New malonate-derived tetraglucoside (MTG) detergents for membrane protein stability

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ABSTRACT: Membrane proteins are widely studied in detergent micelles, a membrane-mimetic system formed by amphiphilic compounds. However, classical detergents have serious limitations in their utility, particularly for unstable proteins such as eukaryotic membrane proteins and membrane protein complexes and thus there is an unmet need for novel amphiphiles with enhanced ability to stabilize membrane proteins. Here, we developed a new class of malonate-derived detergents with four glucosides, designated malonate-derived tetra-glucoside (MTGs) and compared these new detergents with previously reported glucose neopentyl glycol (GNG) and *n*-dodecyl-β-D-maltoside (DDM). When tested with two G-protein coupled receptor (GPCRs) and three transporters, a couple of MTGs consistently conferred enhanced stability to all tested proteins compared to DDM and a GNG. As a result of synthetic convenience and favourable behaviours for a range of membrane proteins, these MTGs have high potential for membrane protein research. This study additionally provides a new detergent design principle regarding the effect of a polar functional group (i.e., ether) on protein stability depending on its position in the detergent scaffold.

Membrane proteins reside in cell membranes, play essential roles in a variety of cellular activities and thus are major targets for a number of marketed pharmaceutical agents. Atomic resolution protein structures provide a fundamental basis for understanding of a protein's mechanism of action in the cellular context and facilitate rational drug design and development.[1a,b] Thus, a tremendous effort has focused on structure determination of membrane proteins using cutting edge biophysical techniques such as cryo-electron microscopy (cryo-EM), X-ray crystallography, and nuclear magnetic resonance (NMR) spectroscopy.[2] However, there is a large difference in the number of membrane proteins of known structure and that of soluble proteins. Despite recent progress in membrane protein structural study; membrane proteins constitute only around 3% of unique protein structures in the protein data bank (PDB) although these bio-macromolecules account for ~30% of the open reading frames (ORFs) in the human genome.[3a-c] The amphiphilic property of membrane proteins is responsible for the relatively limited progress in structural studies of membrane proteins. Due to this intrinsic property, these bio-macromolecules display poor water-solubility and a high propensity to aggregate/denature in aqueous environments. In order to mitigate the issues associated with membrane proteins, diverse membrane mimetic systems have been developed. The traditional yet still the most common system used for membrane protein manipulation are detergent micelles with a hydrophobic core and a hydrophilic exterior.[4a-d] Small amphiphilic molecules, named detergents or surfactants, interact with phospholipid bilayers and the hydrophobic/hydrophilic domains of membrane proteins, and are thus capable of extracting and solubilizing membrane proteins from the membranes. Classical detergents as exemplified by dodecyl-β-D-maltoside (DDM) and octyl-β-D-glucoside (OG) which have a canonical head-to-tail structure and are widely used for membrane protein structural study. However, many unstable proteins such as eukaryotic membrane proteins and oligomeric membrane complexes, readily denature or aggregate over time even in these popular detergents. Micelles formed by a classical detergent are too dynamic to effectively encapsulate membrane proteins. In addition, detergent-extracted membrane proteins tend to lose specific lipid molecules strongly bound to protein surfaces, that are important for maintenance of protein function and stability.[5] Therefore, novel amphiphilic systems need to be developed for effective downstream analyses of membrane protein structure and function.

Over the past two decades, lipid-based self-assemblies such as bicelles,[6] lipidic cubic phase (LCP),[7] and nanodiscs (NDs)[8] have emerged as useful membrane-mimetic systems. In addition, amphiphilic polymers with a polyacrylamide backbone (amphipols)[9] or styrene-maleic acid (SMA) scaffold[10] were invented as innovative approaches, along with amphiphilic peptides (e.g., lipopeptide detergents (LPDs),[11] β-peptides (BPs),[12] peptidisc and Salipro[13]). However, their protein extraction properties are insufficient in most cases. Furthermore, only a couple of systems such as bicelles and LCP have proved effective at generating protein crystals suitable for high resolution structure determination. Practicality is one key issue associated with the peptide-based amphiphiles, while the compositional heterogeneity (i.e., polydispersity) and/or buffer instability (e.g., low pH) are potential problems associated with polymeric amphiphiles.[14] In contrast, small amphiphiles can be used for all the processes of membrane protein research, including protein extraction, purification and crystallization, and are compatible with cutting edge techniques currently used for protein structure determination. Thus, detergent micelles remain currently the optimal system for *in vitro* analysis of

membrane proteins. However, there is substantial scope to improve the stabilization of a wide range of membrane proteins in detergent micelles. Our, and other groups have made substantial efforts to develop novel classes of detergents with enhanced protein stabilization efficacy. Representatives includes glyco-tripod amphiphiles (TPAs),[15] hemifluorinated surfactants (HFSs),[16] cholate or deoxycholate-based facial amphiphiles (FAs),[17] neopentyl glycol amphiphiles with diglucose, dimaltose, or triglucoside head group (GNGs/MNGs/NDTs),[18] mannitol-based amphiphiles (MNAs),[19] mesitylelne-linked glucoside amphiphiles (MGAs),[20] norbornane/resorcinarene/terphenyl-cored amphiphiles (NBMs/RGAs/TPMs),[21-23] rigid hydrophobic group-bearing amphiphiles (e.g., chobimalt,[24] glyco-diosgenin (GDN),[25] and penta-phenylene maltoside (PPM)[26]). Notably, some recent inventions such as penta-saccharide-bearing amphiphiles (PSEs),[27] dendronic trimaltosides (DTMs),[28] and vitamin E-based glucosides (VEGs)[29] showed favourable behaviours for visualization of human β_2-adrenergic receptor (β_2AR) via EM analysis. In the past decade, two NG class amphiphiles, MNG-3 and GNG-3 (commercial names, LMNG and OGNG, respectively) have contributed to the determination of more than 40 new membrane protein structures including the β_2 adrenergic, acetylcholine, opioid G protein-coupled receptors (GPCRs).[30] This successful repertoire underlines the contribution and importance of new micellar systems to membrane protein structural determination. The molecular design introduced in this study was inspired by the suitability of GNG-3 with membrane protein structural study. This glucoside detergent was generally inferior to DDM at stabilizing membrane proteins, yet has contributed to the high resolution 3D structure determinations of several membrane proteins via X-ray crystallographic method, as exemplified by a sodium-pumping pyrophosphatase, human aquaporin 2 (AQP2), and the *N*-Methyl-D-aspartate (NMDA) receptor.[31] Thus, we designed malonate-derived tetra-glucoside detergents (MTGs) using the GNG scaffold, with an intention to improve the protein stabilization efficacy (Scheme 1). Evaluations of these detergents with model membrane protein systems revealed that a few MTGs conferred enhanced stability to all model membrane proteins tested here compared to a gold standard detergent (DDM) and the original GNG (OGNG).

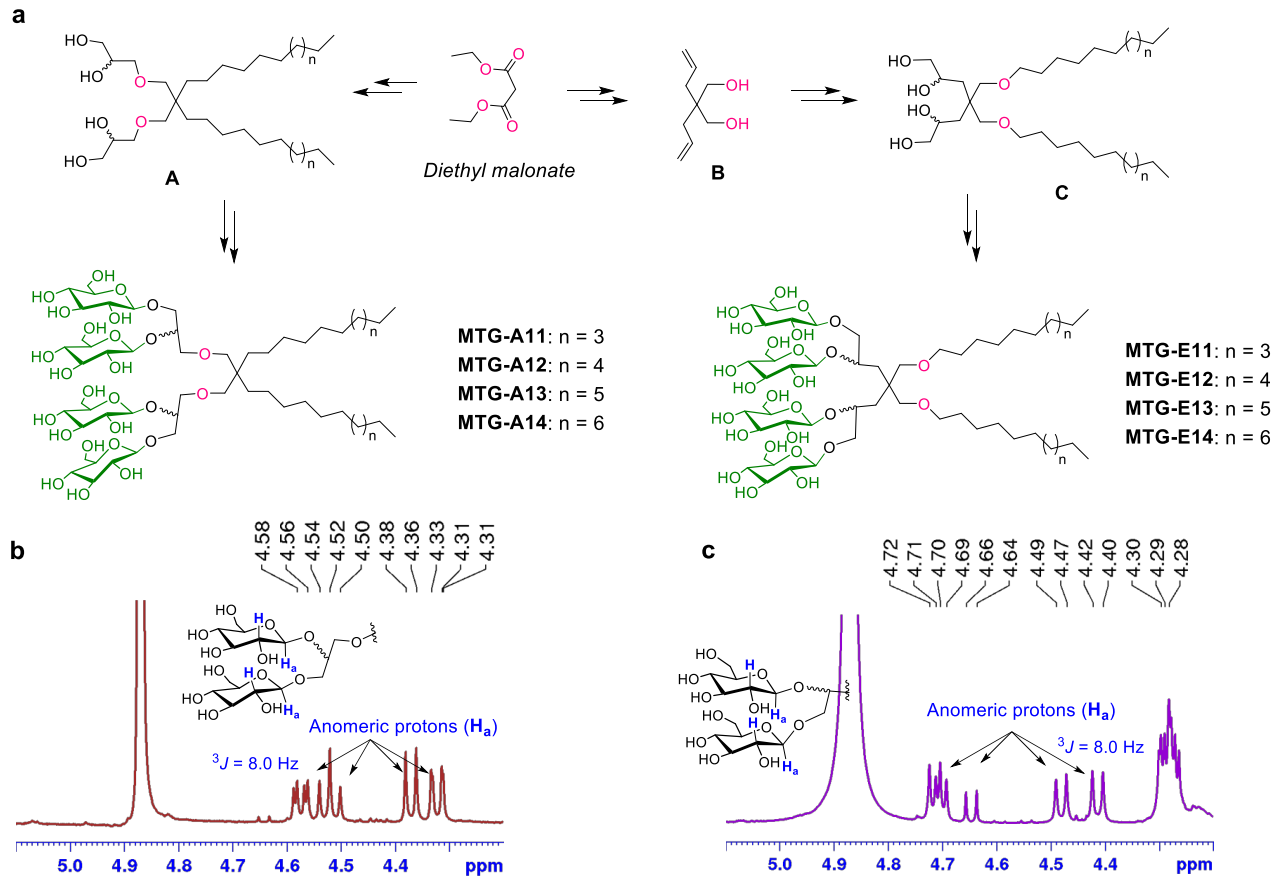
Results

Detergent structures and physical characterizations

The newly designed amphiphiles have two alkyl chains, as seen in GNG-3, but contain four instead of two glucosides in the hydrophilic region (scheme 1). Depending on how the alkyl groups are connected to the central quaternary carbon, the new amphiphiles can be categorized into two sets. One set has the alkyl chains directly attached to the central carbon (MTG-As), while the other set utilizes an ether bonds for this connection (MTG-Es). In both sets, four glucoside units were introduced onto the alkyl chains using two 1,2-diol linkers. The diol linkers were connected to the central carbon via an ether linkage (MTG-As) or directly (MTG-Es). The only difference in the chemical structures between the MTG-As and MTG-Es is the relative location of the ether functional group and thus a comparative study of these two sets is ideal to investigate the effect of the relative location of a polar group (i.e. ether) on protein stability. Because of the presence of four glucosides instead of two, the alkyl chains could be lengthened compared to the previous GNGs. The alkyl chain varied from undecyl (C11) to tetradecyl (C14) for the new agents, significantly longer than the previous GNGs with an alkyl chain ranging from pentyl (C5) to heptyl (C7). This alkyl chain extension is likely beneficial for protein stability because of the enhanced compatibility between the detergent alkyl chain length and the hydrophobic width of membrane proteins (28~32Å). In addition, the variation in the alkyl chain length is necessary to find the optimal balance between hydrophilicity and hydrophobicity (i.e., hydrophile-lipophile balance (HLB)), a critical detergent property for effective protein stabilization.[32]

Both sets of MTG-As and MTG-Es were prepared from inexpensive diethylmalonate according to a synthetic protocol comprising straightforward steps. For preparation of the MTG-As, diethylmalonate was converted to dialkylated tetra-ol derivatives (**A**) *via* four consecutive reactions: alkylation, $LiAlH_4$ reduction, O-allylation and OsO_4-based dihydroxylation. In the case of the MTG-Es, 2,2-diallylpropane-1,3-diol (**B**) was first synthesized by diallylation of diethylmalonate and subsequent reduction of the ester groups with $LiAlH_4$. Next, the alcohol and allyl groups of the compound (**B**) were connected to alkyl chains and transformed into 1,2-diol groups, respectively, to give the tetra-ol derivatives (**C**). Interestingly, the dialkylated tetra-ol derivatives (**A** and **C**) were prepared in a single chromatographic separation by combining the initial synthetic steps together, thereby giving high accessibility to the MTGs. In the following reaction, AgOTf-promoted glycosylation was carried out using the dialkylated tetra-ol compounds (**A** and **C**) and perbenzoylated glucosylbromide as glycosyl acceptors and donor for, respectively (see Scheme S1† for details). Multi-gram quantity preparation of these detergent materials was feasible due to the convenient synthesis and high synthetic yield of every synthetic step.

The four glycosidic bonds newly formed in the glycosylation step are likely to have β-stereochemistry as the benzoyl-protected glucosylbromide was used as a glycosyl donor. β-stereochemistry of the glycosidic bonds was identified from the chemical shifts of anomeric peaks in the ^{1}H NMR spectra of the individual MTGs (Fig. 1b, c & S1†). The presence of peaks in the range of 4.25-4.75 ppm are strong evidence for the formation of the β-selective glycosidic bonds; an α-anomeric proton typically produces an NMR peak around 5.15 ppm. The anomeric peaks observed here showed a rather complex pattern because the two kinds of alcohol (primary and secondary) were used to attach the glucose units. The presence of two epimeric carbons generated in the course of the OsO_4-catalyzed dihydroxylation additionally contributes to the complexity of the β-anomeric peaks. The β-stereochemistry of the glycosidic bonds was further supported by a coupling constant (^3J) between the anomeric (C1; H_a) and neighbouring C2 protons (H), estimated to be 8.0 Hz in all the cases. This J value significantly differs from that of an α-glycosidic bond (^3J = 4.0 Hz).



Scheme 1 Synthetic scheme (a) and NMR characterization (b,c) of newly prepared malonate-derived tetra-glucoside detergents (MTG-As or MTG-Es). (a) Diethylmalonate was used as starting material for generation of the new detergents. (left) Dialkylated tetra-ols (compound **A**) were prepared by a successive operation of multiple reactions of dialkylation, reduction, allylation and syn-dihydroxylation. (right) For the preparation of the dialkylated tetra-ols (compounds **C**), diethylmalonate was first converted into diallylated compounds (**B**) which were then subjected to a synthetic protocol similar to that used for the preparation of compounds shown in **A**. The resulting tetra-ol derivatives containing two alkyl chains (compounds **A** and **C**) were stereo-selectively glycosylated using silver triflate (AgOTf) and benzoyl-protected glucosyl bromide as a promoter and glycosyl donor, respectively. Following glycosylation, the benzoyl protecting groups were removed by global deprotection to afford the two sets of MTGs as the final products. The stereochemistry of the newly formed glycosidic bonds was confirmed by the NMR spectrum of each MTG. As representatives, NMR spectra of (b) MTG-A12 and (c) MTG-E12 focusing on the anomeric region (4.20 ~ 5.10 ppm) are shown with the chemical structures of their head groups. The NMR peaks corresponding to the anomeric protons (H_a) of the MTGs (indicated by the arrows) had coupling constants (^3J) with the neighboring protons (H) of 8.0 Hz in both cases, supporting the β-stereo-chemistry of the glycosidic bonds as expected from neighboring group participation.

High water-solubility is a prerequisite for detergent use in a biophysical study of membrane proteins. All MTGs were highly soluble in water (>10 % w/v), indicative of effective micelle formation. We observed no noticeable precipitation in the individual detergent solutions over a one-month incubation at room temperature. Aggregation behaviours of the MTGs were investigated by measuring critical micelle concentrations (CMCs) and hydrodynamic diameters (D_h) of their micelles in aqueous solution. Critical micelle concentrations (CMCs) were estimated by monitoring inclusion of a water-insoluble fluorescent dye[33] by detergent micelles, while the hydrodynamic diameters (D_h) of detergent micelles were determined through dynamic light scattering (DLS) measurements. The summarized data for the MTGs along with OGNG and DDM are presented in Table 1. As expected from the existence of long double alkyl chains compared to the previous GNGs, all malonate-derived detergents introduced here gave low CMCs ranging from ~10 to ~2 μM, much lower than those of OGNG (~1000 μM) and DDM (170 μM). This result indicates the increased propensity of these agents to self-assemble, which is directly related to micellar stability. When detergent CMC of the two isomeric MTGs (e.g., MTG-E11 *vs.* MTG-A11), were comparable. Detergent CMC values were inversely proportional to the alkyl chain length for both sets of MTGs, as expected from hydrophobicity of the detergent alkyl chains. For instance, the CMC values of the MTG-As were reduced from 12 to 2 μM with an alkyl chain length increase from C11 to C14. Micelles formed by the MTGs varied from 6.1 to 7.7 nm in their sizes with the short alkyl-chained MTGs (MTG-A11, MTG-E11 and MTG-E12) forming smaller micelles than DDM (6.0-6.4 *vs* 6.8 nm), while the long alkyl-chained MTGs (e.g., MTG-A13/A14 or MTG-E14) formed larger micelles than DDM (7.2-7.7 *vs* 6.8 nm). Interestingly, the MTG-Es form micelles substantially smaller than the MTG-A counterparts, as exemplified by MTG-E12 and MTG-A12 (6.4 and 6.8 nm, respectively), a feature which likely influences their protein stabilization properties. For both sets of MTGs the detergent micelle size increases with increasing alkyl chain

length. Further analysis of the DLS data reveals that all MTGs form highly homogeneous micelles (Figure S2†).

Table 1 Molecular weights (MWs), critical micelle concentrations (CMCs) of MTGs along with a previous GNG (OGNG) and a classical detergent (DDM), and hydrodynamic diameter (D_h) (mean±S.D., n = 5) and water-solubility of their micelles.

Detergent	M.W.[a]	CMC (μM)	CMC (wt%)	D_h (nm)[b]	Solubility (wt%)
MTG-A11	1181.4	~ 12	~ 0.0014	6.4±0.4	~10
MTG-A12	1209.5	~ 9	~ 0.0011	6.8±0.5	~10
MTG-A13	1237.5	~ 3.5	~ 0.0004	7.4±0.4	~10
MTG-A14	1265.6	~ 2	~ 0.0003	7.7±0.2	~10
MTG-E11	1181.4	~ 10	~ 0.0012	6.2±0.2	~10
MTG-E12	1209.5	~ 8	~ 0.0010	6.4±0.4	~10
MTG-E13	1237.5	~ 3.5	~ 0.0004	6.7±0.2	~10
MTG-E14	1265.6	~ 2	~ 0.0003	7.2±0.1	~10
OGNG	568.7	~ 1000	~ 0.057	6.1±0.0	~10
DDM	510.6	~ 170	~ 0.0087	6.8±0.0	~10

[a] Molecular weight of detergents. [b]Micelle size determined at 1.0 wt% detergent concentration at room temperature.

Detergent evaluation for membrane protein stability

In order to investigate detergent utility as a biochemical tool for membrane protein study, the *Arabidopsis thaliana* boron transporter 1 (AtBOR1) was first tested with the MTGs.[34] AtBOR1 was expressed in *Saccharomyces cerevisiae* and DDM was used for purification of the transporter. Detergents in DDM-AtBOR1 complexes were then exchanged into the individual MTGs or OGNG via sample dilution. The folded state of the transporter was monitored using a thiol-specific fluorescent dye, *N*-[4-(7-diethylamino-4-methyl-3-coumarinyl)phenyl]maleimide (CPM), during a 120-min incubation at 40 °C.[35] At detergent concentrations of CMC+0.04 wt%, the thermal denaturation profiles showed little difference between the MTG-As and DDM (or OGNG) (Figure S3†). When detergent concentrations were increased to CMC+0.2 wt%, we found improved detergent efficacy for the alkyl versions of the MTGs; the MTG-As were more effective than DDM and OGNG at maintaining AtBOR1 in a stable state (Figure S3†). In the case of the ether versions (MTG-Es), the best efficacy was observed for MTG-E14 at CMC+0.04 wt%, while the other MTG-Es (MTG-E11, MTG-E12 and MTG-E13) were only a little better than DDM and OGNG (Figure S4a†). At increased detergent concentrations of CMC+0.2 wt%, MTG-E11 and MTG-E14 were most effective at stabilizing the transporter under the conditions tested (Figure S4b†). As the MTG-Es were generally better than the MTG-As in the CPM assay, we selected these ether versions (MTG-E11/E12/E13/E14) for further analysis with AtBOR1. In order to monitor protein integrity *via* fluorescence size exclusion chromatography (FSEC), AtBOR1 fused with GFP was produced in *E. coli* membranes treated with the individual MTG-Es/DDM/OGNG at 1.0 wt%. The stability of the detergent-extracted AtBOR1-GFP was estimated by thermally treating the samples at 46 °C for 10 min. Although most effective in the CPM assay, MTG-E14 failed to extract sufficient amounts of the transporter from the membranes, and thus was not included in the heated FSEC study. The other MTG-Es all extracted sufficient amounts of the fusion protein, although their solubilization efficiencies were inferior to DDM (50~60% *vs* ~85%). When the detergent-extracted AtBOR1-GFP was subjected to FSEC before and after heat treatment, the DDM-solubilized transporter showed a large decrease in the main protein peak (fraction number 35), along with a substantial increase in the aggregation peak (fraction number 2) (Figure 1). The use of OGNG resulted in a complete disappearance of the main peak and appearance of a very large aggregation peak under the same conditions. This result indicates that AtBOR1-GFP in OGNG micelles fully aggregated/denatured over the course of thermal treatment. Following heat treatment, all the tested MTG-Es (MTG-E11, MTG-E12 and MTG-E13) also showed a decrease in the intact protein peak, but the reduction was substantially smaller, particularly in the case of MTG-E11, than that observed for DDM (Figure 1a,b). The combined results of CPM and FSEC assays indicate that the MTGs were more effective than DDM and OGNG at stabilizing AtBOR1, with the ether versions superior to the alkyl counterparts.

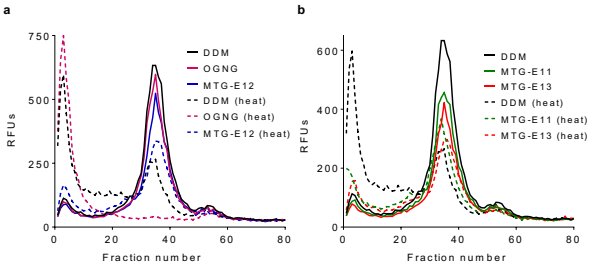


Figure 1. Fluorescence size exclusion chromatography (FSEC) profiles of AtBOR1 fused with GFP (AtBOR1-GFP) before and after thermal treatment. Individual MTGs (MTG-E11, MTG-E12 and MTG-E13), DDM and OGNG were used at 1.0 wt% for extraction of the fusion protein. Detergent-extracted AtBOR1-GFP was treated at 46 °C for 10 min before the samples were loaded onto the SEC column. The data shown is representative of two independent experiments.

Next, we assessed the MTGs with a bacterial leucine transporter (LeuT) from *Aquifex aeolicus*.[36,37] Protein stability was assessed by measuring substrate binding capability, monitored using a radio-active substrate ([^{3}H]-leucine (Leu)) via scintillation proximity assay (SPA).[38] LeuT was first extracted and purified in DDM and the resulting DDM-solubilized LeuT subjected to detergent exchange from DDM into the individual MTGs or OGNG. Using the individual detergents at CMCs+0.04 wt%, detergent effect on LeuT stability was monitored over a 12-day incubation period at room temperature. OGNG completely abolished [^{3}H]-Leu binding ability of the transporter within one day, while DDM showed a gradual decrease in substrate binding activity (Figure 2a). All MTGs were superior to DDM at stabilizing the bacterial transporter long-term, with the best performance observed for MTG-E13. At the higher detergent concentrations of CMC+0.2 wt%, LeuT in some MTGs (MTG-A13/A14/E14) had initial activity lower than the transporter in DDM (t = 0 day), but protein activity recovered within a day of incubation. More importantly, the restored substrate binding ability was better retained over time when LeuT was solubilized in the MTGs rather than DDM (Figure 2b). The initial increase in transporter activity may be due to a slow detergent exchange

from DDM to the MTG. Collectively, the results reveal that all MTGs except two long alkyl chained MTG (MTG-A13 and MTG-A14) are superior to DDM/OGNG at retaining LeuT stability over time.

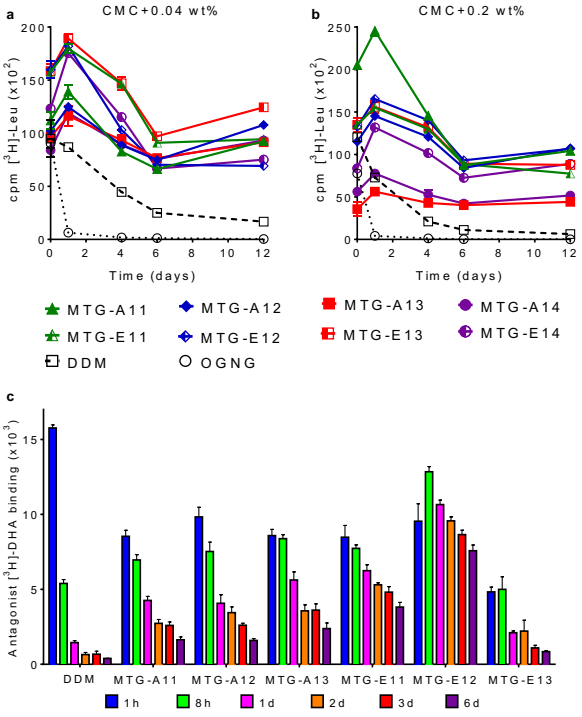


Figure 2. Time course substrate/ligand binding ability of LeuT (a,b) and β_2AR (c) solubilized in MTGs, DDM, and OGNG. Two detergent concentrations (CMC+0.04 wt% (a) or CMC+0.2 wt% (b)) were used for LeuT stability assay, while one detergent concentration (CMC+0.2 wt%) was used for β_2AR stability assay (c). The radio-active substrate ([^{3}H]-leucine (Leu)) and antagonist ([^{3}H]-dihydroalprenolol (DHA)) were utilized for LeuT and β_2AR stability measurements, respectively. Protein samples were incubated for 12 days (LeuT) or 6 days (β_2AR) and substrate/ligand binding ability of the transporter/receptor was monitored at regular intervals during the incubation at room temperature. Error bars: SEM, n = 2-3 (LeuT); n = 3 (β_2AR).

We turned to the human β_2 adrenergic receptor (β_2AR), a GPCR, to further evaluate the MTGs.[39] The receptor purified in DDM was subjected to detergent exchange conducted by a 30-min sample dilution and the final detergent concentrations were CMC+0.2 wt%. A ligand binding assay using a radio-active antagonist ([^{3}H]-dihydroalprenolol (DHA)) was carried out to assess receptor stability.[40] In a preliminary study where receptor capability of ligand binding was measured right after detergent exchange, OGNG-solubilized receptor showed no ligand binding capability (Figure S5†). Intriguingly, the use of the MTGs led to [^{3}H]-DHA-binding capability of the receptor comparable to DDM in all the cases except the two longest alkyl chained detergents (MTG-A14 and MTG-E14). Thus, the six MTGs (MTG-A11/A12/A13 and MTG-E11/E12/E13) were selected to further investigate their effects on receptor stability over time. Receptor activity was monitored at the designated time point over the course of a 6-day incubation at 25 °C (Figure 2c). When solubilized in DDM, receptor activity rapidly dropped to give ~ 10% of the initial activity in two days. In contrast, all the selected detergents except MTG-E13 were significantly more effective than DDM at retaining β_2AR activity long term, with the best performance detected with MTG-E12.

As an additional investigation, we utilized another transporter, melibiose permease from *Salmonella typhimurium* ($MelB_{St}$).[41] The permease was first produced in *E. coli* membranes and then extracted on ice by each detergent (MTG/DDM/OGNG) for 90 min. Following ultracentrifugation, detergent extracts were further thermally treated at a higher temperature (45, 55, or 65 °C) for another 90 min. The amounts of soluble $MelB_{St}$ were estimated *via* Western blot analysis and represented as percentages (%) of total transporter in the untreated membranes. As all tested detergents are mild enough to minimally destroy transporter integrity at 0 °C, the amount of soluble $MelB_{St}$ detected at this low temperature is correlated with how efficiently the individual detergents extract $MelB_{St}$ from the membranes. On the other hand, the amount of soluble $MelB_{St}$ obtained from the thermally treated samples (45, 55, or 65 °C) strongly reflects a detergent that maintains the transporter in a soluble state under the tested conditions. The thermal treatment accelerates protein denaturation/aggregation to a different degree depending on detergent ability to maintain $MelB_{St}$ stability. At 0 °C, of the tested detergents, only OGNG yielded the amount of soluble $MelB_{St}$ comparable to DDM (~100%), while all new detergents except MTG-A14 yielded 60~80% soluble $MelB_{St}$ (Figure 4a). These solubilisation results are consistent with those obtained with AtBOR1. When the detergent extracts were incubated at 45 °C, OGNG gave a much decreased yield of $MelB_{St}$ (~30%), indicating substantial protein denaturation and/or aggregation at this temperature, as seen for AtBOR1, LeuT, and β_2AR. In contrast, use of each MTG resulted in a higher amount of soluble $MelB_{St}$ under the same conditions, likely due to enhanced membrane dynamics or detergent solubility (e.g., MTG-A14). A clear differentiation in detergent efficacy was observed between DDM and the MTGs when the detergents were evaluated at 55 °C. DDM-solubilized $MelB_{St}$ completely lost its solubility in aqueous solution, while a few MTGs such as MTG-A13, MTG-E12, MTG-E13, and MTG-E14 fully maintained the soluble state of the transporter. The MTG-Es were generally better than the MTG-As for $MelB_{St}$ stability, consistent with results for AtBOR1. At 65 °C, a trace amount of soluble $MelB_{St}$ was detected only in the case of MTG-E14. For further verification of $MelB_{St}$ stability, MTG-E13 was selected for assessment of MelB functionality. For this purpose, we utilized fluorescence response of the transporter over the course of the sequential addition of a fluorescent ligand (*i.e.*, 2'-(*N*-dansyl)aminoalkyl-1-thio-β-D-galactopyranoside (D^2G)) and a non-fluorescent substrate (i.e., melibiose).[41,42] Upon addition of D^2G, a functional $MelB_{St}$ would strongly bind to this fluorescent ligand, giving high fluorescence intensity due to the efficient energy transfer from tryptophan to D^2G bound to the active site. The subsequent addition of excess melibiose (a non-fluorescent substrate) leads to reduction in a fluorescent signal as a result of a ligand-substrate exchange in the active site. Thus, $MelB_{St}$ functional state can be unambiguously estimated by monitoring fluorescence intensity with sequential addition of D^2G and melibiose. As expected from a mild property of DDM, DDM-solubilized $MelB_{St}$ showed initial increase and subsequent decrease in the fluorescence signal upon sequential addition of D^2G and melibiose (Figure 4b). However, we found a complete loss in fluorescence response of the transporter when a less

stable homologue, $MelB_{EC}$ obtained from *E. coli*, was used for this functional assay. In contrast, the selected test detergent, MTG-E13, was effective at preserving the functionality of the unstable MelB homologue under the same conditions, indicating that this malonate-based glucoside outperforms DDM with regards to retention of functional MelB.

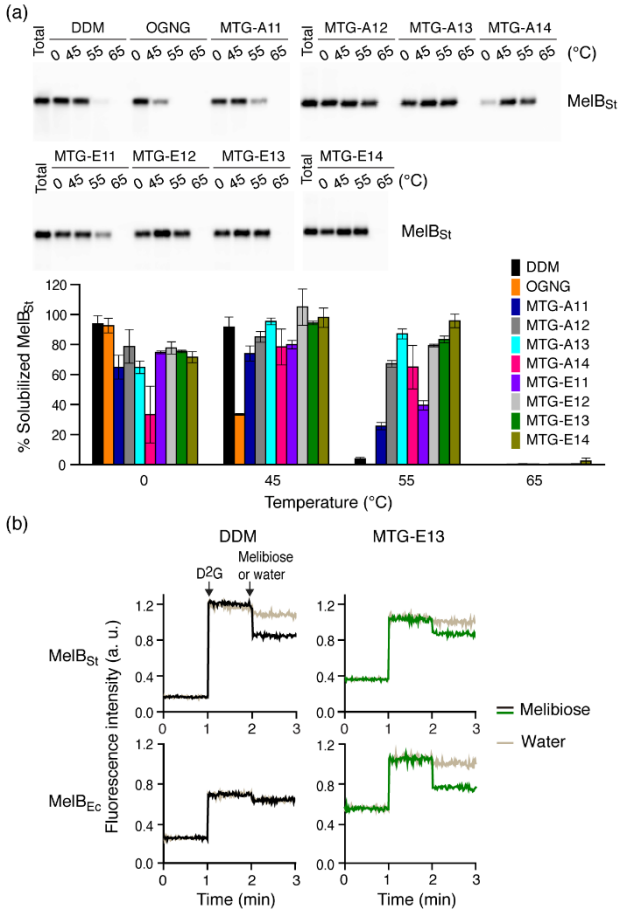


Figure 4. (a) Western blot image of detergent-extracted $MelB_{St}$. MTGs (MTG-A11/A12/A13/A14 and MTG-E11/E12/E13/E14), DDM and OGNG were used at 1.5 wt% for $MelB_{St}$ extraction conducted at 0 °C for 90 min. Detergent-extracted samples were further treated at a higher temperature (45, 55, or 65 °C) for another 90 min. The detergent-extracted samples at 0 °C or thermally treated samples were subjected to ultracentrifugation and then analysed by Western blot (top panel). The amounts of soluble $MelB_{St}$ are represented as relative percentages (%) of total $MelB_{St}$ in histogram (bottom panel). Error bars, SEM, n = 3. (b) Galactoside binding of MelB in MTG-E13. MelB function was assessed using FRET reversal elicited by a ligand-substrate exchange in the active site. Dansyl-2-galacotside (D^2G) and excess melibiose were added to a solution containing DDM/MTG-E13-extracted MelB ($MelB_{St}$ and $MelB_{Ec}$) at 1-min and 2-min time points, respectively. The resulting responses in fluorescence intensity of the samples were then monitored over the course of the additions. Control data was obtained from addition of water instead of melibiose. Melibiose additions are indicated in black (DDM)/green (MTG-E13) and pale brown, respectively with water addition indicated in grey.

As MTG-E11, MTG-E12 and MTG-E13 displayed the most favorable behaviors with multiple membrane proteins, these MTG-Es were further assessed with another GPCR, mouse μ-opioid receptor (MOR). Receptor stability was assessed by measuring melting temperature (T_m) using differential scanning fluorimetry assay. Receptor T_m was estimated by finding the minimum point of negative first order derivative of relative fluorescence units (-dRFU/dT) (Figures 5 and S6). The receptor samples were prepared by diluting DDM-purified receptor into individual detergent-supplemented buffer solutions. The DDM-purified MOR gave a relatively low T_m (28 °C), indicating that the receptor is of limited stability in DDM micelles. When the previous GNG, OGNG, was used in receptor CPM assay, this glucoside detergent showed unusual CPM profiles with a large variation from one experiment to another, strongly suggesting an occurrence of a significant loss in receptor stability during detergent exchange. In contrast, CPM profiles obtained from the tested MTG-Es were highly consistent and receptor T_ms were substantially higher than that of DDM. The receptor in MTG-E11 yielded a T_m of 31 °C, while MTG-E12 and MTG-E13 produced the receptors with T_m of 35 and 37.3 °C, respectively. An increase of 9.3 °C in receptor T_m was observed when MTG-E13 was used as a solubilizing agent instead of DDM. This result is a clear demonstration of enhanced effectiveness of the MTG-Es at stabilizing MOR compared to the conventional detergent.

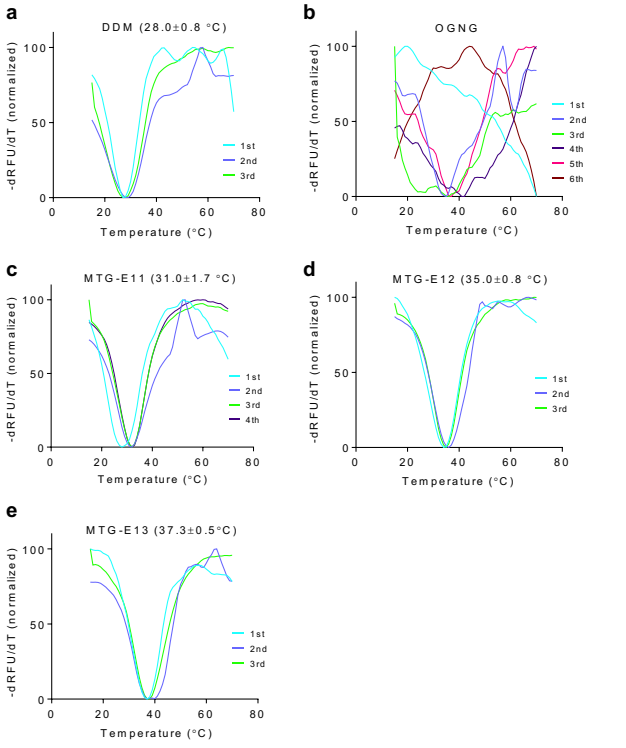


Figure 5. Derivative functions of CPM profiles obtained from CPM assay of MOR solubilized in DDM (a), OGNG (b), MTG-E11 (c), MTG-E12 (d), or MTG-E13 (e). DDM-purified receptor was subjected to detergent exchange and the resulting receptor in the individual detergents thermally denatured in the presence of CPM dye by increasing the temperature from 20 to 70 °C. The value in parenthesis represents average receptor T_m ± SEM. T_m of OGNG-solubilized MOR could not be obtained due to the inconsistency of the data.

Discussion

The current study showed that the most effective detergent for protein stability varied depending on the individual membrane proteins tested here. AtBOR1 and LeuT were most stable in MTG-E14 and MTG-A12/E13, respectively, while MTG-E12 and MTG-A13/E14 conferred the greatest stability to β_2AR and MelB, respectively. This protein-specific nature of detergent efficacy can be further illustrated by the behaviour of MTG-E13. In the case of MTG-E13, the superior efficacy was observed with all the membrane proteins tested here except β_2AR, indicating that this C13 version could be an excellent tool for studying many challenging membrane proteins. Despite the protein-specific nature of detergent efficacy, however, it is noteworthy that MTG-E12 was consistently more effective than DDM at stabilizing all the tested membrane proteins, thereby indicating the presence of a rare detergent attribute, i.e., universal nature of detergent efficacy. This C12 detergent was particularly effective at stabilizing LeuT, β_2AR, and MelB. MTG-E11 was also consistently better than DDM with the individual membrane proteins tested, but this C11 version was slightly inferior to MTG-E12 for stabilization of MelB and two GPCRs (β_2AR and MOR). Importantly, all the MTGs derived from GNG scaffold were markedly superior to a previous version (i.e., OGNG) for protein stabilization. Thus, the current study exemplifies that a single simple modification of a detergent (i.e., a structural change in the detergent head group from diglucoside to tetraglucoside) greatly enhances detergent efficacy. These detergents feature the presence of a compact head group and sufficiently long alkyl chains compatible with the protein hydrophobic dimensions that are likely responsible for their favourable behaviours. Because of their widely applicable stabilising effects and synthetic convenience, MTG-E11, MTG-E12 and MTG-E13 hold significant potential for membrane protein structural study.

The two sets of new agents (MTG-As and MTG-Es) have a minor difference in their chemical structures. The two ether bonds are close to the central quaternary carbon, but these groups were located in the hydrophilic region for the MTG-As, or the lipophilic region for the MTG-Es. As a result, the MTG-As are constitutional isomers of the MTG-E counterparts. Despite the minor differences in their chemical structures, we observed rather large differences in their micelle sizes and efficacies for membrane protein stabilization. The micelles formed by the MTG-As were larger than their isomeric MTG-E counterparts. For example, MTG-A13 formed micelles with a D_h of 7.4 nm, larger than MTG-E13 micelles with a D_h of 6.7 nm. The relatively large micelle sizes of the MTG-As are somewhat unexpected as it seems obvious that the alkyl versions have a larger effective volume of the head group compared to the ether versions mainly due to the presences of the longer spacer between the carbohydrate units and the central quaternary carbon. This unexpected result means that the effective volume of the tail group is substantially smaller for the ether version (vs. the alkyl counterpart) in a micellar environment. This relatively small volume of the MTG-E tail groups is probably due to the presence of the ether functional group in the lipophilic region. As a result, the ether versions (i.e., MTG-Es) likely produce more compact and stable packing of the alkyl chains in the micelle interiors, and this results in greater protein stability in the MTG-Es compared to the MTG-As as seen with AtBOR1 and β_2AR. The superior efficacy of the MTG-Es was also found in the detergent evaluations with LeuT and MelB although the detergent efficacy difference was less clear in these cases. Therefore, this study implies that even a very small variation in detergent structure can significantly impact detergent efficacy for membrane protein stabilization.

Conclusions

In summary, we designed and prepared two sets of tetraglucoside-containing detergents (MTG-As and MTG-Es) differing in the relative position of the ether bonds within the detergent architecture. When these agents were evaluated with a set of model membrane proteins, we identified a few detergents (MTG-E11, MTG-E12 and MTG-E13) that are markedly superior to a gold standard detergent of DDM and the previously reported OGNG at stabilizing almost every protein tested here. Due to universal detergent efficacy for protein stabilization, these malonate-derived glucosides are potential alternatives to classical detergents or other novel detergents for membrane protein study. Furthermore, comparative analysis of the MTG-Es with the MTG-As identified that a small variation in detergent structure has an effect on protein stability, helping rational detergent design and selection for membrane protein research.

ASSOCIATED CONTENT

The supporting information is available free of charge via the internet at http://pubs.acs.org, including Figures S1 through S6, supplementary methods on detergent evaluation with membrane proteins, and synthetic protocols and characterizations of the new detergents.

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Notes

The authors declare no competing financial interest(s).

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New malonate-derived tetraglucoside (MTG) detergents for membrane protein stability

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