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A comparative study of branched and linear mannitol-based amphiphiles on membrane protein stability

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The study of membrane proteins is extremely challenging, mainly because of the incompatibility of the hydrophobic surfaces of membrane proteins with an aqueous medium. Detergents are essential agents used to maintain membrane protein stability in non-native environments. However, many conventional detergents fail to stabilize the native structures of many membrane proteins. Development of new amphipathic agents with enhanced efficacy for membrane protein stabilization are necessary to address this important problem. We have desinged and synthesized linear and branched mannitol-based amphiphiles (MNAs), and comparative studies showed that most of the branched MNAs had advantages over the linear agents in terms of membrane protein stability. In addition, a couple of the new MNAs displayed favorable behaviors compared to *n*-dodecyl-β-D-maltoside and the previously developed MNAs in maintaining the native protein structures, indicating potential utility of these new agents in membrane protein study.

Introduction

Membrane proteins embedded in cellular membranes account for approximately 30% of total cellular proteomes.[1] These bio-macromolecules are important for a range of cellular processes including signal transduction and membrane transport.[2] Additionally, membrane proteins represent more than one-half of current drug targets.[3] Despite their biochemical and pharmaceutical significance, the structural/functional study of membrane proteins lags far behind that of soluble proteins. This slow progress in membrane protein analysis is mainly due to their limited solubility and stability in aqueous environments.[4] Detergent are capable of extracting membrane proteins from the membranes, leading to the formation of protein-detergent complexes (PDCs). The resulting PDCs need to be stable enough for downstream characterization of target proteins outside the native membranes.[5] Conventional detergents, exemplified by *n*-octyl-β-D-glucoside (OG), lauryldimethylamine-*N*-oxide (LDAO), and *n*-dodecyl-β-D-maltoside (DDM), are widely used in membrane protein manipulation including solubilization, stabilization and crystallization.[6] Detergents form self-assemblies, called micelles, with architecture different from lipid bilayers formed by lipid molecules. Because of the inherent difference in self-assembly architecture, detergent micelles differ significantly from the lipid bilayer in many aspects. Lipid bilayers produce lateral pressure necessary for membrane protein function, but detergent micelles are relatively dynamic due to the high conformational flexibility of the hydrophobic groups and fast exchange of detergent molecules between the micellar state and the bulk solvent. They are thus generally less effective at stabilizing membrane proteins than lipid bilayers. However, detergent micelles are generally more suitable for membrane protein crystallization, although some lipidic systems such as bicelles and lipidic cubic phase (LCP) have been successfully used for this purpose.[7] Bicelles have been also used for high resolution NMR-based structural studies of membrane proteins.[8] In contrast to the lipidic systems, detergent micelles have the ability to extract membrane proteins from the membranes by dismantling lipid bilayers.[9] Despite their widespread use for membrane protein manipulation, conventional detergents are often suboptimal for membrane protein stability, resulting in protein denaturation and/or aggregation, hampering advances in membrane protein structural study.[6b,10] This is likely due to the relatively limited molecular architecture of conventional detergents, which typically consist of a large head group and a flexible alkyl chain.

Over the past two decades major effort has focused on the development of novel agents with unique architectures. Some were large molecules and assemblies, as exemplified by the amphipols (Apols)[11] and nanodiscs (NDs),[12] while others were small amphipathic agents with a glycoside head group. These large molecules/assemblies are not efficient at extracting a protein from

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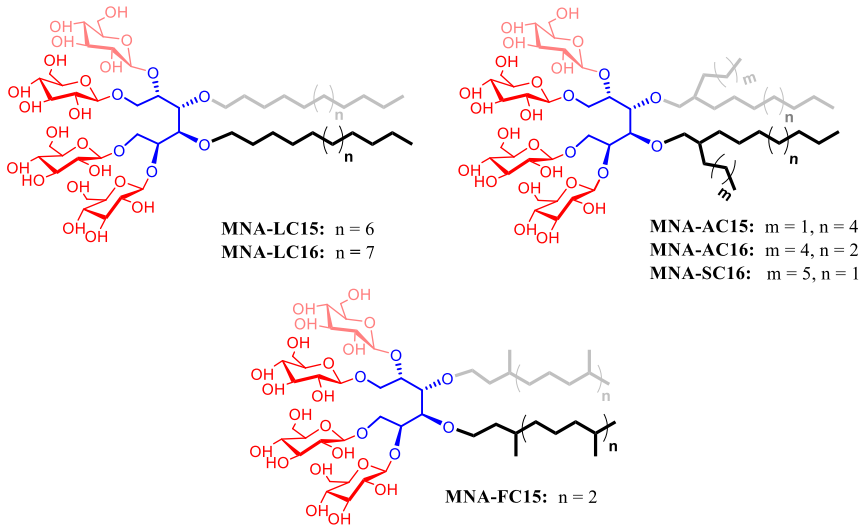
the native membranes, while small molecule detergents are generally very efficient at extraction and have yielded many high-quality protein crystals. A key advantage of these small amphipathic agents compared to the large molecules/assemblies, is that they are relatively straightforward to structurally modify in order to optimize their properties. Representatives include facial amphiphiles (FAs),[13] neopentyl glycol (NG) amphiphiles (GNGs/MNGs/NDTs),[14] mannitol-based amphiphiles (MNAs),[15] penta-saccharide-bearing amphiphiles (PSEs),[16] xylene-based maltosides (XMAs),[17] resorcinarene-based glucosides (RGAs)[18] and norbornane-based maltosides (NBMs).[19] Among these small amphipathic agents, GNG-3 (commercial name: OGNG) and MNG-3 (commercial name: LMNG) have facilitated the elucidation of more than 30 new membrane protein structures in the last seven years,[20] highlighting the importance of small amphiphile development. Previously developed MNAs were shown to be effective at stabilizing a few target membrane proteins.[15] In addition, these agents tend to form small PDCs and associate more tightly to a protein surface than DDM, presumably due to the presence of a glucoside head group and the multiple alkyl chains, respectively. Because this class of amphiphiles is highly flexible and straight forward to modify we prepared new family members in an attempt to optimize both membrane protein extraction and stability in these agents. This study identified some new MNAs with improved behavior compared to the previously reported MNA and DDM, a gold standard conventional detergent.[15] In addition, as linear/branched alkyl chains with different chain length were used as detergent hydrophobic groups, we were able to compare detergent efficacy according to the variations in detergent geometry and/or hydrophobic chain length.



Scheme 1 Chemical structures of newly prepared mannitol-based amphiphiles (MNAs). All of these agents have four glucose units and two alkyl chains as the hydrophilic and hydrophobic groups, respectively. The lipophilic alkyl chains were connected to the hydrophilic groups through a central mannitol linker. The number of carbon units in the alkyl chains were C15 or C16 and the chains could be linear (MNA-LC15 and MNA-LC16), single branched (MNA-AC15, MNA-SC16 and MNA-AC16), or methylated a number of times along the chain (MNA-FC15). The numbers of carbon units in the alkyl chains were used for detergent designation.

Results and discussion

Design and characterization of new MNAs

The newly designed agents commonly contain two alkyl chains as the hydrophobic group, connected to four glucose units *via* a mannitol linker comprising the hydrophilic group (Scheme 1). These mannitol-based amphiphiles (MNAs) can be categorized into three sets: the first set contain two linear alkyl chains similar to the previously developed MNAs, while the second and third sets both have two branched alkyl chains. The second set has one branched point in the individual alkyl chains, while the third set contains the saturated farnesyl chains with three methyl branches. The carbon unit number of these alkyl chains was C15 or C16, which was used for detergent designation. As for the C15 versions, linear (MNA-LC15), branched alkyl chain (MNA-AC15), or saturated farnesyl group (MNA-FC15) was used as a lipophilic group. A linear (MNA-LC16), asymmetrically or symmetrically branched alkyl chain (MNA-AC16 or MNA-SC16) was used for the C16 versions. Of note, C15/C16 alkyl chain MNAs are constitutional isomers of each other. As both MNA-AC15 and MNA-FC15 have the same alkyl chain length (C12), a comparison of these agents with MNA-LC15 having C15 alkyl chain length allowed us to investigate the effect of detergent alkyl chain length on membrane protein stability. MNA-FC15 structurally differs from MNA-AC15 as this agent has three methyl branches located over the dodecyl chain, while MNA-AC15 bears a propyl pendant on the same chain near the interface between the head and tail groups. As a result, the alkyl chains of MNA-FC15 are rather cylindrical, while those of MNA-AC15 are conical. Thus, a comparison of these two MNAs would allow us to investigate the effect of detergent alkyl chain architecture on protein stability. Similar structural effects on protein stability could be investigated with the C16 alkyl chained MNAs. A comparison of MNA-LC16 *vs* MNA-AC16/SC16 would provide insights

into the effect of detergent alkyl chain length (C16 *vs* C10/C9), while that of MNA-SC16 *vs* MNA-AC16 allowed us to study the effect of detergent alkyl chain architecture (cylindrical *vs* conical). It is noteworthy that detergent comparisons here were conducted between the MNAs with the same number of carbon units (C15 or C16) to eliminate detergent efficacy variation depending on hydrophile-lipophile balance (HLB). This balance is known to be an important factor in determining detergent efficacy for protein stabilization.[21,22]

These new agents were prepared in a straight-forward synthetic protocol comprising four to seven efficient steps (see in supplementary scheme S1 and S2). The symmetrically branched alkyl group used for MNA-SC16 preparation was synthesized from dimethylmalonate, while the linear/asymmetrical alkyl groups were purchased from commercial sources. All new agents were water-soluble up to 10 wt% and showed no precipitation during storage for one-month. Detergent assembly behaviors were investigated by measuring critical micelle concentrations (CMCs) and assembly sizes. The critical micelle concentrations (CMCs) of the detergents were estimated using diphenylhexatriene (DPH) *via* a fluorescent dye encapsulation method,[23] and their micelle sizes were determined *via* dynamic light scattering (DLS). The summarized data are shown in Table 1, along with that of DDM. The CMCs of the new MNAs are much lower (1-6 μM) than DDM (170 μM), indicating their marked tendencies to self-assemble. The linear alkyl chained MNAs (MNA-LC15/LC16; 2/1 μM) gave lower CMCs than the branched alkyl chained MNAs (3-6 μM) presumably due to the bulkiness (i.e., large volume) of the branched alkyl chains; a bulky alky group cannot fit well the congested region of the detergent micelle interior and thus has a reduced propensity to form micelles. There was no substantial difference in CMCs between the branched alkyl chain MNAs, although the C16 alkyl chain MNAs are expected to give lower CMCs than the C15 agents based on the increased carbon units (i.e., hydrophobicity). This discrepancy from the expectation is also likely due to the fact that the C16 alkyl chain MNAs has the bulkier hydrophobic groups than do the C15 counterparts.[24] Self-assembly sizes formed by the newly synthesized MNAs were largely dependent on the number of carbon units (C15/C16) and presence/absence of a branching point in the alkyl chain region. All C15 alkyl chain MNAs gave small micelle sizes (R_h; 2.9~3.3 nm), indicating that molecular geometries of these agents are highly conical; their head groups are substantially larger than the tail groups.[25] Interestingly, the C16 alkyl chain MNAs formed larger micelles than the C15 MNA variants, with a large variation observed depending on the presence/absence of a branching point in the alkyl chain region. The branched alkyl chain MNAs (MNA-SC16 and MNA-AC16) produced larger assemblies (e.g., elongated micelles) (17 and 48 nm, respectively) than the linear alkyl chain MNA-LC16 (3.5 nm). This is due to the presence of the relative bulky chains in the branched MNAs, thereby resulting in a cylindrical molecular geometry shape. Notably, MNA-AC15 with the asymmetrical alkyl chains formed the smallest micelles (2.9 nm) of all the new MNAs, with a value close to the previously developed MNA with linear C12 alkyl chains (MNA-LC12). As MNA-AC15 incorporates linear dodecyl chains (C12) with propyl pendants, the comparable micelle size between this agent and MNA-LC12 indicates that the propyl pendant is accommodated well inside the empty spaces of detergent micelle interior. This filling of the empty spaces by the small alkyl pendants is likely to increase micelle stability as well as protein stability as it increases hydrophobic density in the detergent micelles. As a comparison, assemblies formed by MNA-AC16 with the asymmetric alkyl chains were largest (48 nm) and were much larger than those formed by the linear analog of the same alkyl chain length (MNA-LC10; 2.7 nm). This increase in assembly size from MNA-LC10 to MNA-AC16 indicates that the hexyl pendant of MNA-AC16 is too large to fit into the empty spaces present in the detergent assembly interiors, leading to assembly expansion. Similar to DDM, the DLS profiles of MNA assemblies showed a single set of populations in terms of size distribution (Fig. S1). Assembly sizes formed by the MNAs and DDM were further investigated with increasing temperature from 15 °C to 65 °C. Most of the MNAs and DDM tended to show gradual decreases in assembly sizes with increasing temperature. MNA-AC16 displayed a particularly large decrease in self-assembly size with this temperature variation. The exceptions were MNA-LC16, which showed little variation in micelle size over the range of temperatures tested and MNA-LC15 which showed an abrupt enlargement in assembly size from 2.9 nm to 11 nm with temperature elevation from 55 °C to 65 °C (Figure S2).

Table 1 Molecular weights (MWs), critical micelle concentrations (CMCs) of newly prepared MNAs and conventional detergent (DDM) and hydrodynamic radii (R_h) of their micelles at room temperature.

Detergent	*M.W.*[a]	CMC (μM)	CMC (wt%)	R_h (nm)[b]
MNA-FC15	1251.55	~ 6	~ 0.00075	3.2±0.2
MNA-AC15	1251.55	~ 5	~ 0.00063	2.9±0.1
MNA-LC15	1251.55	~ 2	~ 0.00025	3.3±0.1
MNA-AC16	1279.60	~ 5	~ 0.00064	48±4.4
MNA-SC16	1279.60	~ 6	~ 0.00077	17±.1.2
MNA-LC16	1279.60	~ 1	~ 0.00013	3.5±0.2
DDM	510.1	~ 170	~ 0.0087	3.3±0.1

[a] Molecular weight of detergents. [b] Hydrodynamic radius of detergents measured at 1.0 wt% by dynamic light scattering.

Detergent evaluation toward membrane protein solubilization and stabilization

The stabilization efficacy of the newly synthesized MNAs was first evaluated with LeuT, the 12 transmembrane domain leucine transporter from bacteria *Aquifex aeolicus.*[26,27] Protein stability was assessed by measuring the ability of the transporter to bind to the radio-labeled substrate ([^{3}H]-leucine (Leu)) using the scintillation proximity assay (SPA).[28] For detergent evaluation, the transporter was first solubilized with 1.0% DDM and purified in 0.05% of the same detergent. The DDM-purified transporter was diluted with buffer solutions supplemented with the individual MNAs to give final detergent concentrations of CMCs + 0.04 wt%. Substrate binding

activity of the transporter was monitored at regular intervals over the course of a 12-day incubation at room temperature. As shown in Fig. 1a, most MNAs including MNA-LC13 were more effective than DDM at stabilizing LeuT long term. However, the linear alkyl chained MNAs (MNA-LC15 and MNA-LC16) were worse than DDM, indicating that a long alkyl chain detergent is not compatible with LeuT stability. Of the tested detergents, the asymmetric alkyl chain MNAs (MNA-AC15 and MNA-AC16) were the best. The saturated farnesylated and symmetric alkyl chained MNAs (MNA-FC15 and MNA-SC16) were slightly less effective, being more or less comparable to MNA-LC13. A similar detergent efficacy order was observed when detergent concentrations were increased to CMCs + 0.2 wt%. Again, the MNAs with the long alkyl chains (MNA-LC15 and MNA-LC16) were worse than DDM in stabilizing the transporter. Of the new detergents, the asymmetric MNAs (MNA-AC15 and MNA-AC16) were best, followed by MNA-FC15 and MNA-SC16. At these high detergent concentrations, the previously developed MNA (MNA-LC13) was slightly better than the best new MNAs. Interestingly, although initially exhibiting low substrate binding activity, the MNA-SC16-solubilized transporter rapidly increased activity following a 7-day incubation and reached the highest activity at the end of the incubation period (Day 12). The reason for this increase in transporter activity over time is not clear but might be associated with a gradual change in the self-assembly architecture of the detergent molecules from a micellar to liposome arrangement when interacting with the transporter. Overall, the most stabilizing of the agents for LeuT were the asymmetrically branched alkyl chain MNAs, followed by symmetrically branched and linear alkyl chain agents.

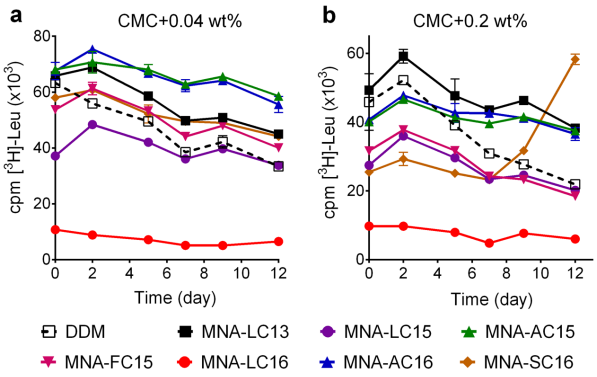


Fig. 1 Change in substrate binding activity for LeuT solubilized in a new MNA (MNA-LC15, MNA-AC15, MNA-FC15, MNA-LC16, MNA-AC16, or MNA-SC16), DDM, or a previously developed MNA (MNA-LC13). Each detergent was used at CMCs + 0.04 wt% (a) and CMCs + 0.2 wt% (b). Protein stability was assessed by monitoring the ability of the transporter to bind the radiolabeled substrate ([^{3}H]-leucine). Transporter activity was monitored at regular intervals during a 12-day incubation at room temperature *via* scintillation proximity assay (SPA). Data points are mean ± SEM, n = 2.

The intriguing results of the MNAs with LeuT prompted us to evaluate these agents with another 12 transmembrane domain transporter, melibiose permease of *Salmonella typhimurium* ($MelB_{St}$).[29] *E. coli* membranes overexpressing $MelB_{St}$ were treated with 1.5% DDM or the individual MNAs (MNA-LC13, MNA-AC15, MNA-FC15, MNA-LC15, MNA-AC16, MNA-LC16 and MNA-SC16) at 0 °C for 90 min. After ultracentrifugation, the amounts of soluble $MelB_{St}$ were analyzed by Western blotting and expressed as percentages of total $MelB_{St}$ detected in untreated membranes (Fig. 2a,b). All MNAs except MNA-SC16 extracted $MelB_{St}$ as efficiently as DDM. This result suggests that these agents could be used as alternatives to conventional detergents for membrane protein extraction. In order to investigate detergent efficacy for MelB thermo-stability, after subjected to ultracentrifugation the membrane extracts were further incubated an an elevated temperature (45, 55, or 65 °C) for 90 min. At 45 °C, $MelB_{St}$ was not only fully extracted by either the MNAs or DDM, but in each case the resulting transporter was stable enough to maintain water-solubility at this temperature. When incubation temperature was further increased to 55 °C, however, the amount of soluble $MelB_{St}$ in DDM dropped down to 25%, indicating a significant protein denaturation/aggregation under these conditions. Similar results were obtained for MNA-FC15 and MNA-AC16, which yielded approximately 40% soluble transporter. In contrast, the other MNAs were markedly better at preserving $MelB_{St}$ solubility with the best results obtained using MNA-AC15 and MNA-LC15. This detergent efficacy variation dependent on temperature might be correlated with thermostability of the detergent assemblies. For example, MNA-AC16 showed a large decrease in assembly size with increasing temperature from 45 °C to 55 °C and an associated decrease of 100% to 40% solubilisation efficiency? of $MelB_{St}$ (Figure S2a and Figure 2b). However, self-assembly of MNA-FC15 and DDM displayed little temperature dependency. Thus, it is unlikely that thermostability of detergent assembly is critical for the detergent efficacy for MelB thermosolubility observed here. This was further supported by the $MelB_{St}$ solubility result at 65 °C. Although all the MNAs except MNA-LC15 gave only a minor decrease in self-assembly size with changing temperature from 55 °C to 65 °C, none of these detergents was able to maintain water-solubility of $MelB_{St}$ at 65 °C. Because of the promising behaviours in retaining $MelB_{St}$ solubility, MNA-AC15 and MNA-LC15 were further assessed for their ability to preserve $MelB_{St}$ functionality.

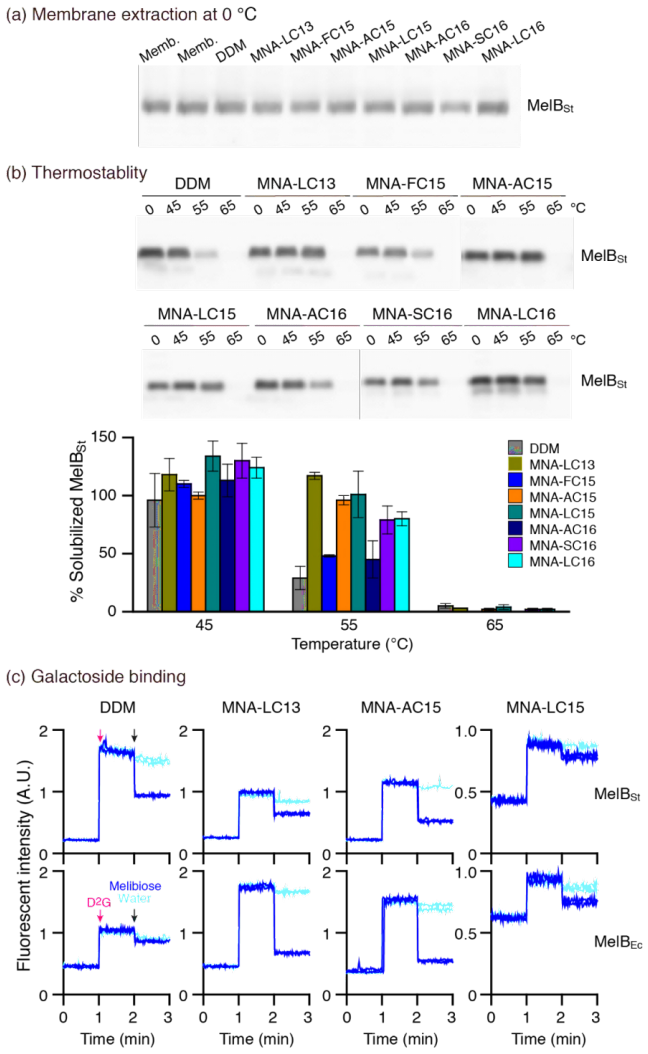


Fig. 2 (a) $MelB_{St}$ extraction and (b) thermo-stability using a new MNA agent (MNA-LC15, MNA-AC15, MNA-FC15, MNA-LC16, MNA-AC16, or MNA-SC16). DDM and MNA-LC13 were respectively used as representatives of conventional detergents and previously developed MNAs. For protein extraction, *E. coli* membranes containing $MelB_{St}$ were incubated with 1.5 wt% individual detergents at 0 °C for 90 min. Protein thermo-stability was addressed by further incubating membrane extratcts at three different temperatures (45, 55, and 65 °C). Supernatants after ultracentrifugation were analyzed by SDS-PAGE and Western blotting after ultracentrifugation ((a) and top panel in (b)). The amounts of soluble $MelB_{St}$ in the individual conditions were expressed as percentages of total $MelB_{St}$ present in untreated membranes (a, Memb) and summarized in histograms (bottom panel in (b)). Error bars, SEM, n = 3. (c) MelB functional analysis using galactoside binding assay. MNA-AC15 and MNA-LC15 were selected for the assay, along with two control agents (DDM and MNA-LC13). Right-side-out (RSO) membrane vesicles containing $MelB_{St}$ or $MelB_{Ec}$ were solubilized with different detergents (DDM, MNA-LC13, MNA-AC15, and MNA-LC15). After ultracentrifugation, the supernatant was used for the functional assay utilizing FRET from Trp to dansyl-2-galacotside (D^2G). MelB functional state was monitored by measuring changes in fluorescence intensity upon addition of D^2G and an excess amount of melibiose at the 1-min and 2-min points, respectively (blue). Water was added instead of melibiose to obtain control data (cyan).

To assess the functional state of $MelB_{St}$ solubilized in the respective detergent, a galactoside binding assay was conducted using the fluorescent ligand, 2'-(*N*-dansyl)aminoalkyl-1-thio-β-D-galactoside (D^2G).[29,30] A functional $MelB_{St}$ should bind this fluorescent ligand, which results in an increase in fluorescence emission intensity *via* Förster resonance energy transfer (FRET) from Trp residues to D^2G. The increased fluorescence emission resulting from D^2G binding is reversed by melibiose addition as ligand-substrate exchange would be detected. Similar to DDM-solubilized $MelB_{St}$, the transporter solubilized in all the tested MNAs exhibited a substantial change in fluoresence intensity following successive addition of D^2G and melibiose (Fig. 2c). When a relatively less stable MelB homologue, MelB of *E. coli* ($MelB_{Ec}$), was used for the assay, DDM-solubilized transporter lost almost all binding to the ligand/substrate (D^2G/melibiose).[31] In contrast, the selected MNAs (MNA-LC13, MNA-AC15 and MNA-LC15) were effective at preserving $MelB_{Ec}$ functionality. Combined together, these results show that MNAs were not only efficient at extracting MelB from the membranes, but were also superior to DDM at retaining MelB in a soluble and functional state.

The new MNAs were further evaluated with a 7 transmembrane domain, G protein-coupled receptor (GPCR), the human β_2 adrenergic receptor (β_2AR).[32] The receptor was extracted in 0.1% DDM and detergent exchange was then carried out by diluting the DDM-purified receptor into each detergent-containing buffer solution, giving final detergent concentrations of CMCs + 0.2 wt%. Receptor stability was assessed by measuring the ability of the receptor to bind the radio-labeled antagonist ([^{3}H]-dihydroalprenolol (DHA)).[33] As a method to preliminarily evaluate detergent efficacy, the ability of the receptor in the individual MNAs to bind [^{3}H]-DHA was measured following 30-min detergent exchange. As expected from a previous study, MNA-LC13 gave receptor activity comparable to DDM (Fig. 3a).[15] As for the new MNAs, all MNAs with C15 alkyl chain unit (MNA-AC15, MNA-FC15 and MNA-LC15) were poor at retaining antagonist binding over the test period, while the MNAs with C16 alkyl chain unit (MNA-AC16, MNA-LC16 and MNA-SC16) were all highly effective at maintaining receptor function. Based on this preliminary result, the three MNAs (MNA-AC16, MNA-LC16, and MNA-SC16) were selected for further evaluation of their effects on long-term receptor stability. In this assay, receptor activity was monitored at regular intervals during an 8-day incubation at room temperature (Fig. 3b). Receptor activity in DDM rapidly dropped to give only 10% of the initial value after a 2-day incubation. A noticeable improvement in detergent efficacy was observed for MNA-AC16 or MNA-LC13 which gave ~10% and ~20% retention of initial receptor activity after the 8-day incubation, respectively. In contrast, use of MNA-LC16 and MNA-SC16 led to markedly improved receptor activity over the course of the incubation. These agents (MNA-LC16 and MNA-SC16) retained ~70% and ~50% initial receptor activity at the end of incubation, indicating their potential utility in GPCR structural study.

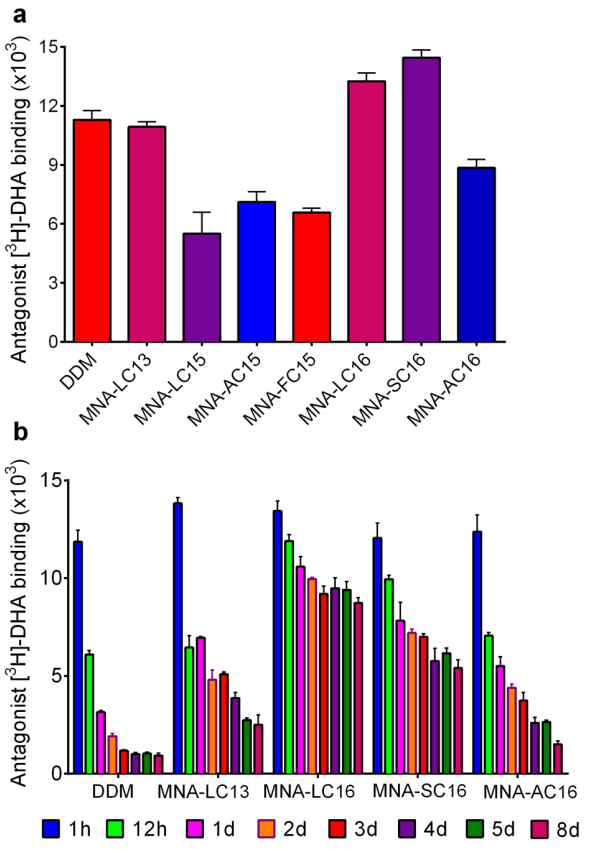


Fig. 3 (a) Initial and (b) long-term stability of β_2AR solubilized in individual new MNAs (MNA-LC15, MNA-AC15, MNA-FC15, MNA-LC16, MNA-AC16, and MNA-SC16). DDM and MNA-LC13 were used as positive controls. DDM-purified β_2AR was diluted into buffer solutions supplemented with individual detergents to give final detergent concentrations of CMCs + 0.2 wt%. Protein stability was assessed by measuring the ability of the receptor to bind the radio-labeled antagonist ([^{3}H]-dihydroalprenolol (DHA)) during an 8-day of incubation at room temperature. Error bars, SEM, n = 3.

We previously reported mannitol-based amphiphiles (MNAs) with two linear alkyl chains varying from C8 to C14 chain length.[15] Based on the promising effects of some of these MNAs, here we have prepared the hydrophobic MNA variants. In the course of these structural modifications, we maintained the number of carbon units in the alkyl chain at C15 or C16 so that we could compare detergent efficacy between detergents with same HLB. Thanks to the presence of the large hydrophilic group (i.e., four glucoside groups combined with the mannitol linker), all new MNAs with C15 or C16 carbon units showed good water-solubility. When these agents were evaluated with membrane proteins containing either 7 or 12 transmembrane helices, it was clear that detergent effects tend to be protein-specific. Specifically, two asymmetric alkyl-chained MNAs (MNA-AC15 and MNA-AC16) were most effective at stabilizing LeuT. MNA-AC15 was also effective for MelB$_{St}$ stability, but MNA-AC16 was inferior to most other MNAs. When tested with β_2AR, MNA-AC15 was most effective of the C15 alkyl chain MNAs, but this agent was inferior to the C16 alkyl chain MNAs. MNA-LC16 and MNA-SC16 showed superiority in retaining activity of this receptor long term. Thus, MNA-AC15 was most favorable for LeuT and MelB$_{St}$ stability although was suboptimal for β_2AR stability. Importantly, the current result indicates that the best agent varies depending on the target membrane protein, probably due to variations in the hydrophobic dimensions and properties of the individual proteins (Table S1). LeuT stability appeared to be sensitive to alkyl chain length of a detergent as the MNAs with the long alkyl chains (MNA-LC15/LC16) showed the worst behaviors in transporter stability. All the other MNAs, except MNA-SC16, have alkyl chain lengths ranging from C10 to C12, which seems to be within an optimal chain length range for LeuT stability. All these agents showed favorable efficacy for transporter stabilization, with the asymmetric MNAs (MNA-AC15 and MNA-AC16) better than MNA-FC15. We conceive that the alkyl pendants (propyl (MNA-AC15) and hexyl chain (MNA-AC16)) of these asymmetric MNAs play a positive role in increasing detergent micelle stability by filling the empty spaces in the micelle interior and increasing the hydrophobic density of the micelles. As with MelB$_{St}$, detergent alkyl chain length was not a main player as MNA-SC16, MNA-LC13, MNA-LC15, and MNA-LC16 with C9, C13, C15, and C16 chain lengths, respectively, were more or less similar with regards to maintaining water-solubility of the transporter. Hydrophobic density in the micelle interior also had little effect as a linear alkyl chain MNA-LC15 was comparable to the branched alkyl chain MNA (MNA-AC15) for MelB$_{St}$ solubility. When it comes to MelB functionality, however, a favorable role of the branched alkyl chain (i.e., the hydrophobic density in micelle interior) was apparent as observed by outperformance of MNA-AC15 compared to MNA-LC15 at retaining this transporter in a functional state. Interestingly enough, a somewhat opposite trend was observed for β_2AR stability. The linear alkyl chain MNA-LC16 was better than the branched alkyl chain MNA (MNA-SC16/AC16). We don't know a precise reason for the different behavior of this GPCR (β_2AR) from the other proteins, but this could be associated with the high tendency of the receptor to aggregate.[34] Thus, as protein aggregation is likely to be effectively prevented by strong associations of detergent molecules with protein surface and sufficient coverage of protein hydrophobic surface, a detergent with a low CMC (strong binding) and a medium micelle size (sufficient surface coverage) would be optimal for receptor stability. MNA-LC16, the best agent for β_2AR stability, has the lowest CMC (1 μM) of the tested MNAs and formed micelles (3.5 nm) larger than the C15 alkyl chain MNAs (2.9~3.3 nm).

One notable result of the current study is the good ability of the MNAs to extract MelB$_{St}$ from the membranes, also being previously observed with another membrane protein.[15] This study showed that most of the MNAs were highly efficient at extracting MelB$_{St}$, in contrast to many recently developed novel detergents such as RGAs,[18] mesitylene-cored glucoside amphiphiles (MGAs),[35] 1,2,3,4-butaneteteraol-based maltosides (BTMs),[36] and dendronic trimaltosides (DTMs).[37] This result is important as there seems a strong correlation between the ability of a detergent to extract a protein from the membrane and their popular use in membrane protein crystallization. For instance, conventional detergents popular in membrane protein crystallization (e.g., DDM, OG and LDAO) are all efficient at membrane protein extraction.[38] In addition, novel agents such as MNG-3 and GNG-3 that showed high efficiency of protein extraction have also been successful at producing well-

diffracting protein crystals.[20] Thus, these examples suggest that detergent ability to efficiently extract target proteins, in addition to effectively stabilizing them, is crucial for a successful outcome of detergent use in crystal structure determination of membrane proteins. Detergents need to strongly interact with target proteins to efficiently extract them from the membranes. Thus, a detergent with high efficiency at protein extraction is likely effective at preventing protein aggregation, which is particularly important under the conditions of protein crystallization. However, achieving both high efficacy for protein stabilization and high efficiency at protein extraction by a single detergent or a set of detergents is challenging as these detergent attributes are often not compatible to each other. For example, LDAO and OG are generally more efficient than DDM at extracting membrane proteins from the membranes, but these agents are inferior to DDM in stabilizing membrane proteins.[38] Thus, the current results are notable as the MNAs introduced here were not only able to extract membrane proteins with high efficiency, but were also effective at stabilizing multiple membrane proteins. Therefore, this study further augments the utility of the MNA detergents for membrane protein research.

Conclusions

In summary, the new MNAs with either linear alkyl chain extensions or branch points in the alkyl chain region were prepared and evaluated with a few membrane proteins including a human GPCR (β_2AR). The C15 branched alkyl chain MNA (MNA-AC15) conferred notable stability to LeuT and MelB while the C16 linear alkyl chain MNA (MNA-LC16) showed the highest efficacy toward β_2AR stabilization. This class also displayed high efficiency at extracting $MelB_{St}$ from the membranes. Along with convenient synthesis and strong binding to membrane proteins, the favorable properties of the MNAs for membrane protein extraction, solubilization and stabilization obtained here are a strong indication of their potential as agents in membrane protein structural study.

Conflicts of interest

P.S.C. and H.H. are inventors on a patent application describing the MNAs.

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