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New penta-saccharide-bearing tripod amphiphiles for membrane protein structure studies†

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Abstract

Integral membrane proteins either alone or as complexes carry out a range of key cellular functions. Detergents are indispensable tools in the isolation of membrane proteins from biological membranes for downstream studies. Although a large number of techniques and tools, including a wide variety of detergents, are available, purification and structural characterization of many membrane proteins remain challenging. In the current study, a new class of tripod amphiphiles bearing two different penta-saccharide head groups, designated TPSs, were developed and evaluated for their ability to extract and stabilize a range of diverse membrane proteins. Variations in the structures of the detergent head and tail groups allowed us to prepare three sets of the novel

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Conflicts of interest

The authors declare the following competing financial interest(s): P. S. C. and M. E. are inventors on a patent application of TPSs/TPS-Ls.

agents with distinctive structures. Some TPSs (TPS-A8 and TPS-E7) were efficient at extracting two proteins in a functional state while others (TPS-E8 and TPS-E10L) conferred marked stability to all membrane proteins (and membrane protein complexes) tested here compared to a conventional detergent. Use of TPS-E10L led to clear visualization of a receptor-G_s complex using electron microscopy, indicating profound potential in membrane protein research.

Introduction

Integral membrane proteins account for approximately one third of total cellular proteomes.¹ These bio-macromolecules are important for a range of biological processes including signal transduction and membrane transport.² Membrane proteins such as G protein-coupled receptors (GPCRs) and ion channels represent more than 50% of all current drug targets.^{3,4} Despite the fundamental importance of membrane proteins in drug discovery, structural and functional studies of these molecules are hampered by their limited solubility and stability in aqueous solution.⁵ Detergents are amphipathic molecules, widely used to maintain the structure and function of target proteins in the course of protein solubilization, purification and crystallization. Conventional detergents, exemplified by *n*-octyl- β -D-glucoside (OG), lauryldimethylamine-*N*-oxide (LDAO) and *n*-dodecyl- β -D-maltoside (DDM), are widely used in membrane protein manipulation.⁶ Both detergents and lipid molecules are amphiphilic, but their self-assemblies have different architectures. Detergents tend to form micelles with a spherical or elliptical shape, while lipid molecules form planar bilayers. Because of this inherent difference in self-assembly, detergent micelles differ significantly from the lipid bilayer in many aspects. Lipid bilayers produce lateral pressure optimal for membrane protein structure and function, but detergent micelles are highly dynamic and are much less effective at providing this stabilizing force. However, detergent micelles are generally a more suitable environment for membrane protein crystallization, although some lipidic systems such as bicelles and lipidic cubic phase (LCP) have been successfully used.⁷ Detergent micelles have the ability to both dismantle the lipid bilayers and associate with the membrane proteins, leading to efficient protein extraction and protein-detergent complex (PDC) formation.⁸ In contrast, the lipidic systems are unable to extract membrane proteins from the membranes.

Conventional detergents typically comprise a single flexible alkyl chain attached to a large hydrophilic head group.⁹ Some notable detergents that deviate from this conventional architecture, such as CHAPS and the Tween detergents, tend to be suitable for only a small number of membrane proteins. Of the more than 120 conventional detergents, only a handful of detergents are widely used for membrane protein study such as DDM and OG. Membrane proteins encapsulated even by these popular detergents often tend to undergo structural and functional degradation over time, hampering advances in membrane protein structural studies.^{6b,10} In contrast to the limited structural diversity and micellar properties of conventional detergents, there are a wide range of membrane proteins with large variability in terms of their tendency to aggregate and denature. To cope with the large diversity of membrane proteins, it is important to develop a range of novel amphiphiles with distinct architectures, making them suitable for use with large numbers of membrane proteins.¹¹

To get around the issues associated with conventional detergents, many novel agents with unique architecture have been actively developed over the last two decades. Large molecules or assemblies such as amphipols (Apol),¹² peptide-based agents (*e.g.*, lipopeptide detergents (LPDs),¹³ β -peptides (BPs)¹⁴ and short peptides),¹⁵ nano-assemblies (*e.g.*, nanodiscs (NDs)¹⁶ and nanolipodiscs¹⁷) have proved to be particularly effective at stabilizing the structures of multiple membrane proteins. In addition, some of these agents were found to be useful in applications such as nuclear magnetic resonance (NMR) spectroscopy and electron microscopy (EM).¹⁸ However, they tend to form large protein-detergent complexes (PDCs) and are often inefficient at extracting membrane proteins from the membranes. Furthermore, these agents are often difficult to synthesize in bulk, preventing their wide spread use for membrane protein study. Most importantly, they are not usually suitable for use in crystallization trials. This limits their application since X-ray crystallography remains the most successful technique for membrane protein structural determination.

In contrast, small amphipathic agents have much greater potential since they are efficient at extracting proteins from the membranes and some representatives have a proven track record in high resolution X-ray crystallographic structure determination. Examples of these novel amphiphiles include tripod amphiphiles (TPAs),¹⁹ facial amphiphiles (FAs),²⁰ neopentyl glycol (NG) amphiphiles (GNGs/MNGs/NDTs),²¹ xylene- or mesitylene-based amphiphiles (XMA/MGAs),²² mannitol-based amphiphiles (MNAs),²³ and penta-saccharide amphiphiles (PSEs).²⁴ Among these agents, the NG class agents (MNG-3 and GNG-3) have contributed to the crystal structure determinations of more than 30 new membrane proteins in the last six years, including several GPCRs.²⁵ Despite the development of dozens of novel agents, progress in membrane protein structural study remains slow. Continuous efforts are necessary to develop novel detergents suitable for high resolution EM and X-ray crystallographic studies. In this study we introduce a class of amphiphiles combining a penta-saccharide hydrophilic head group and three hydrophobic alkyl chains. We designated these agents tripod penta-saccharide amphiphiles (TPSs; Scheme 1). In addition, several membrane proteins including a GPCR, were markedly more stable in selected TPSs than a conventional detergent (DDM). Furthermore, when a representative TPS was utilized for EM-based protein structure analysis, the individual subunits of a GPCR-G_s complex were clearly visualized.

Results and discussion

The amphiphiles described herein are structurally distinct from the classical head-to-tail detergents which have a relatively simple architecture of a comparatively bulky head group attached to a single, long lipophilic tail group. The design of the TPSs features three alkyl chains and a penta-saccharide head group. The alkyl chains were attached to the head groups *via* either an ether linkage (TPS-Es) or through a direct connection (TPS-As). The hydrophilic groups comprise five glucose units: a central glucose core and four peripheral glucose units. The central glucose core is conjugated with the peripheral glucose groups either directly (TPS-A/Es) or *via* propylene spacers (TPS-ELs). Thus, the glucose core serves as an easily accessible platform for generating new hydrophilic groups as shown in Scheme 1. The tripod penta-saccharide amphiphiles described here are distinct from other novel agents we have recently developed. For instance, these agents have a penta-saccharide

as a head group while tripod amphiphiles (TPAs) bear branched diglucosides or maltosides.¹⁹ These two classes are also different in terms of chain length difference between the three alkyl groups in the lipophilic regions; the three alkyl chains of the TPSs are basically identical, but the TPAs have two identical alkyl chains and a single phenyl ring. In comparison with the PSEs, the TPSs share the penta-saccharide head group, but differ in containing three alkyl chains rather than two.²⁴ The TPS-ELs further differ, as these agents also include multiple propylene spacers to generate the new hydrophilic group. As can be seen in the results below, the introduction of the flexible propylene spacers in the detergent head group appears to play a favorable role in stabilizing a GPCR, contributing to improved EM images of the GPCR-G_s complexes. The TPSs (TPS-As, TPS-Es and TPS-ELs) additionally vary in the alkyl chain length. Note that this variation is simple yet essential to find a TPS agent with an optimal hydrophile–lipophile balance (HLB). It is known that an optimal HLB is necessary for effective membrane protein stabilization and crystallization.²⁶ The length of the alkyl chains varied from C6 to C11 (used for detergent designation) depending on the hydrophilicity of the detergent head groups. The relatively short alkyl chains, C6 to C8, were used for the TPS-A/Es. However, longer alkyl chains, C8 to C11, were required to balance the increased hydrophilicity of the TPS-ELs.

These agents were synthesized using straightforward synthetic pathways (see in ESI Schemes I & II†) comprising six or eight steps. To introduce the glucose core or glucose pendants, we employed a glycosylation method utilizing silver triflate (AgOTf) and perbenzoylated glucosylbromide. In the reaction, the protecting group neighboring the anomeric carbon dictates the stereochemistry of the glycosylated products. As the benzoyl protecting group participates in glycosidic bond formation *via* the 5-membered cyclic intermediate (*i.e.*, acyloxonium ion), known as anchimeric assistance, all glycosidic bonds were expected to be β .²⁷ The stereochemistry of the glycosylated products could be readily confirmed by ¹H NMR spectra of the final amphiphiles dissolved in CD₃OD. The proton attached to the anomeric carbon (C1) was coupled to the neighboring C2 proton with a vicinal coupling constant (³*J*) of 8.0 Hz as both protons are in the axial position. This *J* value, typical for β -anomeric protons, is significantly larger than that of the α -anomeric proton (³*J* = 4.0 Hz). The five anomeric protons of TPS-A8 and TPS-E8 with the rigid head group gave five well-dispersed doublet peaks with ³*J* = 8.0 Hz in the range of 4.25–4.95 ppm (Fig. 1a and b). In the case of TPS-E10L, it was hard to precisely determine the coupling constant for the anomeric peaks because of their serious overlap. However, the same coupling constants were estimated for the most prominent doublet peak at 4.22 ppm and a small doublet peak centered at 4.15 ppm. The loss of both the directionality of the terminal glucose units with respect to the central ring and the conformational rigidity of the headgroup, originating from the introduction of the propylene spacers, is likely to be responsible for the poor dispersion of those anomeric peaks. A further evidence for the β -anomeric product is that for TPS-E10L all five anomeric peaks appeared around 4.2 ppm, typical for β -anomeric protons. This NMR analysis supports the fact that five β -glycosidic bonds were formed for TPS-A8 and TPS-E8 as well as TPS-E10L during glycosylation (see Fig. S1† for the full NMR spectra of these amphiphiles). The same β -stereochemistry was observed for the other agents prepared in this study, as shown by their individual ¹H NMR spectra (see ESI†). Thus, β -selective glycosylation allowed preparation of the individual

TPSs to a high enantiomeric purity. Furthermore, the ease of synthesis along with high synthetic yields makes it possible to prepare these agents in multi-gram quantities. Solubility tests showed that all the TPSs were water-soluble at more than 5.0 wt% except TPS-A8, TPS-E8 and TPS-E11L which were soluble up to 1.0 wt%. The critical micelle concentrations (CMCs) of these detergents were measured through encapsulation of a hydrophobic fluorescent dye, diphenylhexatriene (DPH),²⁸ and calculated by determining a cross point of two regression curves, as exemplified by TPS-E10L (Fig. 2a). The micelle sizes of the TPSs were determined as a hydrodynamic radius (R_h) via dynamic light scattering (DLS). The summarized data for these synthetic detergents and a conventional detergent (DDM) are presented in Table 1.

The CMC values of all the TPSs lie within a range from 1 to 70 μ M, all significantly smaller than that of DDM (170 μ M), indicating a strong tendency of these agents to form self-assemblies. The CMC values of the three sets of detergents (TPS-As, TPS-Es and TPS-ELs) decreased with increasing alkyl chain length mainly due to the increasing hydrophobicity of the lipophilic groups. This trend is consistent with the general notion that detergent hydrophobicity is a critical factor in determining detergent CMCs. Interestingly, the TPS-As gave higher CMC values than the TPS-Es. For example, the CMC value of TPS-A6 was 70 μ M, much higher than that of the same alkyl chain length TPS-E (TPS-E6; 20 μ M). The TPS-ELs gave particularly small CMCs, likely due to the long alkyl chain length. TPS-E11L with the longest alkyl chain length (C11) gave the smallest CMC value of 1 μ M, 170 times smaller than that of DDM. The micelle sizes of the TPSs showed a large variation ranging from 2.4 to 43.8 nm. The TPS-As tend to form micelles smaller than those formed by the TPS-E counterparts. For example, micelles formed by TPS-A6 gave an R_h of 2.4 nm, while micelles formed by TPS-E6 gave 2.9 nm. The functional group variation in the linker region (ether *vs.* alkyl) is, at least in part, responsible for differences in micelle size between these two sets. The sizes of micelles formed by the TPS-ELs were smaller than those formed by TPS-Es (*e.g.*, TPS-E8 (43.8 nm) *vs.* TPS-E8L (4.6 nm)). Even long alkyl chain TPS-ELs (TPS-E9L and TPS-E10L) were smaller than TPS-E8 in terms of micelle size. As the head group of the TPS-ELs is much larger than that of TPS-As/Es, the geometry of these agents would be more conical than that of TPS-As/Es, accounting for the relatively small micelles formed by the TPS-ELs compared to the TPS-As/Es. Interestingly, a large increase in micelle size was observed when detergent alkyl chain length increased from C7 to C8 for the TPS-Es or from C10 to C11 for the TPS-ELs. Because of the presence of three alkyl chains, the extension of the individual alkyl chains by one carbon unit causes a significant increase in the volume of the hydrophobic group, resulting in a drastic change in molecular geometry from a conical to a cylindrical shape, and thus associated micelle size. Similar trends have been observed for other novel agents with multiple alkyl chains.^{16,21e}

In all sets of the TPSs, the micelles increased in size with increasing alkyl chain length. For instance, the R_h values of micelles formed by the TPS-As increased from 2.4 to 2.7 to 9.1 nm with increasing alkyl chain length from C6 to C7 to C8. Similar trends were observed for the TPS-Es and TPS-ELs. Detergent micelles were further characterized by TEM analysis. This analysis showed that TPS-E10L tended to form spherical micelles and its estimated micelle size (10–20 nm in diameter) was roughly consistent with that obtained from DLS (6–20 nm) (Fig. 2b and c). A similar consistency in micelle size was obtained from the TEM

images of TPS-E8 or TPS-E11L micelles although a larger variation in particle size was observed in TEM (Fig. S2†). Further analysis of the DLS data revealed that all TPS-As, TPS-Es and TPS-ELs give a single set of micelles in the number-averaged size distribution (Fig. S3†). This minor inconsistency between the DLS and TEM analysis data may be due to the difference in the sample preparation method; wet and dried samples were used for DLS and TEM measurements, respectively.

Detergent evaluation with membrane proteins

The new detergents were first evaluated as biochemical tools with a bacterial leucine transporter (LeuT) from *Aquifex aeolicus*.^{29,30} Protein stability was assessed by measuring the ability of the transporter to bind a radio-labeled substrate ($[^3\text{H}]$ -leucine) using scintillation proximity assay (SPA).³¹ The transporter was initially solubilized with 1.0% DDM and purified in 0.05% of the same detergent. DDM-solubilized LeuT was subsequently diluted into solutions containing individual TPSs or DDM. The final concentration of each detergent (TPS-A/Es, TPS-ELs, or DDM) was CMC + 0.04 wt% or CMC + 0.2%. The residual DDM concentration was estimated to be 0.027 wt%. The effect of the detergents on LeuT stability was monitored at regular intervals over a 12-day incubation period at room temperature. All the tested amphiphiles except TPS-A6 were better than DDM at stabilizing LeuT (Fig. 3a). In the comparison of TPS-As with TPS-Es, the ether versions (TPS-Es) performed better than the alkyl versions (TPS-As), with the best performance observed for TPS-E8, followed by TPS-E7. When the transporter was solubilized in the best agent (TPS-E8), transporter activity was fully retained over the course of the 12-day incubation. The ability of a detergent to maintain transporter function was enhanced with increasing alkyl chain length with the longest alkyl chain containing detergents, TPS-A8 and TPS-E8, being the best of the two sets. When detergent concentration was increased to CMC + 0.2 wt%, a similar stability pattern was obtained. All the TPS-A/Es except TPS-A6 were clearly superior to DDM, while TPS-A6 was just comparable to this conventional detergent (Fig. 3b). However, differences in retaining transporter activity between the novel agents were relatively small; all the TPS-Es and TPS-A8 appeared to be similar to each other in this regard. In the case of the TPS-ELs, detergent efficacy was reduced overall compared to TPS-A/Es. All of these detergents except the longest alkyl chain agent (TPS-E11L) were slightly better than DDM, while TPS-E11L was just comparable to DDM (Fig. S4a and b†). The importance of detergent head group was found in the comparison of TPS-E8 vs. TPS-E8L; these two agents share exactly the same tail and linker groups, but TPS-E8 was superior to TPS-E8L. The absence of the propylene spacers in the penta-saccharide head group of TPS-E8 is likely responsible for the enhanced behavior of this agent. Collectively, the results revealed that the TPS-Es, particularly TPS-E7 and TPS-E8, were superior to the TPS-As and TPS-ELs at retaining the long-term ability of the transporter to bind the substrate.

Next, the TPSs (TPS-As, TPS-Es and TPS-ELs) were evaluated with a eukaryotic transporter, UapA, the uric acid–xanthine/ H^+ symporter from *Aspergillus nidulans*. A thermostabilised UapA construct was used (see ESI† for details).³² Protein stability was assessed using fluorescence spectroscopy with the assistance of a sulfhydryl-specific fluorophore, *N*-[4-(7-diethylamino-4-methyl-3-coumarinyl)phenyl]maleimide (CPM).³³ In

this assay, the maleimide group of the probe is highly reactive with solvent-accessible thiol groups that are generated by protein unfolding, leading to an increase in fluorescence intensity. The assay can therefore be used to estimate the relative amount of protein unfolding by monitoring changes in fluorescence intensity over the course of protein thermal denaturation. UapA was first solubilized in 1.0% DDM and purified in 0.03% DDM. The DDM-purified transporter was diluted into buffer solutions containing individual amphiphiles (DDM, MNG-3, TPS-As, and TPS-Es) to give a final concentration of the test detergents of CMC + 0.04 wt% and a residual DDM concentration of 0.006 wt%. Following the addition of the CPM dye, the fluorescence intensities of the protein-detergent samples were measured every few minutes during a 120 min incubation at 40 °C. As can be seen in Fig. 4a, all the TPSs appeared to be better than DDM at maintaining the folded state of the transporter. No substantial difference was observed between the TPSs at this relatively low detergent concentration. At an increased detergent concentration of CMCs + 0.2 wt%, detergent efficacy differentiation between these novel agents was substantially increased. Among the TPSs, TPS-E8 was most effective at maintaining the folding structure of UapA (Fig. 4b), consistent with the result obtained from detergent evaluation with LeuT. The other TPSs were more or less comparable to each other, yet were significantly better than DDM. Intriguingly, all TPS-A/Es were better than MNG-3, a significantly-optimized novel agent, at stabilizing the transporter at both detergent concentrations. A similar trend was observed for the TPS-ELs; at a detergent concentration of CMC + 0.04 wt%, all these agents behaved more or less similar to each other, but an enhanced differentiation in detergent efficacy was observed at CMC + 0.2 wt%, with the best performance obtained using TPS-E11L (Fig. S5a and b†). At both detergent concentrations, all the TPS-ELs were substantially better than DDM and MNG-3 (Fig. S5a and b†). Taken together, TPS-E8 and TPS-E11L imparted the greatest stability to the transporter, indicative of favorable architecture of these agents for UapA stability.

Encouraged by the results with the prokaryotic and eukaryotic transporters (LeuT and UapA, respectively), these TPSs were further evaluated with a G protein-coupled receptor (GPCR), ³⁴ human β_2 adrenergic receptor (β_2 AR). For this experiment, the receptor was extracted from the membranes using 1.0% DDM and purified in 0.1% of the same detergent. Detergent exchange from DDM to a TPS-A/E was carried out by diluting the DDM-purified receptor into each detergent-containing buffer solution. Following dilution, the residual DDM concentration was 0.0007 wt%. At a final detergent concentration of CMC + 0.2 wt%, stability was assessed by measuring the ability of the receptor to bind the radio-labeled antagonist ([³H]-dihydroalprenolol (DHA)).³⁵ As a preliminary study, we measured initial receptor activity 30 min after detergent exchange. The TPS-Es were substantially more effective than their TPS-A counterparts with the same alkyl chain length (*e.g.*, TPS-E7 *vs.* TPS-A7) at stabilizing the receptor, with TPS-E8 being the best (Fig. S6a†). In addition, detergent efficacy was largely enhanced with increasing alkyl chain length, the same behavior observed for detergent evaluation with LeuT. Only TPS-E8 of the TPS-A/Es was comparable to DDM in this preliminary result. When the TPS-ELs were evaluated, however, all the agents (TPS-E8L, TPS-E9L, TPS-E10L and TPS-E11L) were as effective as DDM (Fig. S6b†). Based on these initial results, the four promising agents (TPS-E8, TPS-E9L, TPS-E10L and TPS-E11L) were selected for further evaluation. For this purpose, ligand

binding activity of the receptor was measured at regular intervals during a 3 or 5-day incubation at room temperature. The DDM-solubilized receptor showed high initial activity, but rapidly lost activity over time (Fig. S7a†). An improved performance was observed for TPS-E8. Following the 3-day incubation, receptor activity solubilized in this agent was three times higher than that in DDM. Protein in TPS-E9L, TPS-E10L and TPS-E11L also resulted in better retention of receptor binding activity compared to DDM or DDM + cholesterol hemisuccinate (CHS), over the test period (Fig. 5). CHS is known to stabilize a number of GPCRs *via* binding with transmembrane regions of the receptors.³⁶ TPS-E10L efficacy was able to preserve ~90% initial receptor activity even after a 5-day incubation (Fig. 5a). Such enhanced long-term stability of the receptor indicates that this agent holds significant potential for GPCR structural study.

The promising behavior of TPS-E10L with the receptor prompted us to assess the efficacy of this agent in stabilising the receptor-G_s complex. For this experiment, β_2 AR and G_s protein individually isolated in DDM were combined to produce DDM-purified β_2 AR-G_s complex. As a next step, DDM was exchanged with TPS-E10L and the stability of the resulting β_2 AR-G_s complex was assessed by size exclusion chromatography (SEC). The complex purified in TPS-E10L maintained its full integrity over a 7-day incubation at room temperature (Fig. 5b), in contrast to a substantial dissociation of the complex purified in DDM within 2 days.³⁷ We next moved to further evaluate this agent in terms of direct visualization of this GPCR complex. Negative stain images of β_2 AR-G_s complexes in TPS-E10L revealed highly homogeneous particles suitable for generation of 2D class averages (Fig. 6a and b). This is in contrast to substantial particle aggregation observed for β_2 AR-G_s complex sample prepared in DDM.²³ The individual domains of the complex (β_2 AR, G_{αS} and G_{βγ}) were discernable by representative class averages (Fig. 6c) and furthermore the subdomains of G_{αS} (the Ras and α-helical domains) and G_{βγ} (G_β and G_γ domains) could be distinctively visualized (the central image in Fig. 6c). Combined together, these results indicate that TPS-E10L has potential in the Cryo-EM-based structural study of both GPCRs and their signaling protein complexes.

In order to investigate the ability of the TPSs to extract membrane proteins from the membranes, we selected some representative TPSs (TPS-A8, TPS-E6, TPS-E7, TPS-E8, and TPS-E10L) and used these for extracting melibiose permease of *Salmonella typhimurium* (MelB_{St}).³⁸ Initially, *E. coli* membranes containing MelB_{St} were treated with individual TPSs at six different temperatures (0, 25, 35, 45, 55, and 65 °C) for 90 minutes. After separation by ultracentrifugation, the resulting protein solutions were analyzed by SDS-PAGE and Western blotting. As expected from a previous study,²⁴ DDM was effective at extracting this protein and maintaining its solubility in a temperature range from 0 to 45 °C, but protein extraction at a higher temperature (55 or 65 °C) yielded little or no soluble MelB_{St}, probably due to protein denaturation/aggregation under those conditions. When the TPSs were used for MelB_{St} extraction under the same conditions, these agents displayed significantly different behaviors from each other. Similar to DDM, TPS-A8, TPS-E6 and TPS-E7 were efficient at extracting MelB_{St} and effective at keeping this protein in a soluble state at a low to medium temperature range (0 to 45 °C). TPS-E8 and TPS-E10L were, however, poor in this context. When incubation temperature was increased to 55 °C, of all the tested TPSs, TPS-A8 generated the largest amount of soluble MelB_{St}, followed by TPS-

E7, indicating that these two agents (TPS-A8 and TPS-E7) are not only efficient at extracting MelB_{St} from the membrane, but are also effective at maintaining the solubility of the protein. These promising results encouraged us to evaluate the effects of the novel TPS agents on MelB_{St} function by monitoring the binding of a fluorescent galactoside, 2'-(*N*-dansyl)aminoalkyl-1-thio- β -D-galactopyranoside (D₂G), *via* melibiose reversal of Förster resonance energy transfer (FRET).^{38a,d,e} Correctly folded and functional MelB binds D₂G and a FRET signal, with an excitation wavelength of 290 nm, is produced between the tryptophans (Trp) of MelB_{St} and the dansyl moiety of D₂G. Upon addition of excess melibiose, D₂G is displaced from the substrate binding site and the FRET signal is lost. MelB_{St} extracted by DDM was responsive to the addition of melibiose, but this conventional agent failed to result in functional protein when a less stable MelB homologue from *Escherichia coli* (MelB_{Ec}) was used.^{38d} In contrast, both MelB_{St} and MelB_{Ec} extracted by TPS-A8 and TPS-E7 exhibited enhanced FRET signals, indicative of improved function in the TPSs compared to DDM.

Detergent efficacy is often substantially affected by a small change in detergent structure. In the current study, TPS-As, TPS-Es and TPS-ELs have the same overall architecture with three flexible alkyl chains connected to the penta-saccharide head groups. The TPS-As and TPS-Es varied in the linker functional group. The TPS-As have one ether bond, while the TPS-Es contain three ether bonds in the linker region. The larger number of ether bonds in the TPS-Es allowed us to increase the length of the hydrophobic group from the central glucose core. For instance, although TPS-A6 and TPS-E6 have the same alkyl chain length (C6), it is notable that the hydrophobic group of TPS-E6 is longer than that of TPS-A6 by roughly two carbon units. This is due to the fact that the alkyl chain length used for detergent designation was determined from different locations: the quaternary carbon for the TPS-As and the ether bond in the linker region for the TPS-Es. For UapA, LeuT and β_2 AR, the overall performance of the TPS-Es was better than that of TPS-As. Furthermore, detergent efficacy tended to be enhanced with increasing alkyl chain length of the TPS-A/Es. This result indicates that the length of the detergent hydrophobic group is mainly responsible for the difference in detergent efficacy observed here. A detergent needs to closely match the dimensions of the target membrane protein for effective protein stability. Of the TPS-A/Es, the longest alkyl chain detergent (*i.e.*, TPS-E8) was most effective at stabilizing the target membrane proteins except MelB_{St}. However, as for β_2 AR, the efficacy of TPS-E8 was only a little improved as compared to that of DDM. By introducing the propylene spacers between the central glucose core and the peripheral glucose units, we could prepare the water-soluble TPS-ELs with the elongated alkyl chains ranging from C8 up to C11. These structural modifications enabled us to further improve detergent efficacy for β_2 AR stability; the TPS-ELs were remarkable at stabilizing the receptor, with the best performance obtained using TPS-E10L. The enhanced efficacy observed for the TPS-ELs with the receptor would be attributed to increased alkyl chain length or the presence of propylene spacers in the head group. The trend observed for β_2 AR was in contrast to that observed for MelB_{St}; for this transporter, a shorter alkyl chain TPS (TPS-A8 or TPS-E7) was superior. It is noteworthy that the modular synthetic approach allowed us to readily vary both the alkyl chain length and the structure of the head group, leading to discovery of the best TPS (TPS-A8, TPS-E8, or TPS-E10L) depending on the individual membrane proteins tested here. Thus, this class

of agents has architecture suitable for optimization and provides chemical tools useful for protein extraction and stabilization as a whole set. This study is also a good example of how detergent efficacy can be significantly enhanced by structural modification and can vary depending on the target membrane protein.

The current study indicates that some TPSs were significantly better than DDM. A further insight can be obtained by a qualitative comparison of the current agents with other novel agents developed so far. In this context, MNG-3 widely used for GPCR structural study is an ideal candidate for the comparison. In a previous study, MNG-3 displayed marginally enhanced behavior compared to DDM in retaining the substrate binding ability of LeuT when tested at CMC + 0.2 wt%.³⁹ In contrast, the markedly enhanced behaviors were observed for a few TPSs (TPS-A8, TPS-E6, TPS-E7, and TPS-E8) under the same conditions in the current study (Fig. 3b). As shown in Fig. 4, TPS-E8 was also clearly superior to MNG-3 at maintaining the integrity of UapA. The CPM result with this transporter showed that MNG-3 was only comparable to or a little better than DDM depending on detergent concentration. As for β_2 AR, the receptor in MNG-3 retained ~60% ligand binding ability after a 5-day incubation at room temperature in a previous study.²⁷ Under the same conditions, the TPS-E10L-solubilized receptor retained >85% activity. In the case of MelB_{St}, the transporter extracted by TPS-E7 was more responsive to galactoside binding than the protein obtained in MNG-3.^{38d} Overall our analyses strongly indicate that TPS-E7, TPS-E8 or TPS-E10L are superior to MNG-3 depending on the target proteins. The exceptional stability of β_2 AR conferred by TPS-E10L could also be found in a comparison with other novel agents including PSE-C11 with a similar head group.²⁴ TPA-5-2 and TFA-1 were clearly worse than DDM at stabilizing the receptor (Fig. S7b†).^{20b,26} As observed before, PSE-C11 showed a largely enhanced efficacy compared to DDM in this regard.²⁴ However, the receptor solubilized in this agent was less stable than that in TPS-E10L; following a 3-day incubation, the PSE-C11-solubilized receptor retained ~60% activity while the TPS-E10L-solubilized receptor showed >90% protein activity (Fig. 7a). Thus, the GPCR stability shown by TPS-E10L was markedly higher than those displayed by a gold standard conventional detergent (DDM) as well as several significantly optimized novel agents (MNG-3, TFA-1, TPA-5-2 and PSE-C11).

Conclusions

In summary, we designed and prepared two types of tripod penta-saccharide agents (TPS-A/Es and TPS-ELs) with distinct architectures both from each other and other previously described novel amphiphiles. When these agents were evaluated with the four membrane proteins, the markedly enhanced protein stability was observed for TPS-A8/E7 (MelB_{St}), TPS-E8 (LeuT and UapA) or TPS-E10L (β_2 AR and UapA) compared to the conventional detergent, DDM. In addition, a couple of agents (TPS-A8 and TPS-E7) have the ability to extract two MelB homologues from the membrane without impairing function. Along with the promising EM result, the ease with which this agent was prepared and the profound efficacy with which this agent stabilizes membrane proteins (and complexes), strongly suggest significant potential for membrane protein structural study. Furthermore, the systematic structural modifications used for detergent property optimization introduced here

provide useful insights into novel detergent design as well as rational detergent selection for membrane protein research.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. Partial ^1H NMR spectra of representative TPSs (TPS-A8 (a), TPS-E8 (b) and TPS-E10L (c)) focusing on the anomeric regions and the chemical structures of the head group of TPS-A8/E8 (d) and TPS-E10L (e) indicating five anomeric protons (red) and their neighbouring protons (blue). Five doublet peaks corresponding to the anomeric protons (H_1 , H_2 , H_3 , H_4 and H_5) were well resolved in a wide range from 4.25 to 4.95 ppm for TPS-A8 and TPS-E8 while five anomeric peaks for TPS-E10L were focused into a small range from 4.15 to 4.30 ppm. The vicinal coupling constants (3J_1 , 3J_2 , 3J_3 , 3J_4 , and 3J_5) for all anomeric peaks of TPS-A8 and TPS-E8 were 8.0 Hz, a typical value for β -anomeric protons. Note small differences in the chemical shifts (δ) of the anomeric peaks between TPS-A8 and TPS-E8: 4.32, 4.61, 4.80, and 4.92 ppm for TPS-A8; 4.34, 4.36, 4.64, 4.83, and 4.94 ppm for TPS-E8.

Fig. 2.

The micellar characterization of TPS-E10L. Change in fluorescence intensity at 430 nm with increasing detergent concentration in the presence of a hydrophobic fluorescent dye (DPH) (a), DLS profile (b) and negative stain TEM image (c) of detergent micelles formed by TPS-E10L. Detergent CMCs were determined from a cross point of two regression curves. Detergent sample for TEM analysis was visualized by staining with 2% phosphotungstic acid. The detergent was used at 1.0 wt% for DLS and TEM experiments.

Fig. 3.

Effect of TPS-A/Es on LeuT stability. Either TPS-As (TPS-A6, TPS-A7, or TPS-A8) or TPS-Es (TPS-E6, TPS-E7, or TPS-E8) were individually tested at concentrations of CMC + 0.04 wt% (a) and CMC + 0.2 wt% (b) and compared to DDM. DDM-purified transporter was mixed with the indicated detergent-containing solutions and LeuT substrate binding activity was assessed at room temperature using $[^3\text{H}]$ -leucine *via* scintillation proximity assay (SPA). Errors, SEM; $n = 3$.

Fig. 4.

Thermal denaturation profile of UapA solubilized in DDM, MNG-3, a TPS-A (TPS-A6, TPS-A7, or TPS-A8), or a TPS-E (TPS-E6, TPS-E7, or TPS-E8). The detergents were evaluated at CMC + 0.04 wt% (a) or CMC + 0.2 wt% (b). Thermally induced protein unfolding was monitored by CPM assay performed at 40 °C for 120 min. The relative amounts of the folded transporter were normalized relative to the most destabilizing condition in this experiment, that is, protein denaturation in DDM (CMC + 0.2 wt%) or MNG-3 (CMC + 0.04 wt%) after 120 min incubation.

Fig. 5.

Long-term stability of β_2 AR purified in DDM and representative TPS-ELs (TPS-E9L, TPS-E10L and TPS-E11L) (a) and time course SEC profiles of β_2 AR- G_s protein complex purified in TPS-E10L (b). For the long-term stability assay, 0.1% DDM-purified β_2 AR was diluted into individual detergent solutions to reach a final detergent concentration of CMC + 0.2 wt%. DDM was evaluated in the absence or presence of cholesteryl hemisuccinate (CHS). Receptor activity was then monitored by measuring the ability of the receptor to bind the radio-labeled antagonist ($[^3H]$ -dihydroalprenolol (DHA)) at regular intervals during a 5-day incubation at room temperature. Errors, SEM; $n = 3$. For the SEC experiment, DDM-purified receptor and G_s protein were combined to produce β_2 AR- G_s protein complex and DDM was then exchanged for TPS-E10L. The integrity of the complex was monitored at 0 (0d) and 7 days (7d) after detergent exchange *via* analytical gel filtration.

Fig. 6. Single-particle EM of negative stained β_2 AR-Gs complex purified in TPS-E10L. Raw image (a), 2D classification (b) and representative class averages in the same orientation (c). The two G_s subunits were designated by $G_{\alpha S}$ and $G_{\beta\gamma}$ in (c).

Fig. 7. Thermostability of MelB_{St} solubilized in DDM or TPSs (TPS-A8, TPS-E6, TPS-E7, TPS-E8, and TPS-E10L). Membranes containing MelB_{St} were treated with the indicated detergent at various temperatures (0, 25, 35, 45, 55, or 65 °C) for 90 min (a) Western blot: MelB_{St} extracted and solubilized by individual detergents was separated by ultracentrifugation and analyzed by SDS-15% PAGE and Western blotting. The total amount of MelB_{St} originally present in the membrane was detected from the untreated membrane sample (Memb). (b) Histogram showing the amount of soluble MelB_{St} detected in panel (a) as a percentage of the total MelB_{St} applied. Error bars, SEM, $n = 3$. (c) Galactoside-binding

assay. Right-side-out (RSO) membrane vesicles containing MelB_{St} or MelB_{Ec} were extracted with DDM, TPS-A8, or TPS-E7, respectively. The detergent extracts were subjected to MelB functional assay (*i.e.*, melibiose reversal of FRET from Trp to dansyl-2-galactoside (D₂G)) as described in the ESI.† D₂G (10 μM, a K_d value for MelB_{St}) and melibiose (saturated concentration) were added at 1 min and 2 min time points, respectively. Water instead of melibiose was used to obtain control data (black lines). Wavelengths for excitation and emission were set at 290 nm and at 490 nm (MelB_{St}) or 465 nm (MelB_{Ec}), respectively.

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Scheme 1.

Chemical structures of tripod penta-saccharide amphiphiles (TPSs and TPS-Ls). All of these agents have five glucose units and three alkyl chains as the hydrophobic and hydrophilic groups, respectively. The head group comprises a glucose core attached to four additional glucose units either directly (TPSs) or *via* a propylene spacer (TPS-Ls). The lipophilic alkyl chains were connected to these hydrophilic groups through ether (TPS-Es and TPS-ELs) or alkyl linkage (TPS-As). The alkyl chain length also varied depending on the hydrophilicity of the head groups (C6 to C8 for TPS-Es and TPS-As; C8 to C11 for TPS-ELs).

Table 1

Molecular weights (MWs), critical micelle concentrations (CMCs) of the novel agents (TPS-A/Es and TPS-ELs) along with a conventional detergent (DDM) and hydrodynamic radii (R_h) (mean $\pm$ S.D., $n = 4$) of their micelles in water measured at room temperature

Detergent	MW ^a	CMC (mM)	CMC (wt%)	R_h^b (nm)
TPS-A6	1111.2	~0.07	~0.0078	2.4 ± 0.05
TPS-A7	1153.3	~0.015	~0.0017	2.7 ± 0.05
TPS-A8	1195.4	~0.007	~0.0008	9.1 ± 0.06
TPS-E6	1199.3	~0.020	~0.0024	2.9 ± 0.05
TPS-E7	1241.4	~0.012	~0.0015	13.0 ± 0.08
TPS-E8	1283.5	~0.007	~0.0009	43.8 ± 16.3
TPS-E8L	1515.8	~0.006	~0.0009	4.5 ± 0.39
TPS-E9L	1557.9	~0.004	~0.0006	4.6 ± 0.18
TPS-E10L	1600.0	~0.002	~0.0003	4.8 ± 0.34
TPS-E11L	1642.1	~0.001	~0.0002	40.6 ± 4.4
DDM	510.1	~0.17	~0.0087	3.4 ± 0.03

^a Molecular weight of detergents.

^b Hydrodynamic radius of detergents measured at 1.0 wt% by dynamic light scattering.