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Article

Rational Approach to Improve Detergent Efficacy for Membrane Protein Stabilization

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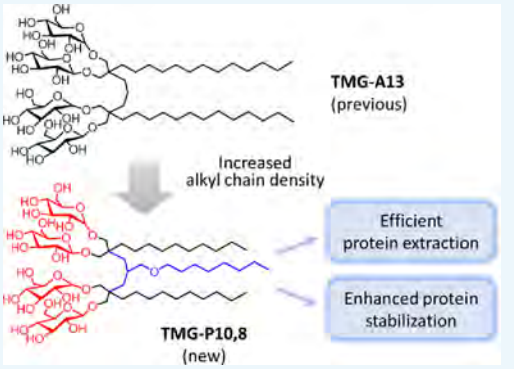
ABSTRACT: Membrane protein structures are essential for the molecular understanding of diverse cellular processes and drug discovery. Detergents are not only widely used to extract membrane proteins from membranes but also utilized to preserve native protein structures in aqueous solution. However, micelles formed by conventional detergents are suboptimal for membrane protein stabilization, necessitating the development of novel amphiphilic molecules with enhanced protein stabilization efficacy. In this study, we prepared two sets of tandem malonate-derived glucoside (TMG) variants, both of which were designed to increase the alkyl chain density in micelle interiors. The alkyl chain density was modulated either by reducing the spacer length (TMG-Ms) or by introducing an additional alkyl chain between the two alkyl chains of the original TMGs (TMG-Ps). When evaluated with a few membrane proteins including a G protein-coupled receptor, TMG-P10,8 was found to be substantially more efficient at extracting membrane proteins and also effective at preserving protein integrity in the long term compared to the previously described TMG-A13. This result reveals that inserting an additional alkyl chain between the two existing alkyl chains is an effective way to optimize detergent properties for membrane protein study. This new biochemical tool and the design principle described have the potential to facilitate membrane protein structure determination.



Membrane proteins play central roles in various biological processes such as cell signaling, membrane trafficking, and material transfer between the cell interior and exterior.[1] Due to their involvement in various diseases, these membrane-associated proteins constitute a significant proportion of drug targets.[2,3] Functional identification and structural analysis of membrane proteins are important steps for understanding how they interact with other proteins, as well as the design of new therapeutic agents. However, the number of membrane proteins with known structures is much smaller than that of soluble proteins.[4] The inherent amphiphilic architecture of membrane proteins, with hydrophobic regions embedded in the membrane and hydrophilic regions exposed to the cell interior and exterior, makes them prone to aggregation and denaturation during protein manipulation, including purification and structure determination. Hence, specific systems are required to preserve their native structures in a soluble and stable state in aqueous solution for downstream characterization.[5,6]

Micelles formed by detergent molecules are widely used as amphiphilic systems that mimic the lipid bilayer. Above a certain concentration, detergent molecules associate into a spherical or elliptical self-assembly, a micelle, with a hydrophilic exterior and hydrophobic interior in aqueous solution. Membrane proteins can integrate into detergent micelles via hydrophobic interactions, forming protein−detergent complexes (PDCs).[7] *n*-Dodecyl-*β*-D-maltoside (DDM), *n*-decyl-*β*-D-maltoside (DM), and *n*-octyl-*β*-D-glucoside (OG) are representatives of classical detergents that are widely used both to extract membrane proteins from native membranes and to stabilize the extracted proteins in aqueous solution. Due to their canonical structure, with single head and tail groups, micelles formed by classical detergents are highly dynamic compared to lipid bilayers. Consequently, membrane proteins encapsulated in detergent micelles tend to denature and/or aggregate over time in aqueous solution.[8,9] Several efforts have been made to provide more cell membrane-like mimetic systems, as exemplified by bicelles,[10] amphipols (e.g., Apols),[11] peptide-based detergents (e.g., lipopeptide detergents and *β*-peptides),[12,13] nanoassemblies stabilized by peptides (e.g., saposin A),[14] proteins (e.g.,

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membrane scaffold protein),[15] or polymers (e.g., styrene-maleic acid[16] and diisobutylene-maleic acid[17]). Due to greater similarity to lipid bilayers than standard detergents, these membrane-mimetic systems were shown to be highly effective at preserving the native conformations of membrane proteins and are increasingly used for membrane protein structure determination via single-particle cryo-EM.[18] However, these systems are largely incompatible with membrane protein crystallization, which remains a major method for protein structure determination. In addition, most of these systems are inefficient at extracting membrane proteins from their membranes. In contrast, detergent micelles are efficient at membrane protein extraction and are also widely used for the crystallization of membrane proteins. Hence, the development of new detergents capable of stabilizing native membrane protein structures is crucial to facilitate a membrane protein structural study.

Over the last 15 years or so, considerable efforts have been made in the development of effective detergents, represented by facial amphiphiles,[19,20] maltose neopentyl glycols,[21,22] glycodiosgenin (GDN),[23] neopentyl glycol-derived triglucosides,[24] mannitol-based amphiphiles,[25] butane-1,2,3,4-tetraol-based maltosides,[26] norbornane-based maltosides,[27] oligoglycerol detergents,[28] cyclopentane-based maltosides (CPMs),[29] and 1,3-acetonedicarboxylate-derived amphiphiles.[30] Among these new amphiphiles, LMNG and GDN have contributed to the elucidation of more than 450 membrane protein structures,[31] highlighting the incredibly important role that novel detergents have played in recent membrane protein structure determination. Previously, we reported tandem malonate-derived glucosides (TMGs) in which two amphiphilic units comprising branched diglucoside head and alkyl tail groups are connected by a propylene spacer.[32] This study identified TMG-A13 as a novel amphiphile effective for stabilizing membrane proteins, including the human β_2 adrenergic receptor (β_2AR). In the current study, we made efforts to improve TMG efficacy for membrane protein stabilization by rationally designing and efficiently preparing two sets of TMG variants, designated TMG-Ms and TMG-Ps (Figure 1). When the new TMG variants were evaluated in terms of protein extraction efficiency and stabilization efficacy using a few model membrane proteins, we identified TMG-P10,8 as a new detergent that was more effective than a gold standard DDM and the parent detergent TMG-A13 for membrane protein stabilization. These results reveal that our rational approach to structural modification is viable for optimizing detergent efficacy for membrane protein study. Both the detergent tool and the design principles introduced here have the potential to contribute to membrane protein structure determination.

RESULTS

Detergent Structures and Physical Characterizations.

We previously developed a class of TMGs that contain two branched diglucoside head groups and two alkyl chains (Figure 1b). These detergents have dimeric structures of two malonate-derived amphiphilic units, with each unit comprising a single branched diglucoside head group and an alkyl chain. The two amphiphilic units are connected using a propylene spacer in the hydrophilic−lipophilic interfaces. The TMGs were shown to be good at stabilizing membrane proteins, but we felt that there was room for further optimizing these detergents by chemical modification. We utilized two approaches to introduce a structural change into the TMG architecture. The first approach was to decrease the distance between the two alkyl chains of the

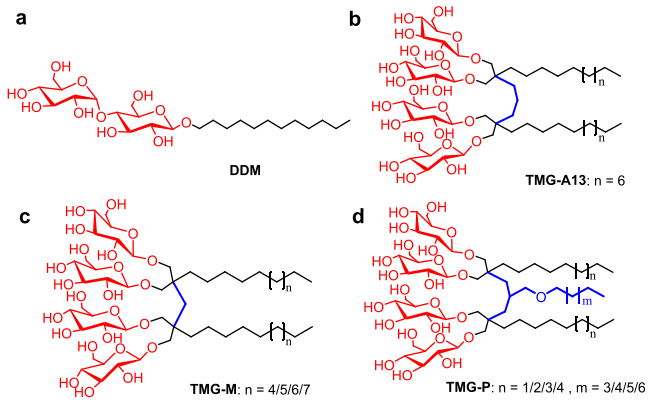

Figure 1. Chemical structures of the conventional detergent DDM (a), the previously developed detergent TMG-A13 (b), and the newly prepared detergents TMG-Ms (c) and TMG-Ps (d). The TMG-Ms and TMG-Ps are derivatives of TMG-A13, but they contain a different spacer between two branched diglucoside head groups. The two head groups are connected by methylene instead of propylene spacer for the TMG-Ms, whereas the TMG-Ps have the same spacer as TMG-A13, but this propylene spacer is conjugated with an additional alkyl chain via an ether linkage. Due to the presence of the shorter spacer or the additional alkyl chain, the TMG-Ms and TMG-Ps have a decreased interalkyl chain distance compared to TMG-A13.

TMGs, which was attained using a methylene (C1) spacer rather than a propylene (C3) spacer for connection of the two amphiphilic units (Figure 1c). The second approach was to insert an additional alkyl chain between the two alkyl chains of the TMGs with no change in the spacer length (Figure 1d). These two approaches generated two sets of TMGs, designated TMG-Ms and TMG-Ps, respectively. Due to either the decrease in the interalkyl chain distance for the TMG-Ms or the presence of an additional alkyl chain for the TMG-Ps, we hypothesized that these new detergents would form detergent micelles with a higher alkyl chain density compared to the original TMGs. The increased alkyl chain density in the detergent micelle interior should increase membrane protein stability.[33−35]

To identify an optimal alkyl chain length, the alkyl chains varied from undecyl (C11) to tetradecyl (C14) for the TMG-Ms. In the case of TMG-Ps, the two outer alkyl chains varied from octyl (C8) to undecyl (C11), while the additional (inner) alkyl chain was introduced to be shorter than the outer alkyl chains by two carbon units, thus varying from hexyl (C6) to nonyl (C9). The inner alkyl chain was attached to the propylene spacer by using an ether linkage. The variations in the alkyl chain length are reflected in the detergent designation. The variation in the detergent alkyl chain lengths is also meaningful in terms of finding an optimal hydrophilic−lipophilic balance (HLB), crucial for membrane protein stability.[36,37] The precise detergent HLB required for protein stability tends to depend on the properties of membrane proteins,[38] but most widely used detergents have a range of HLBs from 11 to 13. When calculated by the Griffin method,[39] the TMG-Ms gave HLB values in a range of 11.9 to 12.8, while the HLB values of the TMG-Ps were in the range of 11.0 to 12.2 (Table S2). Thus, the HLBs of both sets of TMGs appeared to fall into the optimal range for membrane protein stability. It is notable that the HLB values of the TMG-Ps (11.0−11.7), except TMG-P8,6, were smaller than those of the TMG-Ms (11.9−12.8) and TMG-A13 (11.9). This HLB comparison indicates that the TMG-Ps were more hydrophobic than the TMG-Ms and the previously developed TMG-A13, probably due to the presence of the additional alkyl

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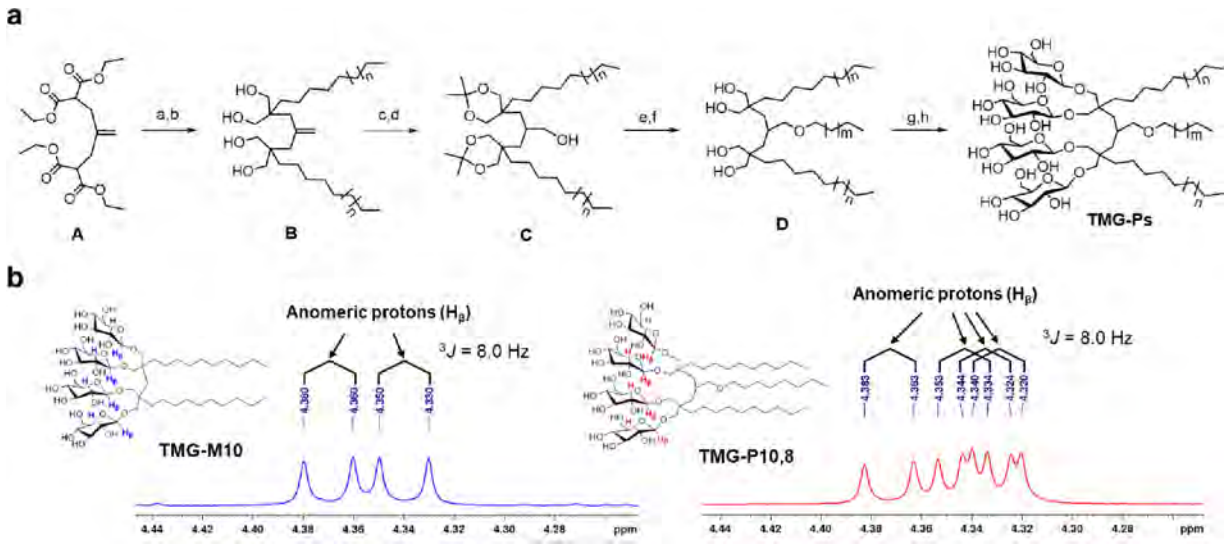

Figure 2. (a) Synthetic scheme for the preparation of the TMG-Ps and (b) ^{1}H NMR characterizations of TMG-M10 (left) and TMG-P10,8 (right). (a) NaH, RI, DMSO, 0 °C → RT; (b) $LiAlH_4$, THF, RT; (c) *p*-TSA·H_2O, 2,2-dimethoxypropane, acetone, RT; (d) BH_3·THF, NaOH, H_2O_2, THF, 0 °C; (e) NaH, RI, DMF, RT; (f) *p*-TSA·H_2O, MeOH, DCM, RT; (g) perbenzoylated glucosyl bromide, AgOTf, CH_2Cl_2, 0 °C → RT; (h) NaOMe, MeOH, RT. Two malonate units were connected to each other by a reaction with methallyl dichloride. The resulting tetraester derivative (A) was subjected to alkylation, followed by ester reduction to give an alkene-functionalized tetraol derivative (B). Following acetonide protection of two 1,3-diol groups, the alkene group was converted to an alcohol via hydroboration (compound **C**). An additional alkyl chain was introduced into the alcohol-functionalized propylene spacer via an ether linkage (compound **D**), which was subjected to glycosylation and deprotection to give the TMG-Ps. The *β*-stereochemistry of the glycosidic bonds in the TMGs was confirmed via an analysis of the anomeric proton signals appearing from 4.25 to 4.45 ppm. The peaks corresponding to the *β*-anomeric protons (H_β) appear at 4.37 and 4.34 ppm for TMG-M10 and 4.37, 4,34, and 4.33 ppm for TMG-P10,8. The coupling constants (^3J) of these doublet peaks were all 8.0 Hz. The black bridges and arrows on the NMR spectra were used to indicate each set of anomeric proton peaks.

chain in the lipophilic region. The HLB values of some TMG-Ps (e.g., TMG-P10,8) are even smaller than those of the classical glucoside detergents OG (12.3) and *n*-nonyl-*β*-D-glucoside (NG) (11.7), indicating that these TMG-Ps are sufficiently hydrophobic to strongly interact with the hydrophobic membrane protein surfaces.

The TMG-Ms were synthesized according to a reported procedure used for the preparation of the original TMGs.[32] 1,3-Diiodomethane was used to link two malonate units instead of 1,3-dibromopropane. As for the preparation of the TMG-Ps, we commenced with a reaction of diethylmalonate with methallyl dichloride, resulting in the generation of two malonate units linked by the alkene-functionalized propylene spacer (compound **A**) (Figure 2a). Following the introduction of an alkyl chain into each malonate unit, the ester groups were reduced by $LiAlH_4$ to generate the dialkylated tetraol derivative (compound **B**). The two 1,3-diol functional groups in compound **B** were protected with acetonide before the alkene group was converted into a primary alcohol via hydroboration. The resulting acetonide-protected alcohol derivative (compound **C**) was subjected to an additional alkylation and subsequent deprotection to produce the trialkylated tetraol derivative (compound **D**). Finally, we introduced four glucose units into compound **D** via AgOTf-promoted glycosylation and NaOMe-mediated removal of the benzoyl protection groups. The chemical structures of all TMG-Ms and TMG-Ps were confirmed by nuclear magnetic resonance (NMR) spectroscopy and mass spectrometry. In the ^{1}H NMR spectra of the new TMGs, we observed multiple doublet signals in the range of 4.45 to 4.25 ppm, with a coupling constant of 8.0 Hz (Figure 2b). The chemical shift and coupling constant corroborate the formation of *β*−glycosidic bonds. A signal corresponding to an *α*-anomeric proton appears at a higher chemical shift (5.10−5.20 ppm) with a smaller coupling constant (4.0 Hz).[40] Due to the difference in molecular symmetry and rigidity, TMG-M10 and TMG-P10,8 showed different patterns of the anomeric proton signals. TMG-M10 gave two doublet signals at 4.37 and 4.34 ppm, while TMG-P10,8 showed four overlapping doublet signals at 4.37, 4.34, and 4.33 ppm in the NMR spectrum (Figure 2b).

The newly synthesized TMGs were all water-soluble (>10 wt %), except TMG-11,9, which formed a gel at 5 wt %. The detergent solutions gave no precipitation over the course of 1-month incubation at room temperature. Critical micelle concentrations (CMCs) of the new TMGs were measured by utilizing a fluorescent dye encapsulation method, while dynamic light scattering (DLS) was used to estimate the hydrodynamic radii (R_h) of the micelles. As summarized in Table 1, the CMCs of the new TMGs were substantially lower than that of DDM (4−20 vs 170 *μ*M). Despite the presence of a smaller number of alkyl chains, the TMG-Ms with two alkyl chains generally form micelles at concentrations lower than those of the TMG-Ps with three alkyl chains. The CMCs of the TMG-Ms were comparable to that of TMG-A13 (4−9 vs 6 *μ*M). In both sets of TMGs, detergent micelles increased in size with an increase in alkyl chain length. In general, the TMG-Ps formed larger micelles than the TMG-Ms, originating from the presence of the additional alkyl chain that increases the volume of the hydrophobic groups, thus making the molecular geometry of the detergent molecules closer to a cylinder.[41]

TMG-P11,9 formed the largest micelles with an average R_h of 8.7 nm, which is likely related to the relatively low water solubility observed for this detergent. Interestingly, micelles formed by the TMG-Ms were smaller in size than those of TMG-A13, indicating that shortening the spacer chain length from propylene (C3) to methylene (C1) decreases the volume of the hydrophobic group more effectively than does the

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Bioconjugate Chem. XXXX, XXX, XXX−XXX

Table 1. Molecular Weight (MW), CMC, Water Solubility of the TMGs (TMG-Ms/Ps) and Control Detergents (TMG-A13 and DDM), and Hydrodynamic Radii (R_h; $n = 5$) of Their Micelles in Water at Room Temperature

detergent	MW[a]	CMC (μM)	CMC (wt %)	R_h (nm)[b]	solubility[c] (wt %)
TMG-M11	1121.4	~9	~0.0010	2.7 ± 0.2	~10
TMG-M12	1149.4	~7	~0.0008	2.9 ± 0.1	~10
TMG-M13	1177.5	~6	~0.0007	3.2 ± 0.3	~10
TMG-M14	1205.5	~4	~0.0005	3.3 ± 0.4	~10
TMG-P8,6	1179.4	~20	~0.0024	2.9 ± 0.1	~10
TMG-P9,7	1221.5	~15	~0.0018	3.8 ± 0.8	~10
TMG-P10,8	1263.6	~10	~0.0013	8.7 ± 2.8	~10
TMG-P11,9	1305.7	ND	ND	ND	~5
TMG-A13[d]	1205.5	~6	~0.0007	3.6 ± 0.2	~10
DDM	510.6	170	0.0087	3.4 ± 0.1	~10

[a]Molecular weight of detergents. [b]Hydrodynamic radius of micelles determined at 1.0 wt % by DLS. [c]Water solubility at room temperature. [d]Data obtained from the literature.[32] ND stands for not determined.

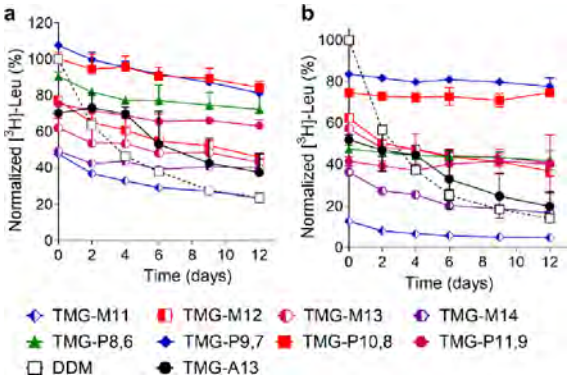

Figure 3. Long-term stability of LeuT solubilized in DDM or new TMGs. The detergents were tested at concentrations of CMC + 0.04 wt % (a) and 0.2 wt % (b). The DDM-purified transporter was mixed with individual detergent-containing solutions and then the resulting detergent exchanged samples were incubated for 12 days at room temperature to monitor the time-course stability of the transporter. LeuT stability was assessed by measuring [^{3}H]-leucine ([^{3}H]-Leu) binding ability at regular intervals during the incubation via a SPA. [^{3}H]-leucine binding to LeuT in the individual detergents is represented as relative to the initial binding activity in DDM at day 0. Data are shown as means ± SEM (error bars) with $n = 3$.

hydrophilic group. Micelles formed by the individual TMGs showed a unimodal size distribution in their number- or volume-weighted DLS profiles, suggesting high homogeneity (Figures S1 and S2). Large aggregates with a size of 50 to 1000 nm were detected in the intensity-weighted DLS profiles of the new TMGs due to the highly sensitive nature of large particles toward light scattering.

Detergent Evaluation with a Set of Membrane Proteins. The new TMGs were first evaluated with the leucine transporter LeuT from the bacterium *Aquifex aeolicus*.[42,43] The transporter was recombinantly expressed in *Escherichia coli* and extracted from the *E. coli* membrane using 1.5 wt % DDM, followed by protein purification in 0.05 wt % of the same detergent. The DDM-purified LeuT was diluted into buffer solutions containing DDM or the respective TMG-M/P to give a final detergent concentration of CMC + 0.04 or 0.2 wt %. Protein stability was assessed by measuring the ability of the transporter to bind a radio-labeled substrate ([^{3}H]-leucine (Leu)) via the scintillation proximity assay (SPA).[44] The Leu binding of the transporter was monitored at regular intervals during a 12-day incubation period at room temperature. As expected, LeuT in DDM rapidly lost the Leu-binding ability over time (Figure 3). Upon detergent exchange from DDM to TMG-A13, the Leu-binding ability of the transporter was substantially decreased, but we found a more gradual loss in the transporter stability than in DDM over time, consistent with the previously reported result.[32] Similar results were obtained when LeuT was subjected to the exchange of detergent from DDM to the individual TMG-Ms. TMG-M11 and TMG-M14 were worse than TMG-A13 in terms of long-term LeuT stability, whereas TMG-M12 and TMG-M13 were slightly better than the latter. In contrast, the TMG-Ps were superior to TMG-A13 in this regard, with the best performance observed for TMG-P9,7 and TMG-P10,8. These two TMG-Ps were highly effective at preserving the Leu-binding ability of the transporter during the 12-day incubation when tested at two different detergent concentrations of CMC + 0.04 and 0.2 wt %. Of note, the effect of residual DDM on LeuT stability was minimal when the new TMGs were evaluated at 0.2 wt %.

We next turned to another prokaryotic transporter, melibiose permease from *Salmonella typhimurium* ($MelB_{St}$), for detergent evaluation.[45−48] $MelB_{St}$ overexpressed in *E. coli* membranes was extracted using 1.5 wt % individual detergents (DDM, TMG-A13, or respective TMG-M/P) for 90 min at 0 °C. The amounts of $MelB_{St}$ solubilized in these samples provided information about detergent extraction efficiency. The samples were then subjected to thermal treatment by incubation at an elevated temperature (45, 55, or 65 °C) for another 90 min, which allowed the evaluation of detergent efficacy for protein stabilization. The amounts of soluble $MelB_{St}$ under the individual conditions were estimated via Western blotting and are presented as percentages (%) of the total transporter in the untreated membranes (Figure 4a). At 0 °C, the amount of soluble $MelB_{St}$ in DDM was nearly 100%, indicating that this classical detergent quantitatively extracted the transporter from the membranes. This result supports the wide use of DDM for membrane protein extraction. Similar to TMG-A13, the TMG-Ms yielded 50−60% soluble $MelB_{St}$, following protein extraction at 0 °C, suggesting that these TMGs may not be ideal for protein extraction (Figure 4a). In contrast, all tested TMG-Ps yielded 90−100% soluble $MelB_{St}$, indicating a much greater efficiency of $MelB_{St}$ extraction. When the $MelB_{St}$ extracts were further treated at 45 °C for an additional 90 min, substantial increases in the amounts of soluble transporter were detected for all of the TMG-Ms, particularly TMG-M13 and TMG-M14. The increased membrane dynamics caused by the elevated temperature is likely responsible for these improved results. In the cases of TMG-Ps, different behaviors were observed depending on detergent identity. TMG-P8,6 yielded decreased amounts of soluble $MelB_{St}$ from ~100 to ~50%, whereas the other two TMG-Ps, TMG-P9,7 and TMG-P10,8, effectively maintained the amounts of the soluble transporter (90−100%). When the protein extracts were incubated at 55 °C, DDM yielded only ~10% soluble $MelB_{St}$. Similar results were obtained for the short

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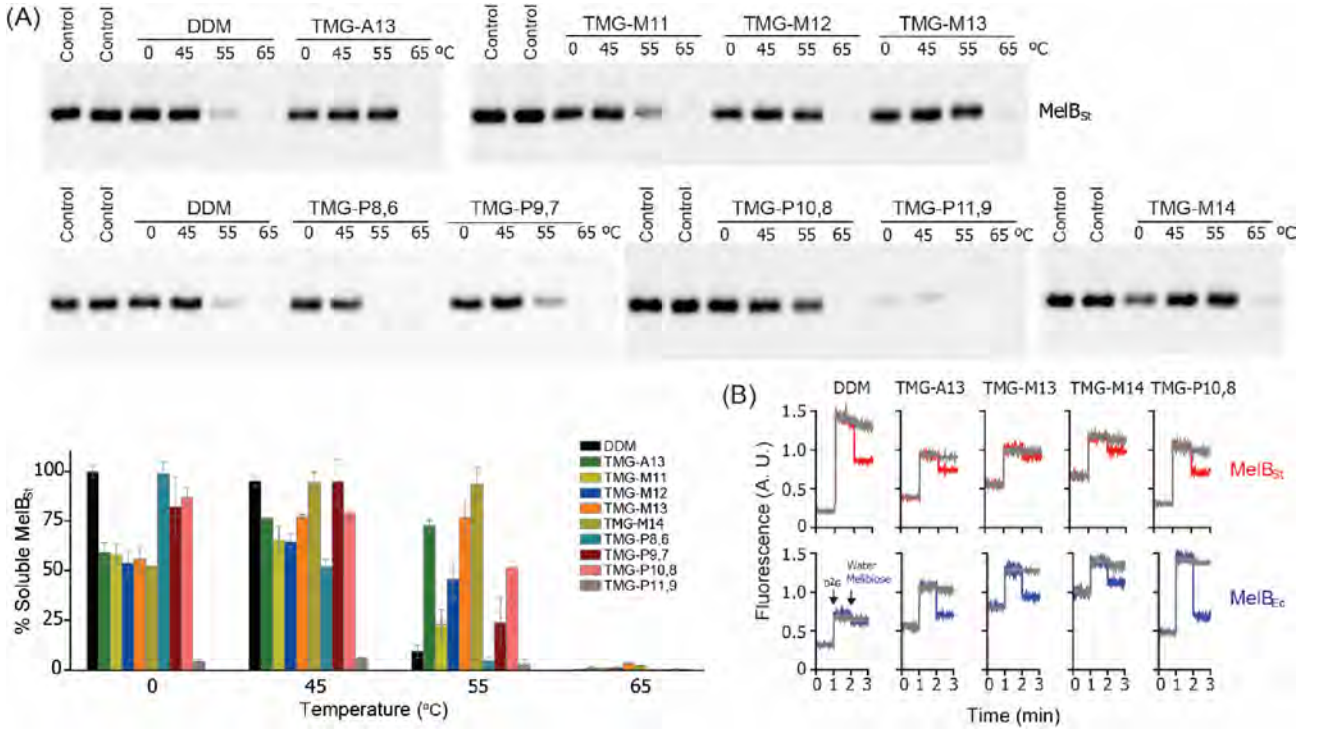

Figure 4. (A) Thermo-solubility of $MelB_{St}$ solubilized in the new TMGs. A conventional detergent (DDM) and the previously described TMG-A13 were used for comparison. $MelB_{St}$ expressed in *E. coli* was extracted from the membranes by the individual detergents at 0 °C for 90 min. The membrane extracts were further incubated at three different elevated temperatures (45, 55, and 65 °C) for 90 min. The resulting $MelB_{St}$ samples were analyzed by SDS-PAGE and Western blotting after ultracentrifugation (top). The amounts of soluble $MelB_{St}$ are expressed as percentages of total $MelB_{St}$ present in the untreated membranes ("Control"), as presented in a histogram (bottom left). Error bars: $n = 3$, SEM. (B) The MelB extracted at 25 °C for 60 min from right-side-out (RSO) vesicles prepared from *E. coli* DW2 cells carrying the wild-type (WT) $MelB_{St}$ was evaluated for galactoside binding through a FRET (Trp → D^2G) measurement. Excitation, 290 nm; emission, 465 or 490 nm for $MelB_{Ec}$ or $MelB_{St}$, respectively.

alkyl-chained TMG-Ms/Ps (i.e., TMG-M11/M12 and TMG-P8,6/P9,7). In contrast, no decrease in the amount of soluble $MelB_{St}$ was observed for TMG-A13 and the two long alkyl-chained TMG-Ms (TMG-M13 and TMG-M14), indicating that these detergents were effective at maintaining the transporter in a soluble state at this elevated temperature. Of the TMG-Ps, TMG-P10,8 was the only detergent that yielded a substantial amount of soluble $MelB_{St}$ (~50%).

Based on the thermo-solubility result, we selected three TMGs (TMG-M13, TMG-M14, and TMG-P10,8), along with the two controls (DDM and TMG-A13), to investigate their effects on the MelB function (Figure 4b). The functionality of $MelB_{St}$ extracted by these selected detergents was evaluated via a galactoside binding assay employing the substrate melibiose and the fluorescent ligand dansyl-2-galactoside (D^2G).[49] Upon the addition of D^2G, functional $MelB_{St}$ binds to this fluorescent ligand, which leads to a strong fluorescence emission via Förster resonance energy transfer (FRET) from the tryptophan (Trp) residue to the dansyl moiety. A subsequent addition of excess melibiose replaces the dansyl ligand with the nonfluorescent substrate in the active site, resulting in a decrease in fluorescence intensity. Therefore, monitoring the fluorescence intensity during the sequential addition of D^2G and melibiose allowed us to assess $MelB_{St}$ functionality. $MelB_{St}$ extracted by DDM was functionally active, as evidenced by the changes in fluorescence intensity upon sequential addition of D^2G and melibiose, respectively (Figure 4c). Similar results were obtained for TMG-A13 (control) and TMG-P10,8. $MelB_{St}$ extracted by either TMG-M13 or TMG-M14 showed a minor change in fluorescence intensity under the same conditions, indicating that these TMGs may be suboptimal at preserving $MelB_{St}$ functionality. When MelB from *E. coli* ($MelB_{Ec}$) was used instead of $MelB_{St}$, DDM yielded a functionally inactive transporter. In contrast, all tested TMGs (TMG-A13, TMG-M13/M14, and TMG-P10,8) were effective in preserving $MelB_{Ec}$ in a functional state. Taken together, these results indicate that TMG-P10,8 is not only efficient at extracting $MelB_{St}$ from the membrane but is also effective at maintaining both MelB homologues ($MelB_{St}$ and $MelB_{Ec}$) in a functional state.

The new TMGs were further evaluated with a purified modified form of the eukaryotic transporter, the uric acid–xanthine/H^+ symporter ($UapA\text{-}G411V_{\Delta 1-11}$) from *Aspergillus nidulans*.[50] As assessed by CPM-based thermal denaturation analysis at 40 °C, all the tested TMGs were superior to DDM, the detergent widely used for extraction, isolation, and structure determination of this protein (Figure S3a,b).[51] In addition, these new detergents were better than LMNG and at least as good as TMG-A13 at maintaining the protein in a stable state. Importantly, when selected TMG-M/Ps were assessed for their ability to extract $UapA\text{-}G411V_{\Delta 1-11}$ from *Saccharomyces cerevisiae* membranes, all of the tested TMGs, apart from TMG-P9,7 (59%), gave extraction efficiencies of greater than 70%, with the best results achieved by TMG-P10,8 (94%) and TMG-M13 (86%) (Table S2). These efficiencies were comparable to DDM (96%) and LMNG (96%). This is reflected in the FSEC profiles for these detergents (Figure S3c).

To further evaluate the new detergents for a more challenging membrane protein, we employed a G protein-coupled receptor, the human β_2AR.[52] The LMNG-purified receptor was diluted

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Bioconjugate Chem. XXXX, XXX, XXX−XXX

into buffer solutions containing the individual detergents (DDM, TMG-A13, and the TMG-M/P) to give a final detergent concentration of 0.2 wt %. The receptor stability was assessed by monitoring the ability to bind a radioactive antagonist ([^{3}H]-dihydroalprenolol (DHA)) at room temperature.[53,54] Following detergent exchange via dilution, the ability of the receptor to bind DHA was measured (Figure S4). This preliminary study showed that some new TMGs including TMG-A13 were comparable to DDM at yielding active receptor, which prompted us to select those TMGs (TMG-A13, TMG-M12/M13, TMG-P10,8, and TMG-P11,9) for a long-term β_2AR stability study. When the receptor stability was monitored during a 5-day incubation at room temperature, all tested TMGs were more effective than DDM at stabilizing the receptor long term (Figure 5). Furthermore, TMG-M12 was comparable to

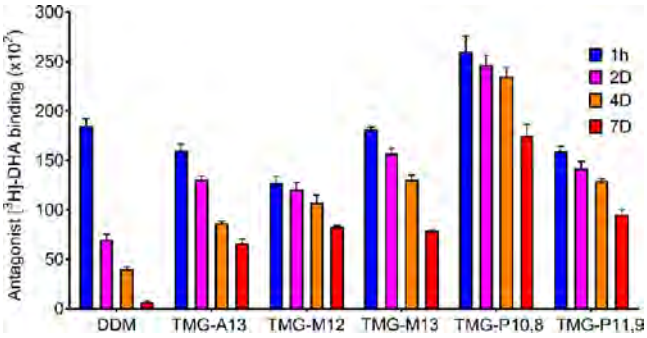

Figure 5. Time-course stability of β_2AR solubilized in the TMGs. The detergents were tested at 0.2 wt % using DDM and TMG-A13 as controls. The receptor stability was assessed by measuring the ability of the protein to bind the radiolabeled antagonist ([^{3}H]-dihydroalprenolol (DHA)) during a 7-day incubation at room temperature. Error bars: SEM, n = 3.

TMG-A13, while TMG-M13 and TMG-P11,9 were both better than TMG-A13. TMG-P10,8 stood out among the tested detergents, as β_2AR in this detergent exhibited nearly twice the ability to bind DHA relative to the receptor in TMG-A13 over the entire incubation period.

DISCUSSION

Membrane proteins are highly diverse in their structures and functions, and thus detergent efficacy toward membrane protein stabilization tends to be protein-specific. A similar trend was found in the current study. For instance, TMG-P9,7 was one of the best TMGs for stabilizing LeuT, but this TMG was inferior to DDM in stabilizing β_2AR. On the other hand, TMG-M14 outperformed DDM for MelB thermo-stability, but this long alkyl-chained detergent was suboptimal for stabilizing LeuT and β_2AR. The short alkyl-chained TMG-M11 was the best of the TMGs for UapA stability but was among the worst detergents for the stabilization of the other membrane proteins. Despite the protein-specific nature of detergent efficacy, TMG-P10,8 conferred greater stability to all tested membrane proteins in comparison with DDM and the previously developed TMG-A13. Specifically, TMG-P10,8 was the best TMG for LeuT and β_2AR stability and this TMG displayed favorable behaviors in the $MelB_{St}$ and UapA-G411$V_{\Delta 1-11}$ extraction/stability studies compared to DDM and/or TMG-A13. These results indicate that TMG-P10,8 can be used for all the steps of membrane protein manipulation including protein extraction, purification, and structure determination.

Only a limited number of detergent design principles for enhanced protein stability have been described, mainly due to difficulties associated with studying molecular interactions in PDCs. The current study differs from other detergent studies in that a rational approach was used to alter the chemical structure of the TMGs in an attempt to increase the stability of membrane proteins solubilized in these agents. Specifically, we hypothesized that an increased alkyl chain density in detergent micelles would enhance the detergent efficacy for protein stabilization. For the TMG-Ms, use of the methylene spacer instead of the propylene shortened the interalkyl chain distance compared to the previous TMGs. Consequently, micelles formed by the new detergents would have an increased alkyl chain density in the micelle interiors compared to those formed by the previous TMGs, thereby resulting in an enhanced protein stability. Contrary to our expectation, TMG-M13 conferred very similar protein stability to TMG-A13, suggesting that there is little or no merit in the shortened interalkyl chain distance in the TMG scaffold. It is important to note, however, that the structural change from TMG-A13 to TMG-M13 not only varies the interalkyl chain distance but also affects other detergent properties important for membrane protein stability. Notably, TMG-M13 is more hydrophilic than TMG-A13 due to the presence of the shortened alkyl spacer, as demonstrated by their HLB values (12.2 vs 11.9) (Table S1). Thus, the comparable efficacy of these two detergents for protein stabilization observed here likely results from the compromise between the favorable (short interalkyl chain distance) and unfavorable properties (hydrophilic nature) of TMG-M13 compared to TMG-A13.

In the case of TMG-Ps, however, we were able to increase the detergent alkyl chain density without increasing detergent hydrophilicity. The insertion of an additional alkyl chain between the two existing chains reduces detergent hydrophilicity of the TMG-Ps compared to TMG-A13, as supported by the decreased HLB values from 11.9 (TMG-A13) to 11.7 (TMG-P9,7) or 11.3 (TMG-P10,8). The superiority of TMG-P10,8 to TMG-P9,7 seems to originate from the alkyl chain length, in addition to detergent hydrophobicity. In general, detergents effective for membrane protein stability have an alkyl chain length ranging from C10 to C13, suggesting that TMG-P9,7 is a little too short to sufficiently stabilize membrane proteins. It is noteworthy that the insertion of an additional alkyl chain in the lipophilic region alone is unlikely to provide an enhanced membrane protein stability. To effectively increase the alkyl chain density in detergent micelles, it is crucial to introduce an additional alkyl chain between the existing alkyl chains as we did with the TMG-Ps. Therefore, the TMG-P is an ideal platform to attain optimal detergent properties in terms of alkyl chain length, HLB and alkyl chain density in the micelle interiors.

Many recently described detergents contain a maltoside head group, as exemplified by LMNG, 1,3,5-triazine-based maltosides,[55] glycerol-decorated tris(hydroxymethyl)methane-cored maltosides,[35] and tandem 1,3,5-triazine-based maltosides.[56] Maltoside detergents are generally more effective than glucoside detergents for membrane protein stability, as can be seen in the detergent comparison between DDM vs OG or LMNG vs OGNG (Figure S5). However, maltoside detergents may not be ideal when it comes to a membrane protein structural study. Due to their tendency to form large PDCs, maltoside detergents are generally suboptimal for structure determination of membrane proteins via X-ray crystallography, NMR spectroscopy, and

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Bioconjugate Chem. XXXX, XXX, XXX−XXX

single-particle cryo-EM. Thus, glucoside detergents that form smaller PDCs are considered more suitable for membrane protein structural study. As observed with OG, however, many proteins encapsulated by glucoside detergents are not stable enough for structure elucidation and in some cases insufficiently stable for isolation. Detergents that combine the stabilizing properties of maltoside detergents and the compact PDC size characteristic of glucoside detergents could significantly contribute to a membrane protein structural study. OGNG, a glucoside detergent that we previously designed and characterized, conferred similar stability to DDM and has been successfully used for structural studies of 17 membrane proteins.[31] Thus, the new glucoside detergent (TMG-P10,8) described here may have significant use for membrane protein structure determination.

CONCLUSIONS

We modulated the alkyl chain density by introducing two types of structural modifications into the original TMG architecture: shortening the alkyl spacer used to connect the two amphiphilic units from propylene (C3) to methylene (C1) and inserting an additional alkyl chain between two existing alkyl chains. Shortening the alkyl spacer to generate the TMG-Ms had little effect on detergent efficacy for protein stabilization, while the addition of an additional alkyl chain used for the preparation of the TMG-Ps was found to be effective at enhancing protein stability. As a result, TMG-P10,8 was identified as a new detergent not only efficient at extracting membrane proteins from the membranes but also effective at stabilizing membrane proteins long term. Incorporating multiple favorable factors in a small detergent structure, such as optimal alkyl chain length, HLB, alkyl chain density, and glucoside head group, is highly challenging. This result indicates that such a challenge can be achieved via a rational approach to detergent structural modification. As a result, the current study not only affords a detergent tool effective for membrane protein study but also provides an insight into detergent development and optimization. The detergent design principle discussed here ignites future detergent development for membrane protein analysis.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.bioconjchem.3c00507.

Additional experimental details including methods on detergent evaluation with membrane proteins and synthetic procedures and characterizations of the new materials (PDF)

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Notes

The authors declare the following competing financial interest(s): P.S.C., S.Y., H.E.B and A.S. are inventors on a patent application that covers the TMG-M/Ps.

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