prevent membrane protein precipitation in the absence of membranes, detergent concentrations in purification buffers are traditionally adjusted to a multiple of the detergent's cac [18]. In contrast, higher detergent concentrations, usually in the amount of 1% *w/v*, are used to dissolve membranes. However, high detergent concentrations can lead to membrane protein denaturation and/or precipitation. Therefore, after extraction, detergent concentrations are often reduced to a multiple of the detergent's cac. For example, for low-cac detergents, such as DDM, a decrease in detergent concentration is readily accomplished by lowering the detergent concentration to two times of its cac during affinity purification. The DDM concentration applied during extraction (19.6 mM) is significantly higher than its cac (100 μM). For high-cac detergents, such C8E4, the concentration used for extraction (32.6 mM) and the cac (8 mM) are both high compared to the values obtained from the low-cac detergent DDM before. Therefore, high-cac detergents typically provide a harsher solution environment for membrane proteins throughout purification.

To investigate how mixing the head groups of low- and high-cac detergents affect their aggregation properties, the cac values of DDM, C8E4, and the hybrid detergent were analyzed by means of dynamic light scattering (DLS) (Table S1 and S2). The cac of detergents depends on temperature, solvents, and salts [19,20]. For practical reasons, since the required temperature and composition of protein purification buffers are difficult to generalize, cac values are routinely determined in pure water [1,12]. The cac of the hybrid detergent (210 μM) in water was similar to the cac of DDM (100 μM) and significantly lower than the cac of C8E4 (8 mM) (Table S1). The finding that the cac of the hybrid detergent is significantly lower than the cac of C8E4 is expected since it is known that increasing the size of the hydrophilic head of a detergent can reduce the *cac* [21]. Furthermore, the diffusion coefficients obtained from the aggregates formed by the hybrid detergent in solution are similar to values obtained from micelle-forming detergents DDM and



Scheme 1. Schematic showing the steps involved in the synthesis of the hybrid detergent: Synthesis of the nonpolar backbone (a-c), asymmetric head group coupling (d-g), and deprotection (hybrid detergent). Structural changes are highlighted in blue. O-Benzyl, O-tetrahydropyranyl, and *O*-acetyl protecting groups are abbreviated with BnO, THPO, and AcO, respectively.

C8E4. The diffusion coefficients correspond to hydrodynamic diameters of 4.8–5.6 nm (Fig. S2). Our data indicate that mixing the head groups of different, micelle-forming detergents *via* a gemini-based design approach can lead to new micelle-forming detergent with low *cac* values, even if one of the initial detergents is a high-cac detergent. Generally, this is an interesting detergent design strategy for nMS and other biophysical techniques in membrane protein research. Low detergent concentrations are more likely to provide mild solution

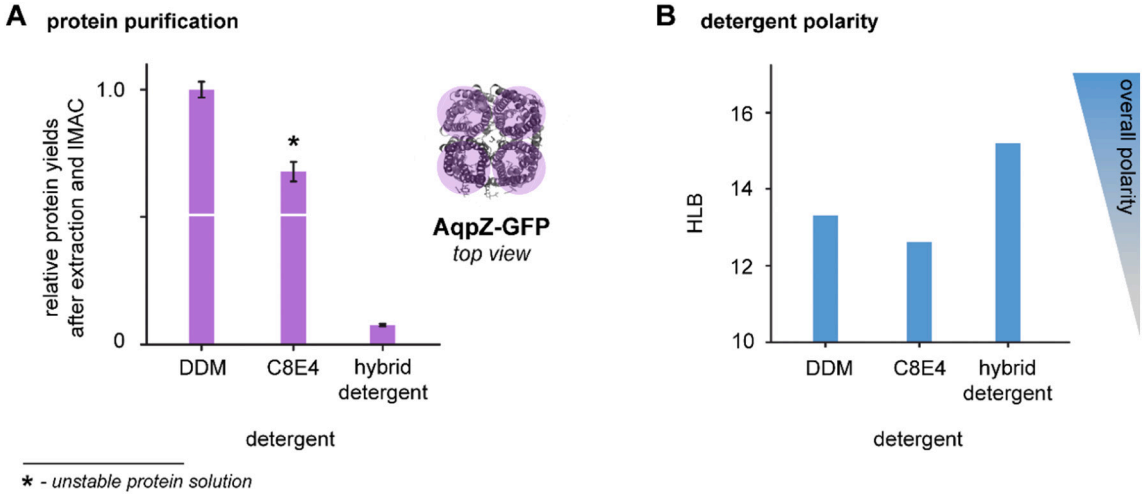


Fig. 2. Comparing protein yields and detergent polarity. A) Bar chart showing protein yields of AqpZ-GFP obtained upon extraction and IMAC purification with different detergents. Higher relative protein yields were obtained from detergents DDM and C8E4. Proteins obtained from C8E4 readily precipitated during further purification steps, *e.g.* buffer exchange. Lower relative protein yields, but no protein precipitation was obtained from the hybrid detergent. B) Bar chart showing HLB values of detergents. Higher overall polarity is obtained for the hybrid detergent. Combining the head groups of DDM and C8E4 leads to a more polar hybrid detergent that is less efficient for membrane protein extraction but better in keeping the protein stable in solution than C8E4. Relative protein yields are shown with standard deviation from three repeats ($n = 3$).

conditions for membrane proteins and are less likely to produce electrospray instability or other experimental artifacts [22].

Having determined the cac of the hybrid detergent, we explored its utility for membrane protein purification and delipidation by following an established protocol [1]. We selected the tetrameric aquaporin channel (AqpZ) from *Escherichia coli* (*E. coli*) as model system, because it is compatible with a broad range of detergents [1]. The protein was solubilized from *E. coli* membranes with high detergent concentrations (1% *w/v*) and purified by immobilized metal ion affinity chromatography (IMAC). Protein yields were normalized to DDM and C8E4 (Fig. 2). Assessing first the relative yields, higher protein quantities were obtained from DDM and C8E4. However, most of the protein obtained from C8E4 precipitated in subsequent purification steps, *e.g.*, during buffer exchange. In this case, no protein could be detected by UV/VIS spectroscopy after supernatant clarification. This indicates that C8E4 is efficient in disrupting membranes and solubilizing AqpZ, but less efficient in keeping the protein soluble. This result agrees with the observation that short-chain PEG detergents denature membrane proteins more efficiently than saccharide detergents [23]. While lower protein yields were obtained from the hybrid detergent, the isolated protein was stable over multiple freeze thaw cycles and no protein precipitation was obtained after buffer exchange. Our data indicate that the hybrid detergent preserved AqpZ better than C8E4. We rationalize this outcome with the cac of the hybrid detergent (210 μM), which is lower than the high-cac detergent C8E4 (8 mM) and leads likely to a milder solution environment for AqpZ.

The reduced protein quantities obtained from the hybrid detergent can be explained by means of the overall polarity of detergents. To compare the overall polarity of the hybrid detergent with DDM and C8E4, we applied the hydrophilic-lipophilic balance (HLB) which was introduced by Griffin [24]. The HLB scale describes the relative polarity of a detergent in relation to the mass balance between hydrophilic head and lipophilic tail [24]. It is a metric that is used to rank the overall polarity of detergents on the basis of HLB numbers, which range from zero to 20. The larger the molecular weight of a detergent head compared to its tail, the larger the HLB value. Detergents that are good in solubilizing membranes commonly have a HLB between 12 and 14, such as DDM (13.3) and C8E4 (12.6) (Table S1 and S3) [25]. The hybrid

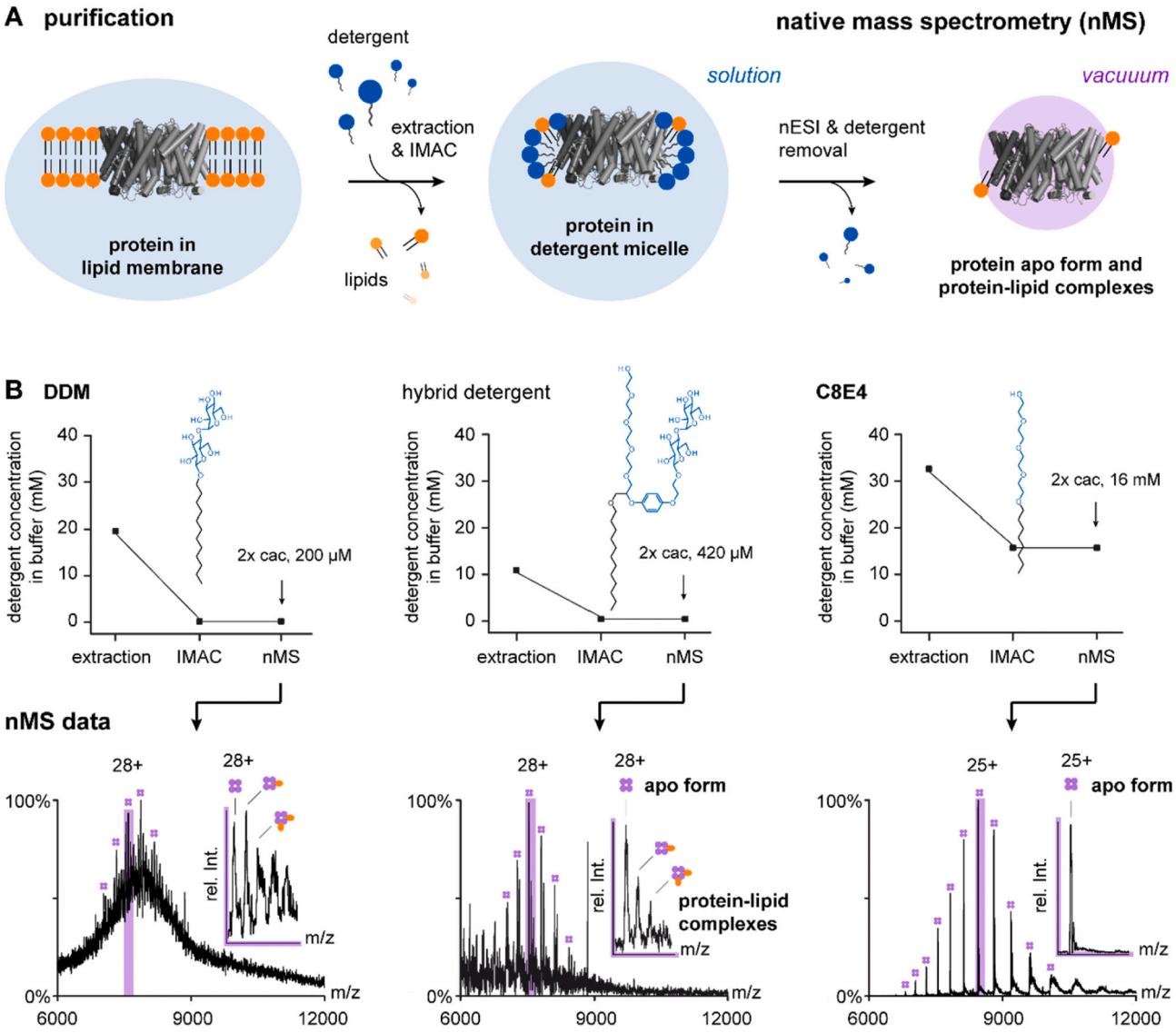


Fig. 3. Investigating delipidation outcomes with native mass spectrometry. a) Schematic showing steps involved in the purification and native mass spectrometry (nMS) analysis of the membrane protein AqpZ. Plots showing the detergent concentration in buffers at different stages of purification and the mass spectra obtained following purification with DDM (left), hybrid detergent (middle), and C8E4 (right). Membrane protein delipidation increases the higher the detergent concentration in purification buffers. Spectra were obtained using similar instrument conditions.

detergent has a larger head and is therefore more polar compared to DDM and C8E4, as indicated by a larger HLB (15.2). Considering this background, this indicates that increasing the polarity of detergents, as indicated by a larger HLB, decreases their ability to solubilize membranes [25]. Although the hybrid detergent can stabilize AqpZ in solution, it is less efficient in disrupting lipid membranes and lower protein yields are obtained.

To investigate how mixing the head groups of DDM and C8E4 affect the preservation of oligomeric states and ligand binding, we investigated the extracted samples by nMS [26]. The samples were transferred into the vacuum of a Q Exactive mass spectrometer following nano-electrospray ionization (nESI) and the detergent environment was removed by collisional activation using comparable instrument settings (Fig. 3A) [26]. Mass spectra obtained following detergent removal with DDM, the hybrid detergent, and C8E4 revealed well-resolved protein charge states corresponding to the apo form of AqpZ (Fig. 3B). The expected tetrameric state of AqpZ could be retained in all three detergent environments during purification [1]. In addition, lipid-bound states were detected in the cases of DDM and the hybrid detergent (Fig. 3B). In the case of C8E4, no protein-lipid complexes were obtained. Comparing the relative intensities of protein-lipid complexes by nMS can inform about the relative degree of protein delipidation, since all three protein samples were purified and analyzed under comparable conditions [1]. Considering this hypothesis, the nMS data indicate that C8E4 exhibits stronger delipidating properties than DDM and the hybrid detergent under the experimental conditions employed (Fig. 3B).

To rationalize delipidation outcomes, we compared changes of detergent concentrations in purification buffers among purification and analysis, including extraction, IMAC, and nMS. As mentioned above, detergent concentrations during IMAC and nMS experiments are commonly adjusted to a multiple of the detergent's cac. Therefore, the concentrations of low-cac detergents, including DDM and the hybrid detergent, are lower during IMAC and nMS compared to the high-cac detergent C8E4 (Fig. 3B) (Table S1). Detergents compete with lipid molecules for binding to membrane proteins in a concentration-dependent manner. Results obtained from Ilgü et al. showed that purifying membrane proteins in the presence of high detergent concentrations can lead to efficient delipidation, such as in the cases of high-cac detergents [27]. This agrees with our results, which show that more lipid interactions were retained throughout IMAC and nMS with the low-cac hybrid detergent and DDM compared to the high-cac detergent C8E4 (Fig. 3B) (Table S1). More broadly, lipids are important for maintaining the structure and function of AqpZ stable in solution [28]. Considering this hypothesis, we speculate that protein precipitation obtained from C8E4 is linked to its strong delipidating properties. The ability of C8E4 to delipidate membrane proteins under the experimental conditions employed goes hand in hand with its high cac [27]. The ability to produce low-cac detergents by combining detergent head groups of low- and high-cac detergents provides a new detergent design strategy for tuning membrane protein delipidation throughout purifications.

Having investigated the delipidating properties of detergents, we finally investigated their charge-reducing properties in the vacuum of a mass spectrometer. Maintaining non-covalent interactions in protein assemblies is key for successful nMS experiments. Lowering the charge of protein ions reduces the disruptive impact that repulsive Coulomb forces can have on native protein structures in vacuum [29]. The average charge state (z_{ave}) of AqpZ obtained from the hybrid detergent (28+) is similar to data obtained from DDM, which is classified as non-charge-reducing detergent (Fig. 3B) (Table S1) [1,15]. In contrast, a lower z_{ave} was obtained when AqpZ was liberated from C8E4 micelles (25+). Considering the hypothesis that gas-phase properties of detergents are mainly governed by the properties of their polar groups [1,11,15], we initially expected that the hybrid detergent would exhibit an average of the properties of DDM and C8E4. Our data indicate that this is valid for the ease of the detergent removal, but not necessarily for charge-reducing properties (Fig. 3B).

The charge-reducing properties of C8E4 have been rationalized in two ways: Similar to the head group of previously reported OGDs [11], tetraethylene glycol (E4) can complex cations [30] and reduce the charge of protein ions during detergent removal [15]. In addition, clusters formed by C8E4 during the electrospray process can compete with charging of proteomicelles during nESI [15]. In contrast, the head group of DDM and its detergent clusters formed during the electrospray process are not capable of reducing the charge of proteins in a comparable manner. However, the binding affinity of a non-charge-reducing detergent towards proteins in vacuum increases with the number of hydroxyl groups in its head group [11]. In light of these explanations, we speculate that the head group of DDM in the hybrid detergent preferentially binds to the protein surface and sterically shields the E4 head, thus limiting its ability to capture and reduce the charge of membrane proteins. Therefore, the hybrid detergent does not exhibit charge-reducing properties.

Since the proteomicelles formed by DDM and the hybrid detergent show similar z_{ave}'s, it is very likely that they experience similar activation conditions during detergent removal insight the mass spectrometer [1]. This supports the hypothesis that the relative difference in intensity of protein-lipid complexes depend more on their stabilization in solution rather than on charge effects in vacuum [1]. The reduction in relative intensity of protein-lipid complexes obtained from the hybrid detergent can be explained by the higher detergent concentration applied in purification buffers during IMAC and nMS (2× cac, 420 μM) compared to DDM (2× cac, 200 μM) (Fig. 3B). Considering differences in detergent concentrations during purification can also help to rationalize delipidation outcomes previously obtained from strongly delipidating [G1] OGDs and mildly delipidating [G2] OGDs (Fig. S3).

Another important question prompted by these results is as to whether delipidation is also affected by the overall polarity of the detergents. It is well established that the cac decreases when the polarity of detergents decreases, for example, by increasing the molecular weight of the lipophilic tail in relation to the hydrophilic head group [31]. However, in the case of the hybrid detergent, a low cac was obtained although the overall polarity was increased compared to DDM and C8E4 (Fig. 2). This finding agrees with results obtained from Thota et al. who confirmed that the cac is not only affected by the overall polarity but also the overall size and geometry of detergents [21]. For example, the hybrid detergent is almost three times heavier and has a more conical shape than C8E4 (Table S1) (Fig. 1). Therefore, it is likely that a low number of hybrid detergent monomers is required to form a micelle, which is reflected in a low cac. The interplay between overall polarity, size, and shape affects cac values of detergents. The ability to tune these parameters through combining the head groups of different detergents will be important for the ability to design detergents with defined delipidating properties.

The idea that membrane protein delipidation changes with the concentration of a detergent in purification buffers is well established [16,27] and can be addressed experimentally in different ways. Examples include (i) varying the concentration of a detergent in purification steps, including extraction, affinity chromatography, or size-exclusion chromatography [3], (ii) varying the time with which protein-lipid complexes are exposed to a detergent in solution [3], and (iii) applying purification steps with the same detergent and detergent concentration repetitively, such as in the cases of multiple ultrafiltration steps [32,33]. Considering this background, we expect that designing hybrid detergents enables a new strategy to lower the detergent concentration that is required in existing purification protocols for the benefit of a mild delipidation. However, techniques that are commonly applied to monitor delipidation outcomes, *e.g.* colorimetric assays, size-exclusion chromatography, and omics methods, provide average results from analyte populations in solution. These methods can be used to assess how the composition and overall amount of co-purified lipids change in response to different detergents and purification conditions [27]. Complementary to other techniques, nMS enables to visualize the

relative amount of co-purified lipids in contact with membrane proteins after removing the detergent micelle insight a mass spectrometer [34]. Here, the combination of a newly designed hybrid detergent and state-of-the-art nMS technology allows us to show how membrane protein delipidation is driven by detergent concentrations on the level of individual protein-lipid complexes.

3. Conclusion

Taken together, we evaluated how detergent properties and membrane protein purification outcomes change when the head groups of different detergents get combined into a hybrid detergent. We present a new synthetic route for the synthesis of asymmetric, hybrid detergents. Furthermore, we show that fusing the head groups of low- and high-cac detergents can lead to hybrid detergents with comparatively low cac values. In the case of DDM and C8E4, the hybrid detergent has a higher overall polarity, which is associated with lower proteins yields during membrane protein extraction. However, our data indictate that compared to C8E4, the hybrid detergent can better provide membrane protein solubility and retain more protein-lipid interactions throughout purification. The central question addressed throughout this work is as to whether the properties of detergent micelles or the detergent concentration in purification buffers drive membrane protein delipidation. We conclude that both aspects matter. We show that protein stability and delipidation are linked to the concentration of detergents in purification buffers which in turn depend on the aggregation properties of detergent micelles. Since detergent concentrations in purification buffers are commonly adjusted to a multiple of the detergent's cac, the ability to produce low-cac detergents by combining different head groups is a meaningful opportunity for tuning delipidating properties of detergents in membrane protein research. We anticipate that hybrid detergents will facilitate the rational design of detergents for the analysis of challenging membrane proteins in the future.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Leonhard H. Urner reports financial support was provided by Fonds der Chemischen Industrie, Focus Area Nanoscale of the Freie Universität Berlin, and the Ministry of Culture and Science of the German state of North Rhine-Westphalia (NRW return program). Carol V. Robinson reports financial support was provided by European Research Council (ERC Advanced Grant No. 695511, ENABLE). C.V.R. provides consultancy services for OMass Therapeutics and I. L. is employed by that company.

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Data availability

Data supporting the findings of this work are provided with the manuscript as electronic supporting information.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbamem.2022.183958.

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