Novel Tripod Amphiphiles for Membrane Protein Analysis


Abstract: Integral membrane proteins play central roles in controlling the flow of information and molecules across membranes. Our understanding of membrane protein structures and functions, however, is seriously limited, mainly due to difficulties in handling and analysing these proteins in aqueous solution. The use of a detergent or other amphiphatic agents is required to overcome the intrinsic incompatibility between the large lipophilic surfaces displayed by the membrane proteins in their native forms and the polar solvent molecules. Here, we introduce new tripod amphiphiles displaying favourable behaviours toward several membrane protein systems, leading to an enhanced protein solubilisation and stabilisation compared to both conventional detergents and previously described tripod amphiphiles.

Keywords: amphiphiles • membrane proteins • molecular design • protein structures • stabilization

Introduction

Integral membrane proteins (IMPs) perform a wide range of functions including the transfer of biologically relevant molecules and information across the lipid bilayer of cells and organelles. Membrane proteins constitute about a third of the open reading frames in the human genome and are the targets of more than half of current pharmaceuticals.[1] Detailed structural and functional information for IMPs are essential to provide a fundamental understanding of their mechanism of action as well as to facilitate the rational design of new drug molecules. Despite extensive efforts, however, our understanding of the structure and function of IMPs lags far behind that of soluble proteins. Only a few hundred IMP structures are known, corresponding to less than 1.0% of the total number of membrane proteins. This low number is attributed to difficulties associated with the handling these proteins.[2] A key requirement of isolation and structural studies of IMPs is that they must be maintained in solution by amphipathic additives, which shield their large hydrophobic surface area from the aqueous environment. Conventional detergents such as dodecyl-\(\beta\)-d-maltoside (DDM), lauryldimethylamine-N-oxide (LDAO) and \(n\)-octyl-\(\beta\)-d-glucopyranoside (OG) are widely used to both extract IMPs from the lipid bilayer and to maintain their native state in solution.[3] However, many membrane proteins solubilised with even these popular reagents have a tendency to denature and/or aggregate,[4] excluding them from further studies.

Various classes of novel amphiphiles have been developed to tackle this challenging problem.[5] Examples include amphipols,[6,7] lipopeptide detergents (LPDs),[5] hemifluorinated surfactants (HFSs),[5e] nanodiscs (NDs),[5f] short peptide designers[5g] cholate-based facial amphiphiles[5h,i] and cholesterol-derived agents (chobimalt).[5j] The published studies, which use these agents, tend to focus on membrane protein stabilisation. In contrast, we have focused on the development of novel agents with favourable membrane protein solubilisation properties in addition to stabilisation, exemplified by tripod amphiphiles (TPAs),[5k]\[\text{m} \] maltose-neopentyl glycol (MNG)[5p] and glucose-neopentyl glycol (GNG) amphiphiles.[5q] It is noteworthy that members of the NG class have facilitated the crystal-structure determinations of more than ten new membrane proteins including several independent G-protein-coupled receptors (GPCRs).[5r]
Our previous research has shown the potential of tripod amphiphiles to both solubilise and stabilise IMPs.\(^{[5\text{n}]}\) A TPA with an N-oxide head group (the commercial name is TRIPAO) efficiently extracted bacteriorhodopsin (bR) and Rho protein from membranes (Figure S1 in the Supporting Information). TPA-solubilised bR and the potassium channel from Streptomyces lividans have been crystallised, although their structures have not yet been solved.\(^{[5\text{n}]}\) In a recent study, glyco-tripod amphiphiles (namely TPA-2 and TPA-2-S, commercially available) displayed favourable behaviours for the solubilisation and stabilisation of the photosynthetic superassembly comprised of the light-harvesting centre I (LHI) and the reaction centre (RC) complex from Rhodobacter capsulatus (Scheme 1).\(^{[5\text{n}]}\) Such generally favourable behaviours of these molecules have prompted us to expand the set of tripod amphiphiles by introducing structural variation into the previously described agents. Here, we prepared two new tripod amphiphiles and evaluated their properties with several membrane protein systems including a G-protein-coupled receptor (GPCR). We found that the new agents displayed superior properties in the solubilisation and stabilisation of membrane proteins compared to conventional detergents and previously described TPAs.

**Results**

The chemical structures of previously reported TPAs (TPA-2, TPA-2-S and TPA-5) and newly-synthesised tripod amphiphiles (TPA-5-1 and TPA-5-2) are illustrated in Scheme 1. The new amphiphiles share their hydrophilic head group with TPA-5 but have a variable hydrophobic group; the alkyl chain of TPA-5 was extended from a butyl to a hexyl chain to give TPA-5-1, whereas further modification was introduced by directly attaching the hydrophilic group to the hydrophobic group without an amide linkage to produce TPA-5-2. These molecules were easily prepared on a multigram scale that would support biochemical research (see the Supporting Information for details). All new TPAs were highly water soluble (up to \(\approx 20\) wt%). Critical micelle concentration (CMC) values were estimated by solubilisation experiments employing the hydrophobic fluorescent dye diphenylhexatriene\(^{[7]}\) and the hydrodynamic radii (\(R_\text{h}\)) of the micelles were estimated through dynamic light scattering (DLS) measurements. The data for the tripod amphiphiles (TPA-2, TPA-2-S, TPA-5, TPA-5-1 and TPA-5-2) and two conventional detergents (DDM and OG\(^{[8]}\)) are presented in Table 1. The CMC values of TPA-5-1 and TPA-5-2 are approximately 0.30 \(\text{mM}\) (0.031 wt%) and about 0.021 \(\text{mM}\) (0.002 wt%), respectively. These values are much smaller than those of TPA-5 (\(\approx 8.0\) \(\text{mM}\); 0.78 wt%) and the two branched diglucoside-bearing TPAs, TPA-2 and TPA-2-S (3.6 \(\text{mM}\), 0.24 wt% and 1.8 \(\text{mM}\), 0.12 wt%, respectively). This is likely due to the increased hydrophobicity induced by the alkyl-chain extension and the removal of the polar amide group. The small CMC values of the new agents indicate their strong aggregation tendency, which may imply the formation of protein–detergent complexes (PDCs) with enhanced stability. All TPAs except TPA-5 form smaller micelles than DDM and form a single micelle size population as assessed by DLS (see Figure S2 in the Supporting Information). [d] These values were obtained from the reference [8].

![Scheme 1. Chemical structures of previously reported (TPA-2, TPA-2-S and TPA-5) and new tripod amphiphiles (TPA-5-1 and TPA-5-2).](Image)

<table>
<thead>
<tr>
<th>Amphiphiles</th>
<th>(M_w)</th>
<th>CMC [(\text{mM})]</th>
<th>CMC [wt %]</th>
<th>(R_\text{h}) [(\text{nm})]</th>
</tr>
</thead>
<tbody>
<tr>
<td>TPA-2</td>
<td>659.8</td>
<td>(\approx 3.6)</td>
<td>(0.24)</td>
<td>(2.0±0.06)</td>
</tr>
<tr>
<td>TPA-2-S</td>
<td>665.8</td>
<td>(\approx 1.8)</td>
<td>(0.12)</td>
<td>(3.3±0.06)</td>
</tr>
<tr>
<td>TPA-5</td>
<td>984.0</td>
<td>(\approx 8.0)</td>
<td>(0.78)</td>
<td>(6.0±0.12)</td>
</tr>
<tr>
<td>TPA-5-1</td>
<td>1040.0</td>
<td>(\approx 0.30)</td>
<td>(0.031)</td>
<td>(2.7±0.02)</td>
</tr>
<tr>
<td>TPA-5-2</td>
<td>1011.1</td>
<td>(\approx 0.021)</td>
<td>(0.002)</td>
<td>(2.8±0.01)</td>
</tr>
<tr>
<td>DDM</td>
<td>510.1</td>
<td>(\approx 0.17)</td>
<td>(0.0087)</td>
<td>(3.5±0.03)</td>
</tr>
<tr>
<td>OG</td>
<td>292.4</td>
<td>(\approx 25)</td>
<td>(0.73)</td>
<td>1.5–2.3(^{[9]})</td>
</tr>
</tbody>
</table>

[a] Molecular weight of the detergents. [b] The hydrodynamic radius of the micelles as determined at 1.0 wt%. [c] Two forms of aggregates were observed with hydrodynamic radii of approximately 2.2 and 75 nm (see Figure S2 in the Supporting Information). [d] These values were obtained from the reference [8].

}\(^{[2]}\)
perassembly from the membrane and thus this amphiphile was not included in this experiment. The structural stability of SQR was assessed with a reactive probe, N-[4-(7-diethylamino-4-methyl-3-coumarinyl)phenyl]maleimide (CPM).\[10] In this assay, the probe molecules react with thiol groups that become solvent-accessible upon protein unfolding, leading to fluorescence emission. The assay can therefore be used to estimate the relative amount of protein unfolding by monitoring the fluorescence change of the assay solutions. SQR with four subunits (SdhA, SdhB, SdhC and SdhD) was initially extracted from the native membrane with DDM. The pure protein was diluted to generate solutions containing CMC = 0.04 wt% of the amphiphatic agents. The protein integrity was monitored at the elevated temperature (30°C) for 130 min. Under these conditions, the previously reported tripod amphiphile TPA-2 was comparable to DDM at maintaining the folding structure of SQR, whereas TPA-2-S and TPA-5-1 were worse than DDM (Figure 1a). Notably, TPA-5-2 was better than DDM. When we increased the detergent concentration to CMC = 0.2 wt%, all TPAs except TPA-2-S were comparable to DDM (Figure S3a in the Supporting Information). Similar results were observed for another membrane protein, the rhomboid intramembrane serine protease GlpG (Figure S3b in the Supporting Information).\[11]

We next turned to the bacterial leucine transporter (LeuT) from *Aquifex aeolicus*.[12] The protein activity was estimated by assessment of radiolabelled leucine binding using the scintillation proximity assay (SPA).[13] The transporter was initially extracted with 1.0 wt% DDM and then diluted in solutions containing the individual amphiphiles (DDM, TPA-5-1, TPA-5-2, TPA-2 and TPA-2-S). Figure 1b shows that at lower amphiphile concentration (i.e., CMC + 0.04 wt%) TPA-2- and TPA-2-S-solubilised LeuT showed a rapid activity loss, similarly to our previous observations for LDAO- and OG-solubilised samples.[16] TPA-5-1, the amphiphile with an amide linkage to the hydrophobic group, was more efficient than the glucose-bearing TPA agents (TPA-2 and TPA-2-S), yet inferior to DDM, in maintaining the transporter activity during the twelve day experimental period. In contrast, TPA-5-2, the amphiphile with a direct connection to the hydrophobic group, displayed a favourable stabilising effect comparable to DDM. When we increased the detergent concentration to CMC = 0.2 wt%, similar results were observed (Figure S4a in the Supporting Information). For a continuing evaluation of the TPAs with bacteriorhodopsin (bR), the protein was initially solubilised from the native purple membrane with 2.0 wt% octyl-β-D-thiogluco side (OTG), the most commonly used detergent for manipulation of this protein.[14] Following ultracentrifugation to remove insoluble debris, the bR solution was diluted with amphiphile-containing solutions to give the final concentration of 0.2 wt% OTG and 0.8 wt% TPA; TPA-2-S was not included in this experiment because the agent consistently showed the worst results among the current TPA set for the stability of the proteins investigated. The stability of bR was assessed by spectrophotometry measuring the strong absorbance at λ = 554 nm of the native structure. When the absorbance was monitored over a period of twenty days, two of the new agents, TPA-5-1 and TPA-5-2, were observed to be more effective than OTG and TPA-2 at maintaining the native structure of bR, with TPA-5-2 being the best agent (Figure S4b in the Supporting Information).

We then turned to a photosynthetic superassembly, composed of the light-harvesting complex I (LHI) and the reaction centre (RC), from *R. capsulatus*.[15] This LHI–RC superassembly contains a large number of protein subunits and multiple cofactors such as bacteriochlorophyll and carotenoids, thereby giving a highly characteristic UV/Vis absorption spectrum. Thus, the protein integrity can be unambiguously assessed by spectrophotometry. We monitored the absorbance ratio (A875/A680) of protein solutions to investigate the integrity of the LHI–RC complexes (the absorbances at λ = 875 and 680 nm arise from cofactors embedded in the native conformation of the complexes and the oxidation of bacteriochlorophyll freed from LHI upon denaturation, respectively). The superassembly was extracted from the native membrane with 1.0 wt% DDM and purified with the same detergent at the CMC (0.009 wt%) through Ni²⁺-NTA affinity chromatography, utilising a seven-membered
histidine tag on the C terminus of the M-subunit of the RC. This protein was diluted with amphiphile-containing solutions, so that the final concentration of each agent is CMC+0.04 wt%, with residual DDM far below its CMC (0.0004 wt%). As expected from a previous study, the LHI-RC complex in the two conventional detergents (OG and LDAO) was rapidly destroyed. In contrast, TPA-2-, TPA-5-1- and TPA-5-2-solubilised complexes showed enhanced stability over twenty days as compared to DDM-solubilised sample with the best results achieved by using TPA-5-2 (Figure 2a). Similar results were obtained by increasing detergent concentrations (Figure S5 in the Supporting Information).

![Figure 2](image)

**Figure 2. a) Stability of the R. capsulata superassembly (● = TPA-2, ○ = TPA-5-2, □ = DDM, ▼ = LDAO and ▲ = OG) and b) activity (white bars = 0, black dashed bars = 24 and black bars = 48 h) of β2AR WT in TPAs and conventional detergents (1%) as a function of time. The superassembly was solubilised in individual detergents at CMC+0.04 wt% and the stability was assessed by measuring the absorbance ratio (A875/A680) over twenty days. β2AR WT was extracted with 1.0 wt% DDM, TPA-5-1 or TPA-5-2, and its activity was measured over time by a radioligand binding assay by using the antagonist [3H]-dihydroalprenolol during the storage of the receptor samples at 4°C.**

In the examples discussed so far, we used conventional detergents (DDM or OTG) to solubilise and isolate the individual IMPs from the membranes. The purified proteins were then diluted in amphiphile-containing solutions for the evaluation of the novel agents. Within this protocol, there is a possibility that the small residual conventional detergent could influence the protein stability. To avoid this possibility, the newly synthesised TPAs, TPA-5-1 and TPA-5-2, and DDM were used to extract the wild-type β2 adrenergic receptor (β2AR WT, a representative of the human G-protein-coupled receptor (GPCR) family), from the membrane and the receptor stability was monitored over 48 h at 4°C. The agents were selected based on a preliminary experiment with β2AR-T4L, showing that these TPAs resulted in similar Tm values for the purified receptor to that of DDM. A radioligand binding assay employing the antagonist [3H]-dihydroalprenolol was utilised to measure the receptor activity. The DDM-solubilised receptor showed high initial activity but rapidly lost almost all activity (Figure 2b). TPA-5-1-solubilised receptor showed a low initial activity as well as a rapid reduction in activity. In contrast, the receptor solubilised in TPA-5-2 displayed almost a third higher initial activity compared to DDM and maintained two-thirds of the maximal activity after 48 h.

Because TPA-5-2 consistently displayed the best behaviours for several membrane protein systems, we further characterised this agent by using a fusion protein of the human transporter CMP-Sia with a C-terminal GFP expressed in *Saccharomyces cerevisiae*. The protein solubilised by 1.0 wt% DDM, TPA-5-1 or TPA-5-2 was analysed by fluorescent size exclusion chromatography (FSEC). TPA-5-1 and TPA-5-2 gave solubilisation efficiencies comparable to that displayed by DDM. In addition the TPA-solubilised protein yielded a single monodispersed peak almost identical to that obtained by using DDM (Figure S6 in the Supporting Information). The favourable property of TPA-5-2 at extracting β2AR WT and the CMP-Sia fusion protein from the membranes led us to examine this agent with *Salmonella typhimurium* melibiose permease (MelB) expressed in *E. coli*. For protein extraction, 1.5 wt% TPA-5-2 and DDM were used at 0°C for 10 min, and then the aggregated material was removed through ultracentrifugation. The amount of solubilised MelB was determined after ultracentrifugation by using a Western blot (Figure 3). Both TPA-5-2 and DDM efficiently extracted MelB under these conditions, indicating comparably good solubilising properties of these agents. To investigate the detergent efficacy on the MelB thermostability, we elevated the temperature of the solubilising solution to 45, 55 and 65°C with an increased incubation time of 90 min. DDM yields detectable MelB at 45°C, but soluble protein could not be detected at 55°C after ultracentrifugation; we assume that DDM-solubilised proteins denatured and/or aggregated at this temperature. In contrast, TPA-5-2-solubilised MelB retained its soluble form even at 55°C, further demonstrating the superior properties of the agent to conventional detergents for membrane protein stabilisation. Taken together, these data indicate that TPA-5-2 is useful for both efficient solubilisation and enhanced stabilisation of IMPs.

**Discussion**

Detergents with an appropriate hydrophile–lipophile balance (HLB) are known to be useful for membrane protein manipulation. Conceivably, the strength of the interaction...

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between the detergent molecules and the hydrophobic surfaces of membrane proteins could be optimised to effectively stabilise encapsulated protein structures. Specifically, a relatively hydrophilic detergent would weakly bind to membrane proteins, leading to protein aggregation, whereas a rather hydrophobic detergent will interact too strongly with the proteins, resulting in the destruction of forces stabilising the protein structure. In a previous study, we found that the TPA architecture with branched head and tail groups has an advantage over conventional detergent architectures, that is, linear structures.[5] However, TPA-5 was previously shown to be unsuccessful in solubilising the R. capsulatus superassembly from the native membrane.[5m] We reasoned that the poor behaviour of this agent could be attributed to an imbalance between the hydrophobic and hydrophilic domains. For example, TPA-5 has a large hydrophilic group and a comparatively small hydrophobic group. Therefore, we increased the length of the alkyl chain and, in addition, removed the polar amide group to give TPA-5-1 and TPA-5-2, respectively. Thus, these new TPAs were rationally designed to attain an optimum HLB, without disruption of the favourable architectural features. The current results show that TPA-5-2, with the largest hydrophobic region was the best, supporting this design principle.

Detergents with a good track record for membrane protein crystallisation such as OG and DDM appear to have some common attributes: good solubilisation and stabilisation efficacy. Similar properties have been demonstrated for novel classes of amphiphiles including the TPA, GNG and MNG amphiphiles; all of these classes have produced high quality membrane protein crystals.[5n-4] Novel agents with a good stabilisation efficacy but an unfavourable solubilisation efficiency, on the other hand, have failed in providing new crystal structures of membrane proteins. On the basis of this analysis, we reached the conclusion that the detergent stabilisation characteristics, TPA-5-2 holds significant potential for the crystallisation of membrane proteins.

The detergent properties can be significantly improved by introducing small variations in their hydrophilic and/or hydrophobic regions. For instance, glucose-containing conventional detergents display a significantly different behaviour from maltose-containing detergents. The widely used detergents OG and DDM are structurally similar, but it is known that these agents display quite different properties in terms of solubilisation, stabilisation and crystallisation of membrane proteins. A similar trend was observed for the GNG and MNG cases.[5q] GNG amphiphiles tend to form smaller PDCs but are less stabilising, whereas MNG amphiphiles were previously shown to be more stabilising but tend to form large PDCs. Another instance can be seen in our very recent report on carbohydrate analogues of triton X-100, designated as CGTs; by the replacement of the oxyethylene glycol units of triton X-100 with glucose or maltose, the stabilisation propensity was significantly enhanced (Figure S7 in the Supporting Information).[20] In addition to the effect of hydrophilic group variation on detergent properties discussed above, a hydrophobic variation may also result in a big change in the detergent properties. Examples include the HF-MNGs (vs. MNGs; Figure S8 in the Supporting Information).[24] The current study provides further supportive data; the small structural variation in the TPA architecture, exemplified by the small differences among TPA-5, TPA-5-1 and TPA-5-2, leads to significant differences in the detergent behaviour toward diverse membrane proteins.

There are a large number of membrane proteins yet to be structurally characterised. This is particularly true for many classes of eukaryotic IMPs, which are much less stable in solution than are the prokaryotic homologues. Thus, it is necessary to develop a number of alternative tools, including the TPAs and MNGs, for membrane protein studies. In ad-
dation, it is essential to assess these tools by using multiple membrane protein systems as described here in order to establish utility across membrane protein groups.

**Conclusion**

We have conveniently prepared new tripod amphiphiles by taking advantage of a modular synthetic approach. These new agents, especially TPA-5-2, confer a favourable solubilisation efficiency and an enhanced stabilisation efficacy for several membrane protein systems, relative to previously reported examples. Additional questions should be addressed to maximise the utility of these new agents. For instance, the physicochemical properties of the amphiphiles, such as the aggregation number and the aqueous surface tension, need to be determined. A concerted effort also needs to be made to evaluate TPA-5-2 for membrane protein crystallisation. However, the current study clearly shows that TPA-5-2 has significant potential for membrane protein manipulation. More importantly, it is significant that this promising TPA was obtained through rational design based on the general principle that the hydrophilic–lipophilic balance plays a critical role in membrane protein solubilisation and stabilisation properties. This design principle should be useful for future development of novel amphiphiles for biochemical applications.

**Experimental Section**

**Synthesis and characterisation of the amphiphiles and membrane protein solubilisation:** Details can be found in the Supporting Information.

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