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Article

Tris(hydroxymethyl)aminomethane Linker-Bearing Triazine-Based Triglucosides for Solubilization and Stabilization of Membrane Proteins

Lubna Ghani, Xiang Zhang, Chastine F. Munk, Parameswaran Hariharan, Baoliang Lan, Hong Sik Yun, Bernadette Byrne, Lan Guan, Claus J. Loland, Xiangyu Liu, and Pil Seok Chae*

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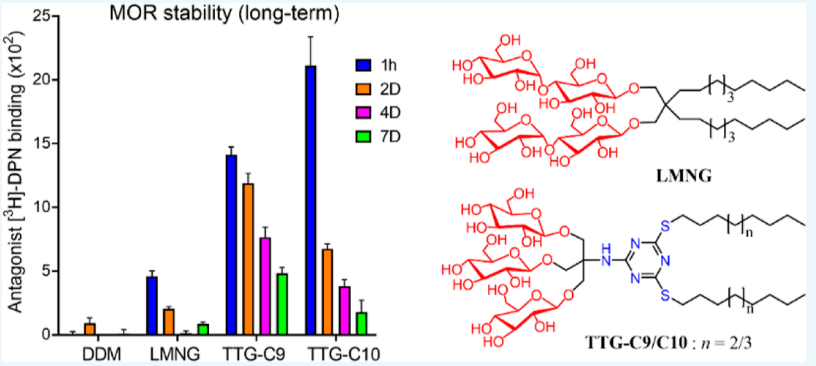

ABSTRACT: High-resolution membrane protein structures are essential for a fundamental understanding of the molecular basis of diverse cellular processes and for drug discovery. Detergents are widely used to extract membrane-spanning proteins from membranes and maintain them in a functional state for downstream characterization. Due to limited long-term stability of membrane proteins encapsulated in conventional detergents, development of novel agents is required to facilitate membrane protein structural study. In the current study, we designed and synthesized tris(hydroxymethyl)aminomethane linker-bearing triazine-based triglucosides (TTGs) for solubilization and stabilization of membrane proteins. When these glucoside detergents were evaluated for four membrane proteins including two G protein-coupled receptors, a few TTGs including TTG-C10 and TTG-C11 displayed markedly enhanced behaviors toward membrane protein stability relative to two maltoside detergents [DDM (*n*-dodecyl-*β*-D-maltoside) and LMNG (lauryl maltose neopentyl glycol)]. This is a notable feature of the TTGs as glucoside detergents tend to be inferior to maltoside detergents at stabilizing membrane proteins. The favorable behavior of the TTGs for membrane protein stability is likely due to the high hydrophobicity of the lipophilic groups, an optimal range of hydrophilic−lipophilic balance, and the absence of cis−trans isomerism.

Integral membrane proteins play crucial roles in various cellular functions, such as material and signal transfer between the cell interior and exterior, catalytic conversion of biomolecules, cell adhesion, and cell-to-cell communication.[1] These bio-macromolecules are major human drug targets.[2,3] In particular, G protein-coupled receptors (GPCRs) are the targets of more than 40% of currently available drug molecules.[4] In order to fully understand cellular processes at the membrane and to facilitate drug discovery, high-resolution membrane protein structures are of critical importance. However, structural and functional characterization of membrane proteins remains extremely challenging due to a number of features including low natural abundance, amphiphilic characteristics, and high conformational flexibility. Despite recent advances in protein structure determination methods, there are still a small number of membrane proteins of known structure compared to soluble proteins. The main difficulty is in finding an appropriate membrane mimetic system that is highly efficient/effective for membrane protein solubilization and stabilization. Detergents, amphipathic agents, serve as essential tools for structural study of membrane proteins. These are widely used not only to extract membrane proteins from the membrane but also to maintain protein integrity over the course of protein purification, necessary for downstream characterization.[5−7] Many conventional deter-

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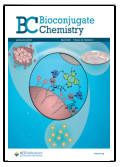

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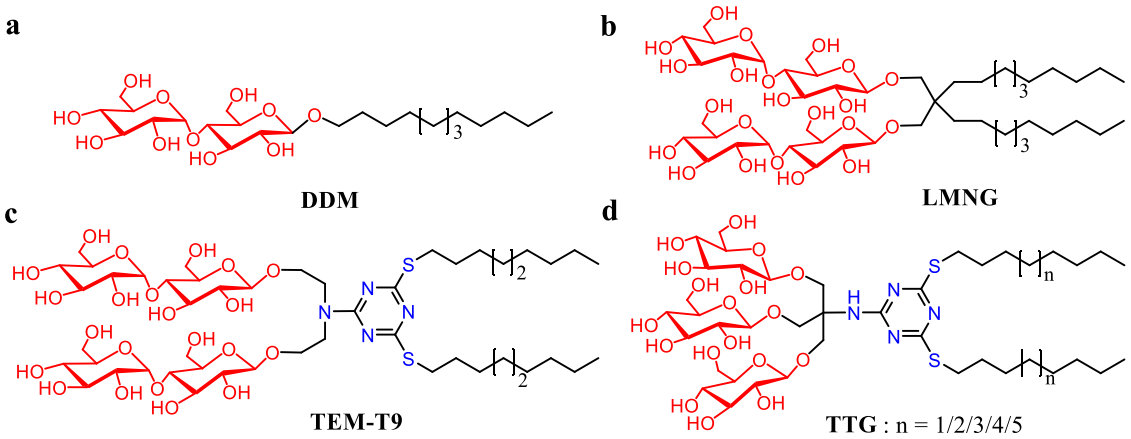

Figure 1. Chemical structures of the conventional detergent [DDM (a)], two previously developed detergents [LMNG (b) and TEM-T9 (c)], and the newly prepared detergents [TTGs (d)]. TEM-T9 and TTGs differ from DDM and LMNG in terms of the presence of the 1,3,5-triazine core between the head group and the alkyl chains. The newly prepared TTGs are distinct from the other detergents including TEM-T9, in terms of containing a glucoside, rather than a maltoside, head group.

gents such as OG (*n*-octyl-*β*-D-glucoside), DM (*n*-decyl-*β*-D-maltoside), and DDM (*n*-dodecyl-*β*-D-maltoside) remain widely used for membrane protein solubilization, purification, and structural study. However, these conventional detergents are often unsuitable for the study of biologically and pharmaceutically important membrane proteins such as eukaryotic proteins and protein complexes with multiple subunits. These fragile membrane proteins have the tendency to aggregate and/or denature over time when solubilized in these conventional detergents.[8] The limited properties of conventional detergents for membrane protein study likely originate from their canonical architecture of single head and tail groups. Micelles formed by these detergents are much more dynamic compared to phospholipid bilayers and are thus less effective at maintaining the native structure of membrane proteins.[9] In addition, detergent-extracted membrane proteins often lose lipid molecules specifically bound to the protein surface that are essential for preserving protein integrity.[10,11] Therefore, it is necessary to find novel amphiphilic systems that confer enhanced stability to membrane proteins and thus facilitate downstream analysis of these important bio-macromolecules.

Over the past 2 decades, several membrane-mimetic systems have been developed, as exemplified by amphiphilic polymers [amphipols (Apols)[12] and styrene−maleic acid (SMA) copolymers][13] and amphiphilic peptides/proteins [lipopeptide detergents (LPDs),[14] *β*-peptides (BPs),[15] peptidiscs,[16] membrane scaffold proteins (MSPs)[17] and saposin A].[18] Among these membrane-mimetic systems, MSPs, SMA copolymers, and saposin A are capable of forming nano-sized superassemblies with membrane proteins and lipids, MSP-based-nanodiscs (NDs), styrene−maleic acid lipid particles (SMALPs) and Salipro, respectively. In these nano-assemblies, membrane proteins are surrounded by a small segment of lipid bilayer stabilized by an amphiphilic protein/peptide/polymer. As a result, membrane proteins in these nano-assemblies are, in principle, highly stable and thus have been successfully used for cryo-electron microscopy (cryo-EM)-based structural studies.[19] However, these protein/polymer-based amphiphilic molecules are either unsuitable for or poor at extracting membrane proteins from the membranes and have had limited success at crystallization of membrane proteins. In contrast, small amphiphiles, called detergents, are highly compatible with all membrane protein manipulations (protein extraction, purification, and crystallization) as well as all protein structure determination methods including nuclear magnetic resonance (NMR) spectroscopy, X-ray crystallography, and cryo-EM. The single major disadvantage of detergent micelle systems is suboptimal behavior in terms of membrane protein stability. Thus, as long as they display enhanced membrane protein stabilization efficacy, detergent micelles can be extremely useful for membrane protein research. A great deal of effort has been devoted to the development of novel classes of amphiphiles for this purpose. These include facial amphiphiles (FAs),[20,21] neopentyl glycol-based amphiphiles (GNGs/MNGs/NDTs),[22−25] mannitol-based or mesitylene-linked glucoside amphiphiles (MNAs and MGAs),[26,27] rigid core [norbornane/resorcinarene/cyclopentane-bearing amphiphiles (NBMs/RGAs/CPMs)],[28−30] rigid hydrophobic group-bearing amphiphiles [e.g., chobimalt,[31] glyco-diosgenin (GDN),[32] scyllo-inositol-based glycosides (SIGs),[33] and penta-phenylene maltoside (PPM)].[34] Pendant-bearing amphiphiles, as exemplified by pendant-bearing GNGs (P-GNGs),[35] lithocholate-based FAs (LFA),[36] and glycerol-decorated trimaltosides (GTM),[37] are noteworthy as the pendant introduced into the hydrophilic−hydrophobic interface of these detergent molecules has a favorable influence on membrane protein stability. Very recently, foldable detergents highlighted that the detergent ability to dramatically change conformations when interacting with membrane protein surfaces can result in enhanced protein stability.[38] Among these newly developed amphiphiles, it is notable that MNG-3 [commercial name: lauryl maltose neopentyl glycol (LMNG)] has been successfully used for structural studies of more than 300 membrane proteins over the last 11 years (Figure 1).[39] The wide use of LMNG in membrane protein research underlines the pivotal role of novel amphiphiles in membrane protein structural studies. In the current study, we prepared a class of detergents containing a 1,3,5-triazine core, two alkyl chains, and three glucose units, designated tris(hydroxymethyl)aminomethane (TRIS) linker-bearing triazine-based triglucosides (TTGs). When evaluated with a few membrane proteins [leucine transporter (LeuT), melibiose permease (MelB), β_2 adrenergic receptor (β_2AR), and μ-opioid receptor (MOR)], these glucoside detergents displayed favorable behaviors toward membrane protein stabilization compared to two widely used

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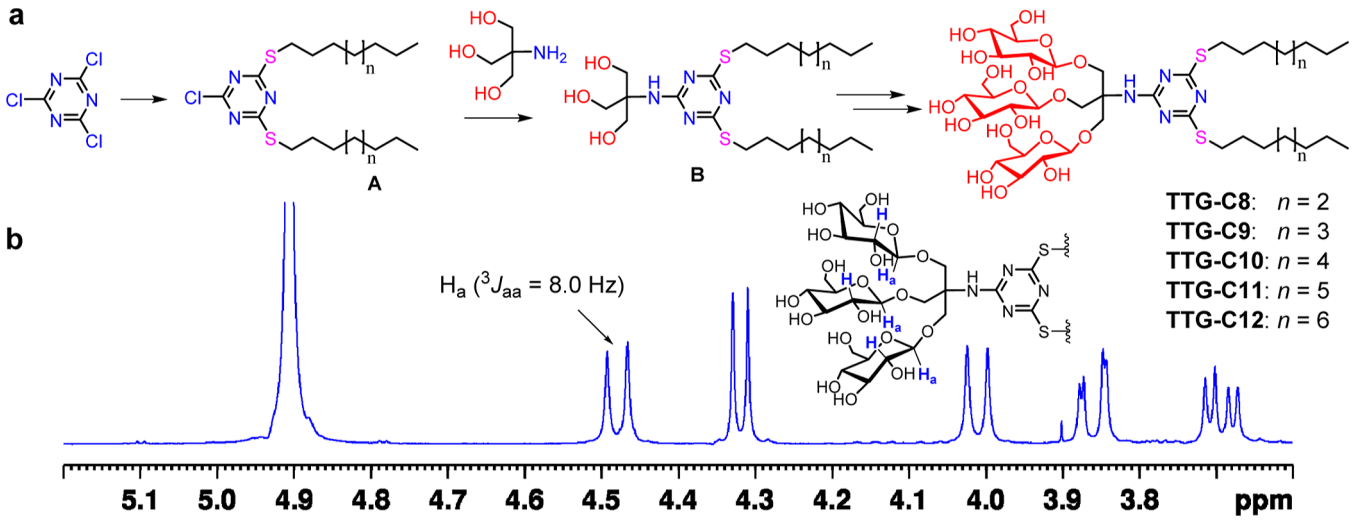

Figure 2. (a) Synthetic scheme for preparation of TTGs and (b) partial ^{1}H NMR spectrum of TTG-C10 focusing on the anomeric region (4.1−5.2 ppm). The new detergents (TTGs) were prepared using cyanuric chloride as a starting material. Two alkyl chains were introduced into the triazine scaffold via a thioether linkage to generate dialkylated triazine derivatives (**A**), followed by attachment of TRIS onto the ring as a linker through an amino linkage. The resulting TRIS-bearing triazine derivatives (**B**) were used to attach the three glucoside moieties via glycosylation and subsequent global deprotection. The ^{1}H NMR spectra of TTG-C10 were obtained using CD_3OD as the NMR solvent. The NMR peak corresponding to the β-anomeric protons of TTG-C10 (H_a), as indicated in blue in the chemical structure of the detergent head group (inset), appears as a doublet at 4.48 ppm with a vicinal coupling constant (J) of 8.0 Hz.

maltoside detergents (LMNG and DDM) (Figure 1). In addition, these detergents showed reasonable efficiency at extracting membrane proteins from the membranes. Thus, the TTGs serve as useful tools for membrane protein manipulation including protein extraction, purification, and structure determination.

RESULTS

Detergent Structures and Physical Characterizations.

We previously developed 1,3,5-triazine-based maltosides with a diethanolamine linker, named TEMs, by introducing the 1,3,5-triazine ring as a central core (Figure 1c).[40] In the current study, the same central core and alkyl hydrophobic groups were used for preparation of the new detergents. However, the new 1,3,5-triazine-core detergents contain three glucose units instead of two maltose units present in the TEMs (Figure 1d). A thioether linkage was used to introduce the alkyl chains as this bond is hydrophobic compared to other functional groups such as amide and ether that have often been used for alkyl chain introduction into a detergent scaffold.[23,26,33] The high hydrophobicity in the lipophilic region is necessary for effective encapsulation of membrane proteins. The three glucose units were attached to the 1,3,5-triazine core using a TRIS linker. The TRIS linker contains a single primary amine group for attachment to the triazine ring and three alcoholic groups for glycosylation. This linker was previously used in the detergent scaffolds of the TPAs and TDTs.[25,41] Detergent preparation containing pentaerythritol instead of TRIS as a linker failed, probably due to the low nucleophilicity of the alcohol group compared to the amine group. The set of amphiphiles (TTGs) vary in the carbon chain length, ranging from octyl (C8) to dodecyl (C12), which was used for detergent designation. This alkyl chain length variation is necessary for the following two reasons. First, the detergent alkyl chain needs to be compatible with the hydrophobic dimensions of membrane proteins for protein stability.[21,22] Membrane proteins have a range of hydrophobic width (~30 Å), and thus a specific alkyl chain length may be required depending on the exact hydrophobic width of a given membrane protein.[42] Second, detergent alkyl chain length varies the hydrophilic−lipophilic balance (HLB) value, important for membrane protein stability.[43] The HLB values of the TTGs calculated from Griffin's method are given in Table S1.[44] The HLB values vary from 11.2 (TTG-C12) to 12.4 (TTG-C8), which falls into the optimal range (11−13) for membrane protein stability.[45] The exact HLB necessary for protein stability would also differ depending on the properties of individual membrane proteins.

The new TTGs were synthesized via a straightforward protocol comprising four synthetic steps (Figure 2a): dialkylation with an alkanethiol (R−SH), TRIS coupling, glycosylation, and global deprotection. An inexpensive starting material, 2,4,6-trichloro-1,3,5-triazine (a.k.a., cyanuric chloride), was used to attach the two alkyl chains to the two electrophilic sites of cyanuric chloride. Using an alkanethiol and diisopropylethylamine (DIPEA) as the nucleophile and the base, respectively, these reactions produced the dialkylated products (**A**) in high synthetic yields (~80%). The resulting di-alkylated 1,3,5-triazine derivatives (**A**) retain one reactive site on the triazine ring used to introduce a TRIS linker into the scaffold. This operation produced the triol-functionalized triazine derivatives (**B**; ~80%) that were used as substrates for β-selective glycosylation (~85%). Finally, a global removal of the benzoyl protecting groups on the glucose ring resulted in the TTG amphiphiles (~90%; see Supporting Information for details). NMR spectroscopic analysis was used to confirm the stereochemistry of the newly formed glycosidic bonds. For example, the NMR spectrum of TTG-C10 showed a doublet signal at 4.48 ppm with a vicinal coupling constant ($^3J_{aa}$) of 8.0 Hz, indicating that only β-selective glycosylation occurred (Figure 2b).

All of the newly synthesized amphiphiles are water-soluble (>10%), except TTG-C12 which gave ~5% water solubility. High water solubility is a prerequisite for bio-applications of these molecules. The solutions containing the individual TTGs

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did not yield any precipitates during a 30-day incubation at room temperature. The critical micelle concentration (CMC) for each new agent was determined using a fluorescent dye, diphenylhexatriene (DPH).[46] The sizes of micelles formed by the new agents were estimated via hydrodynamic radii (R_h) measured by dynamic light scattering (DLS) analysis. A summary of the results is shown in Table 1. All of the TTGs form micelles at concentrations much lower than DDM (3−15 vs 170 μM), indicating strong hydrophobic interactions between the TTG molecules in the interior of detergent micelles. The micelle size increases with the stepwise elongation of detergent alkyl chains from C8 (2.9 nm) to C12 (5.5 nm). Micelles formed by the individual TTGs showed a unimodal size distribution based on their number- or volume-weighted DLS profiles (Figure S2a,b). By contrast, their intensity-weighted DLS profiles showed a bimodal distribution of detergent self-assemblies; large assemblies formed by these TTGs reached a size of 50−1000 nm (Figure S2c).

Table 1. Molecular Weights, Critical Micelle Concentrations of the TTGs and Two Control Detergents, LMNG and DDM, and Hydrodynamic Radii (R_h) (Mean ± S.D., n = 4) of Their Micelles in Water at Room Temperature

detergent	MW (Da)[a]	CMC (μM)	R_h[b] (nm)	solubility (wt %)
TTG-C8	975.1	∼15	2.9 ± 0.1	∼10
TTG-C9	1003.2	∼10	3.3 ± 0.1	∼10
TTG-C10	1031.2	∼6	3.6 ± 0.2	∼10
TTG-C11	1059.3	∼4	3.7 ± 0.1	∼10
TTG-C12	1087.3	∼3	5.5 ± 0.3	∼5
LMNG	1005.2	∼10	9.8 ± 0.2	∼10
DDM	510.6	∼170	3.5 ± 0.1	∼10

[a]Molecular weight of detergents. [b]Hydrodynamic radius of detergent self-assemblies measured at 1.0 wt % by DLS experiments.

Detergent Evaluation with a Set of Membrane Proteins. The TTGs were first evaluated with LeuT from the bacterium, *Aquifex aeolicus*.[47] The transporter was first extracted from *Escherichia coli* using 1.0 wt % DDM, followed by purification with 0.05 wt % of the same detergent. The DDM-purified LeuT was diluted into buffer solutions containing DDM or the respective TTG to give a final detergent concentration of 0.04 or 0.2 wt % (Figure 3). Protein stability was assessed by measuring the ability of the transporter to bind a radio-labeled substrate [[^{3}H]−leucine (Leu)] via a scintillation proximity assay (SPA).[48] The ability to bind Leu was monitored at regular intervals over a 13 day incubation period at room temperature. At a detergent concentration of 0.04 wt %, the LeuT in DDM exhibited a marked ability to bind the radio-labeled substrate, but this decreased rapidly over the incubation period. LeuT in all TTGs yielded comparable initial Leu binding to protein in DDM. However, the transporter in all individual TTGs retained higher levels of Leu-binding ability long term compared to the protein in DDM. A similar trend was observed when the detergents were used at an increased concentration of 0.2 wt %. At this higher concentration, the transporter in DDM initially exhibited high levels of Leu binding, but this rapidly decayed over the incubation period. LeuT in some TTGs at 0.2 wt %, particularly TTG-C9, exhibited initial Leu-binding levels lower than DDM, but this initial Leu binding was maintained over the whole incubation period for all TTGs, even TTG-C9. Overall, all TTGs except TTG-C9 outperformed over DDM for LeuT stability. The best performance was observed with TTG-C8 (0.2 wt %) and TTG-C11 (0.04 wt %).

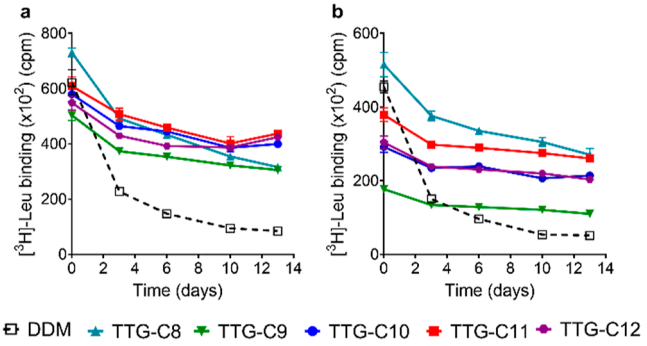

Figure 3. Stability of LeuT in the new detergents (TTGs) over time. DDM was used as a control. The detergents were tested at 0.04 (a) or 0.2 wt % (b). LeuT stability was assessed by measuring the ability of the transporter to bind the radio-labeled substrate [[^{3}H]-leucine (Leu)] at regular intervals during a 13 day incubation at room temperature via SPA. Results are from two individual experiments performed in triplicate. Error bars: SEM.

For further evaluation of the new detergents, we employed melibiose permease from *Salmonella enterica serovar typhimurium* ($MelB_{St}$).[49−53] $MelB_{St}$ overexpressed in *E. coli* membranes was extracted using 1.5 wt % of DDM or the individual TTGs for 90 min at 0 °C. Detergent efficiency for membrane protein extraction can be estimated by measuring the amount of soluble $MelB_{St}$ in these extracts. The $MelB_{St}$ extracts were further incubated at an elevated temperature (45, 55 or 65 °C) for another 90 min, which gives information about detergent efficacy for protein stabilization. The amounts of soluble $MelB_{St}$ obtained following this thermal treatment vary depending on the ability of a given detergent to stabilize the transporter. The amounts of soluble $MelB_{St}$ in the individual conditions were obtained by SDS-PAGE and Western blotting and are presented as percentages (%) of the amount of the transporter in the untreated membranes (Figure 4). At 0 °C, DDM gave quantitative $MelB_{St}$ extraction from the membrane. With the exception of TTG-C12, the TTGs yielded substantial amounts of soluble $MelB_{St}$ (70−90%), indicating that TTG-C8/C9/C10/C11 can be used for membrane protein extraction, although they are slightly less efficient than DDM. The low $MelB_{St}$ extraction efficiency observed for TTG-C12 is likely to be associated with the low solubility of this long alkyl-chained detergent under the conditions tested. When the detergent extracts were further incubated at 45 °C, all tested TTGs showed improved efficiency for protein extraction compared to detergent efficiency obtained at 0 °C. TTG-C8/C9/C10/C11 yielded 90−100% soluble $MelB_{St}$, while MelB extraction efficiency was dramatically increased from 0 to ∼50% for TTG-C12. The enhanced protein extraction efficiency observed here is likely due to the increased membrane dynamics and/or improved detergent solubility at this elevated temperature. The efficacy of the tested detergents for $MelB_{St}$ stabilization could be differentiated when we increased the solution temperature to 55 °C. DDM, TTG-C8, and TTG-C9 failed to retain $MelB_{St}$ in a soluble state at this temperature. In contrast, TTG-C10 and TTG-C11 were highly effective at retaining protein stability at this high temperature, yielding ∼75 and ∼65% soluble $MelB_{St}$,

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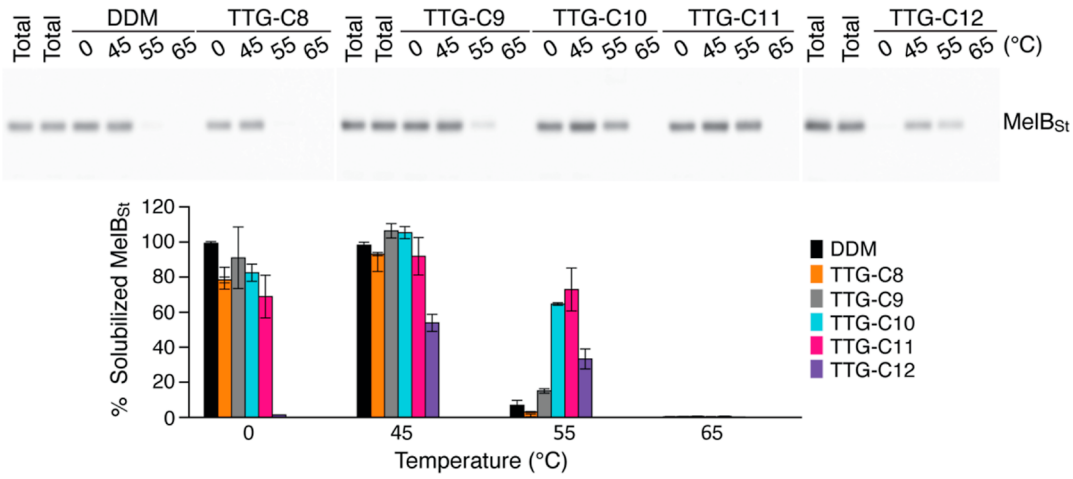

Figure 4. Thermo-solubility of $MelB_{St}$ solubilized in the TTGs. A conventional detergent (DDM) was used as a control. *E. coli* membranes containing $MelB_{St}$ were treated with individual detergents at 0 °C for 90 min. The resulting membrane extracts were further incubated at three different elevated temperatures (45, 55, and 65 °C) for 90 min. The extracts and thermally treated $MelB_{St}$ samples were subjected to ultracentrifugation before being analyzed by SDS-PAGE and detected by Western blotting (top panel). The amounts of soluble $MelB_{St}$ are expressed as a percentage of total $MelB_{St}$ present in the untreated membranes ("total") and are presented in a histogram (bottom panel). The experiment was carried out in triplicate. Error bars: SEM.

respectively. This result indicates that these TTGs are superior to DDM at stabilizing $MelB_{St}$ in a soluble state (Figure 4). Interestingly, TTG-C12 with the longest alkyl chain gave ~35% soluble $MelB_{St}$, although this detergent was the least efficient at extracting the transporter from the membrane at 0 °C. Thus, this C12 alkyl-chained detergent might be suitable for the purpose of protein stabilization rather than protein extraction.

The new detergents were further assessed with a GPCR, the human β_2AR.[54] The DDM-solubilized receptor was purified in LMNG and then exchanged into the individual detergents via sample dilution. LMNG was used for receptor purification as this NG-based detergent has been shown to be particularly effective for GPCR stability.[38] Among the TTGs, four detergents (TTG-C9/C10/C11/C12) were selected for this study as these detergents were effective at stabilizing the two transporters (LeuT and $MelB_{St}$) long-term. The receptor purified in LMNG was diluted in the different detergents to a final concentration of 0.1 wt % and then incubated for 7 days at room temperature. We monitored the specific ligand-binding ability of the receptor at regular intervals using the radio-active antagonist [[^{3}H]-dihydroalprenolol (DHA)] (Figure 5a).[55−57] The receptor in DDM showed low DHA binding shortly after dilution, a clear indication of receptor instability in DDM micelles.

As expected, LMNG was superior; the receptor in this NG class detergent initially showed an increased ability to bind DHA compared to DDM. However, the receptor in LMNG exhibited a gradual decrease in ligand-binding ability over the incubation period, with ~50% retention of the initial ligand-binding capability after the 7 day incubation. Remarkably, all tested TTGs were comparable to or better than LMNG at maintaining β_2AR stability, with the best performance observed for TTG-C10/C11/C12. There were only small decreases (~10%) in the ligand-binding ability when the receptor was diluted using TTG-C11 and TTG-C12. When compared with a previously developed triazine-based detergent (TEM-T8), these TTGs were superior to the latter for β_2AR stability long-term (Figure S3).

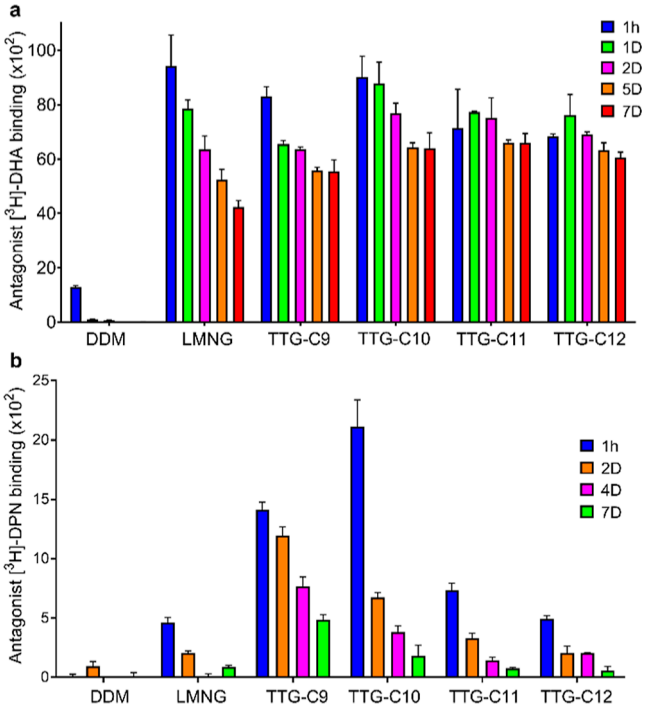

Figure 5. Stability of β_2AR (a) and MOR (b) solubilized in the TTGs over time. DDM and LMNG were used as controls and tested at 0.1 wt %. Stability of β_2AR and MOR was assessed by measuring the ability of the receptor to bind the radio-labeled antagonist [[^{3}H]-dihydroalprenolol (DHA) or [^{3}H]-diprenorphine (DPN), respectively] during a 7 day incubation at room temperature (β_2AR) or 4 °C (MOR). The receptor stability assay was performed in duplicate (β_2AR) or triplicate (MOR). Error bars: SEM.

We continued evaluation of the new detergents with another GPCR, the mouse MOR.[58] The DDM-solubilized receptor was purified in LMNG and then exchanged into the respective TTGs via sample dilution to give a final detergent concentration of 0.1 wt %. The stability of the receptor in the individual detergents was assessed by measuring the ability to bind the radioactive antagonist [[^{3}H]-diprenorphine

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(DPN)]. The MOR samples were incubated for 7 days at 4 °C, and the antagonist-binding ability was monitored at regular intervals (Figure 5b).[59] The MOR in DDM completely lost the ability to specifically bind DPN upon detergent exchange, whereas the receptor in LMNG showed a relatively low initial ability to bind the ligand, followed by a substantial decrease in binding activity over the incubation time period. TTG-C11 and TTG-C12 were more or less comparable to LMNG for MOR stability. In contrast, TTG-C9 and TTG-C10 were markedly more effective than LMNG, yielding ∼3 and ∼4 times greater initial DPN binding than LMNG, respectively. TTG-C9 was the most effective at retaining long-term stability of the receptor. Combined with the results obtained for β_2AR, these findings indicate that both TTG-C9 and TTG-C10 hold significant potential for GPCR structural study.

DISCUSSION

We demonstrated that the TTG amphiphiles are beneficial for solubilization and/or stabilization of two transporters (LeuT and $MelB_{St}$) and two GPCRs (β_2AR and MOR). Detergent efficacy for protein stabilization tends to be protein-specific. For instance, TTG-C8 and TTG-C11 were best for LeuT stability, while TTG-C10 and TTG-C11 outperformed the other TTGs with respect to $MelB_{St}$ stability. For GPCR stability, all tested TTGs (TTG-C9, TTG-C10, TTG-C11, and TTG-C12) were effective at stabilizing β_2AR, while two, TTG-C9 and TTG-C10, were clearly better than the other TTGs at stabilizing MOR. Although we observed the protein-specific nature of detergent efficacy for protein stabilization, it is important to note that TTG-C10 was significantly more effective than DDM, a gold standard for membrane protein study, at stabilizing all tested membrane proteins. Notably, this C10 alkyl-chained glucoside detergent (TTG-C10) was even superior to LMNG in preserving the ligand-binding ability of the two GPCRs (β_2AR and MOR). This result is remarkable considering the fact that LMNG is a novel detergent that is significantly optimized for GPCR stability and has been used for structural studies of 301 GPCRs over the past 11 years.[39] In addition, TTG-C11 was one of the best novel amphiphiles for stabilization of three membrane proteins (LeuT, $MelB_{St}$, and β_2AR), although this C11 alkyl-chained TTG was inferior to the C9/C10 version of the TTGs in stabilizing MOR. Both TTG-C10 and TTG-C11 were also effective in protein extraction. Thus, TTG-C10 and TTG-C11 have significant potential for membrane protein structural study. Interestingly, both β_2AR and MOR were stabilized by TTG-C9 more effectively than by LMNG, suggesting that this TTG may also find its use in GPCR structural study. It is important to note that the results were obtained using protein samples containing low residual amounts of DDM or LMNG as the proteins purified in these detergents were diluted into buffers supplemented by the individual TTGs.

Of the five TTGs prepared in the current study, three detergents (TTG-C9, TTG-C10, and TTG-T11) conferred enhanced stability to a few membrane proteins, indicating favorable features of this new class. One important feature is the high hydrophobicity of the lipophilic group, illustrated by the low CMCs of the TTGs (∼4 vs 10 μM for LMNG), that results in strong detergent−detergent and detergent−protein interactions in the protein-detergent complex (PDC) environment. The high hydrophobicity of the TTG lipophilic group was attained via the thio-ether connection of the alkyl chains to the triazine core. Another important feature of these TTGs is the balance of detergent hydrophilicity and hydrophobicity, as reflected by their HLB values (11.5−12.1). The three glucose head groups were introduced to the detergent scaffold using the TRIS linker in order to balance the high hydrophobicity of the two alkyl chains. The TRIS linker was previously used to introduce three glucose units into other detergent scaffolds such as TPA and TDT.[25,41] However, the amino group was generated by the introduction of this linker into the detergent scaffold for the TTGs, while an amide group results from the corresponding connection in the TPAs and TDTs. Due to the presence of cis−trans isomerism, the amide linkage of the detergent scaffold could be detrimental for membrane protein stability, which may explain the comparatively suboptimal performance of the TPAs and TDTs with respect to protein stability.[48] In contrast, the TTGs containing amino linkage without cis−trans isomerism proved highly effective for membrane protein stability.

The additionally notable feature of the TTGs is the presence of the glucoside rather than a maltoside head group. Glucoside detergents are generally inferior to their maltoside counterparts, as exemplified by comparisons of OG vs DDM or OGNG vs LMNG. There are few glucoside detergents more effective than LMNG at stabilizing membrane proteins, particularly GPCRs. For instance, previously reported glucoside detergents such as SIGs, P-GNGs, and malonate-derived tetraglucoside detergents (MTGs) were generally inferior to LMNG at stabilizing the two GPCRs (β_2AR and MOR).[33,35,60] Thus, the superior efficacy of the TTGs for GPCR stabilization compared to LMNG observed here is a significant achievement. Due to the presence of the small head group, glucoside detergents tend to form smaller PDCs than maltoside detergents, a characteristic favorable for the generation of high-quality protein crystals via vapor diffusion. For example, despite its overall inferior properties compared to DDM, OGNG has contributed to 17 high-resolution crystal structures of membrane proteins, including enzymes (e.g., CAAX prenyl protease 1 homolog (ZMPSTE24) [4AW6], a Na^+ -translocating pyrophosphatase (PPase) [4AV3], a superoxide oxidase [5OC0]), channels (two-pore domain potassium channel $K_{2P}2.1$ [6CQ6], trimeric intracellular cation [TRIC] channel [TREK-1; 6IYU], and transient receptor potential (TRP) channels [TRPV2; 6BWJ]), and aquaporins [AQP2 and AQP7; 4NEF].[39] The TTGs contain glucoside head groups, and some (TTG-C9, TTG-C10 and TTG-C11) proved markedly more effective for membrane protein stability than maltoside detergents (DDM and/or LMNG). Thus, it is likely that these detergents will find wide use in membrane protein structural study. Further evaluation with membrane protein super-assemblies such as respiratory chain and photosynthetic super-complexes would be interesting. These super-assemblies remain among the most challenging targets for *in vitro* structural studies of membrane proteins. In addition, these TTGs may find utility in *in vitro* reconstitution of membrane proteins. There are only a few detergents (e.g., LMNG) effective for functional reconstitution of membrane proteins into lipid bilayers.[61]

CONCLUSIONS

In summary, we designed and prepared a set of TRIS linker-bearing triazine-based glucoside (TTGs) detergents by implanting two alkyl chains and three glucose units into opposite sides of a triazine core. When these new agents were evaluated with a set of model membrane proteins including

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two GPCRs (β_2AR and MOR), we identified three detergents (TTG-C9, TTG-C10, and TTG-C11) that were markedly superior to a gold standard (DDM) at stabilizing all membrane proteins tested. These glucoside detergents were better than or at least comparable to LMNG, a maltoside detergent significantly optimized for GPCR stability. The high hydrophobicity of the thioether linkage and removal of cis−trans isomerism associated with an amide linkage, along with an optimal range of hydrophilic-lipophilic balance are likely responsible for the favorable behaviors of the TTGs for membrane protein stability. Because of the high synthetic accessibility, small PDC formation and marked protein stabilization efficacy, the TTGs introduced here have the potential for wide use in membrane protein structural study.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.bioconjchem.3c00042.

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

■ AUTHOR INFORMATION

Corresponding Author

Pil Seok Chae − *Department of Bionano Engineering, Hanyang University, Ansan 155-88, South Korea;* orcid.org/0000-0003-1799-3304; Email: pchae@hanyang.ac.kr

Authors

Lubna Ghani − *Department of Bionano Engineering, Hanyang University, Ansan 155-88, South Korea;* Present Address: Department of Chemistry, Women University of Azad Jammu & Kashmir Bagh, (WUAJK), Bagh-12500 (AJK), Pakistan

Xiang Zhang − *Tsinghua-Peking Center for Life Sciences, Beijing Frontier Research Center for Biological Structure, Beijing Advanced Innovation Center for Structural Biology, School of Medicine, School of Pharmaceutical Sciences, Tsinghua University, Beijing 100084, China*

Chastine F. Munk − *Department of Neuroscience, University of Copenhagen, Copenhagen DK-2200, Denmark*

Parameswaran Hariharan − *Department of Cell Physiology and Molecular Biophysics, Center for Membrane Protein Research, School of Medicine, Texas Tech University Health Sciences Center, Lubbock, Texas 79430, United States*

Baoliang Lan − *Tsinghua-Peking Center for Life Sciences, Beijing Frontier Research Center for Biological Structure, Beijing Advanced Innovation Center for Structural Biology, School of Medicine, School of Pharmaceutical Sciences, Tsinghua University, Beijing 100084, China*

Hong Sik Yun − *Department of Bionano Engineering, Hanyang University, Ansan 155-88, South Korea;* orcid.org/0000-0002-2187-6840

Bernadette Byrne − *Department of Life Sciences, Imperial College London, London SW7 2AZ, U.K.;* orcid.org/0000-0001-9598-9832

Lan Guan − *Department of Cell Physiology and Molecular Biophysics, Center for Membrane Protein Research, School of Medicine, Texas Tech University Health Sciences Center, Lubbock, Texas 79430, United States;* orcid.org/0000-0002-2274-361X

Claus J. Loland − *Department of Neuroscience, University of Copenhagen, Copenhagen DK-2200, Denmark;* orcid.org/0000-0002-1773-1446

Xiangyu Liu − *Tsinghua-Peking Center for Life Sciences, Beijing Frontier Research Center for Biological Structure, Beijing Advanced Innovation Center for Structural Biology, School of Medicine, School of Pharmaceutical Sciences, Tsinghua University, Beijing 100084, China*

Complete contact information is available at: https://pubs.acs.org/10.1021/acs.bioconjchem.3c00042

Notes

The authors declare the following competing financial interest(s): P.S.C. and L.G. are inventors on a patent application that covers the TTGs.

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