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Tandem neopentyl glycol maltosides (TNMs) for membrane protein stabilisation†

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Abstract

A novel class of detergents, designated tandem neopentyl glycol maltosides (TNMs), were evaluated with four target membrane proteins. The best detergent varied depending on the target, but TNM-C12L and TNM-C11S were notable for their ability to confer increased membrane protein stability compared to DDM. These agents have potential for use in membrane protein research.

Detergents are widely used for membrane protein extraction from membranes.¹ In addition, they are required to maintain the proteins in solution during protein purification and further downstream characterization.² Due to the presence of both a hydrophilic head and a hydrophobic tail, detergent molecules, in their micellar form, are able to effectively replace the membrane lipids surrounding membrane proteins to form protein–detergent complexes (PDCs). These PDCs are often stable in an aqueous medium, an essential requirement for successful characterization of the membrane protein structure and function. The most widely used conventional detergents are lauryldimethylamine-*N*-oxide (LDAO), *n*-octyl-β-D-glucoside (OG) and *n*-dodecyl-β-D-maltoside (DDM).³ OG is useful for membrane protein crystallization due to its characteristic of small PDC formation, while DDM is known to be particularly effective at stabilizing multiple membrane proteins including GPCRs and transporters.⁴ However, many membrane proteins, particularly eukaryotic proteins, encapsulated even in DDM are not stable enough for downstream characterization.⁵ Thus, it is of great interest to develop novel detergents that impart enhanced stability to a range of membrane proteins.⁶ Although acting as a membrane mimetic system, it is notable that the detergent micelle differs from biological membranes in many aspects. For instance, the asymmetrical distribution of lipid molecules across the inner and outer leaflets of the membrane plays critical roles in determining the membrane protein orientation and

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function.^{7a} In addition, animal cell membranes contain a large amount of cholesterol, essential for controlling membrane fluidity.^{7b} Detergent micelles lack these membrane characteristics.

Several small agents with non-conventional architectures have been successfully developed, as exemplified by tripod amphiphiles (TPAs),^{8a,b} hemifluorinated surfactants (HFSs),^{8c,d} peptide-based amphiphiles,^{8e,f} and cholate- or cholesterol-based amphiphiles.^{8g,h} Recent representatives include β -peptides (BPs),^{9a} facial amphiphiles (FAs),^{9b,c} mannitol-based amphiphiles (MNAs)^{9d} and penta-saccharide amphiphiles (PSEs).^{9e} Most of these agents contain multiple carbohydrates as the head groups, but a trimaltoside detergent is rare. Only one FA (FA-4) contains three maltose units.^{9b} In the current study, we introduced a novel class of amphiphiles with three identical alkyl chains as the hydrophobic group and a branched trimaltoside as the hydrophilic group, designated tandem neopentyl glycol maltosides (TNMs) (Scheme 1). These agents differ from the trimaltoside FA primarily because of the presence of a lipophilic group comprised of flexible alkyl chains rather than a rigid steroidal scaffold. They also deviate from the TPAs with respect to the presence of a branched trimaltoside headgroup attached *via* a neopentyl glycol (NG) linker instead of the dimaltoside group attached *via* a serinol linker.¹⁰ Also note that the previously reported TPAs have two identical alkyl chains and one cyclic ring in the lipophilic region, while the currently-developed TNMs contain three identical alkyl chains. These distinct agents were evaluated with four membrane proteins to assess their ability to stabilise membrane proteins in solution. For each of the four membrane proteins, at least one TNM was found to be markedly superior to a representative conventional detergent (DDM) at stabilizing the native structure of the protein in the long term.

The new agents feature the presence of two consecutive NG units in the central region, which is indicated in the detergent designation. The TNMs developed in this study can be categorized into two classes according to the identity of the spacer used to connect the NG units. One class, TNM-Ls, contains a propyl chain (long) between these units, while the other, TNM-Ss, has a direct connection (short). In order to find a detergent with optimal hydrophile–lipophile balance (HLB), necessary for effective membrane protein stabilisation, the alkyl chain length varied from C9 to C14 for TNM-Ls and from C11 to C14 for TNM-Ss. The chain length variation is indicated in the detergent designation. All synthetic agents (TNM-Ls and TNM-Ss) were prepared to high purity (>95%) *via* a synthetic protocol comprising eight steps with good to high synthetic yield (50–95%) (see the ESI[†] for details). Synthetic efficiency could be further improved using neopentyl glycol or dipentaerythritol as a starting material (see the ESI[†] for details). All of the agents, except TNM-C13L and TNM-C14L, were water-soluble up to 20%. TNM-C13L and TNM-C14L tend to form a hydrogel at high detergent concentration (>5 wt%). In contrast, their short linker counterparts (*i.e.*, TNM-C13S and TNM-C14S) did not show hydrogel formation even at detergent concentrations of >10%, indicative of a clear difference in the detergent self-assembly behaviour of the two classes (TNM-Ls and TNM-Ss).

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The novel detergents were characterized in terms of the critical micelle concentration (CMC) and hydrodynamic radius (R_h) of their micelles. Diphenylhexatriene (DPH) was used as a hydrophobic fluorophore for CMC determination,¹¹ and dynamic light scattering (DLS) was used to estimate the size of the detergent micelles. The results are summarized in Table S1 (ESI[†]). The CMC values of TNM-Ls tend to decrease with increasing alkyl chain length. For instance, the TNM-L with the shortest alkyl chain length, TNM-C9L, has a CMC of ~2 μ M, while that of TNM-C14L, with the longest alkyl chain, is ~0.4 μ M. A similar trend was observed for the short versions (*i.e.*, TNM-Ss). In a comparison of TNM-Ls and TNM-Ss, TNM-Ss gave higher CMC values than the TNM-L counterparts. Specifically, the TNM-Ss had CMC values ~two times higher than the TNM-L counterparts (TNM-C12S (~1.5 μ M) *vs.* TNM-C12L (~0.8 μ M)). The CMC values of all the TNMs were at least 70-fold smaller than DDM (170 μ M). All TNM-Ls except TNM-C9L and TNM-C10L tend to form large self-assemblies with hydrodynamic radii (R_h) more than 29 nm; TNM-C9L and TNM-C10L form small micelles with R_h of ~4.5 nm. In contrast, all the self-assemblies formed by the TNM-Ss were rather small, giving the maximum R_h (11.6 nm) for the longest alkyl chain agent (TNM-C14S). Overall, the micelles formed by the TNMs were larger than those formed by DDM (3.4 nm). Detergent micelles were further analysed in terms of the number-based size distribution. This analysis showed that the individual TNMs (TNM-Ls and TNM-Ss) form micelles with a single set of populations (Fig. S1, ESI[†]).

The novel agents were first evaluated with the *Rhodobacter (R.) capsulatus* superassembly, comprising the light harvesting complex I (LHI) and the reaction centre (RC).¹² This complex (LHI-RC) is known to be sensitive to protein denaturation.¹³ To begin with, DDM-purified LHI-RC complexes were diluted into buffer solutions containing individual detergents to reach a final detergent concentration of CMC +0.04 wt%. These protein samples were incubated at 15 °C for TNM-Ls and 20 °C for TNM-Ss for the first 10 days and then incubated at 32 °C for the next 10 days to investigate the effect of temperature variation on protein stability. Absorbance at 875 nm (A_{875}) was used as a criterion to assess the integrity of the complexes solubilised in the individual detergents; the native complexes display an intense absorption signal at this wavelength arising from cofactors embedded in the protein interior (*e.g.*, chlorophylls and carotenoids).^{8b} As observed in a previous study, the complex in DDM showed a gradual decrease in A_{875} with time during the incubation at room temperature (Fig. 1a and b).^{8b} The rate of decrease was more rapid when the incubation temperature was increased to 32 °C. The protein in OG was more unstable compared to DDM, consistent with a previous result.^{8b} When TNM-Ls or TNM-Ss were used under the same conditions, all novel agents except for TNM-C14S were superior to the two conventional detergents (DDM and OG) at stabilising the complex. Of the TNMs, the shortest alkyl chain TNMs for both classes (TNM-C9L and TNM-C11S, respectively) were the most effective, while the longest alkyl chain agents (TNM-C14L and TNM-C14S, respectively) were the least effective. An increase in the detergent concentration from CMC +0.04 wt% to CMC +0.2 wt% resulted in less stable proteins in both DDM and OG (Fig. S2, ESI[†]). Similar results were obtained for the TNMs, particularly for the longest alkyl chain TNMs such as TNM-C14L and TNM-C14S; TNM-C14S was comparable to DDM at stabilising the complexes at this high concentration. However, all other TNMs were clearly superior to DDM under the same condition.

The TNMs continued to be evaluated with a protein transporter, the uric acid–xanthine/H⁺ symporter (UapA) from *Aspergillus nidulans*.¹⁴ In this case, we also used DDM as a benchmark for conventional detergents. The folded state of the protein was monitored over the course of a 120 min incubation at 40 °C using a small dye molecule, *N*-[4-(7-diethylamino-4-methyl-3-coumarinyl)-phenyl]maleimide (CPM).¹⁵ This molecule contains both the fluorophore (coumarin) and the quencher (maleimide), and thus fluoresces upon reaction with free thiol residues. When individual detergents (TNM-Ls, TNM-Ss and DDM) were used at CMC +0.2 wt%, all the TNM-Ls were better than DDM at retaining the folded state of UapA (Fig. 2a). TNM-C12L appeared to be the most effective of the TNM-Ls, followed by TNM-C14L. In the case of TNM-Ss, all the new agents were better than DDM, with the best performance obtained for TNM-C14S (Fig. 2b). This agent was markedly superior to DDM and the other TNM-Ss (TNM-C11S, TNM-C12S and TNM-C13S). When the detergents were used at CMC +0.04 wt%, the detergent efficacy order of the TNM-Ls and TNM-Ss was similar to that obtained at CMC +0.2 wt%. TNM-C12L (and TNM-C14L) and TNM-C14S were the best of the TNM-Ls and TNM-Ss, respectively, at retaining the folded state of UapA (Fig. S3a and b, ESI[†]). Thus, overall TNM-C12L and TNM-C14S imparted the greatest stability to the transporter. Note that most of the TNM-Ls and TNM-Ss were significantly better than DDM at both detergent concentrations.

The TNMs were further evaluated with the bacterial leucine transporter (LeuT) from *Aquifex aeolicus*.¹⁶ The transporter was first extracted from the membrane with 1.0% DDM and purified in the same detergent (0.05%). The DDM-purified LeuT was diluted with buffer solutions containing the individual TNMs or DDM so that the detergent concentration reached CMC +0.04 wt%. The transporter solubilised in the individual detergents was incubated for 10 days at room temperature. During the incubation, the substrate binding activity of the transporter was measured at regular intervals using a radiolabelled substrate ([³H]-leucine (Leu)) *via* scintillation proximity assay (SPA).¹⁷ Upon detergent dilution, most of the TNM-Ls were inferior to DDM at preserving the high activity of the transporter (Fig. 3a). In contrast, TNM-C10L clearly outperformed DDM and the other TNM-Ls in this regard. Overall, we observed better results for the TNM-Ss than the TNM-Ls (Fig. 3b). Interestingly, the activity of the transporter solubilised in the respective TNM-Ss was retained over time although a large variation was found in the initial activity. Notably, TNM-C11S showed the best behaviour of the TNM-Ss; the TNM-C11S-solubilised transporter not only had enhanced initial substrate binding activity relative to the DDM-solubilised protein, but also showed almost full retention of the high initial activity over a 10 day incubation. TNM-C12S, with one additional carbon unit in all three alkyl chains compared to TNM-C11S, was worse than DDM during the first 4 days of incubation, but better over the later incubation (4–10 days). When the detergent concentration was increased to CMC +0.2 wt%, a similar trend in the detergent efficacy order was observed; TNM-C10L and TNM-C11S were the best agents of their respective groups and both agents were better than DDM (Fig. S4a and b, ESI[†]).

Next we assessed the detergents using a G protein-coupled receptor (GPCR), the β_2 adrenergic receptor (β_2 AR).¹⁸ DDM was also used as a positive control as this agent is the most effective of the conventional detergents for receptor stability. For this experiment, β_2 AR was extracted and purified using DDM and in the presence of 0.01% cholesteryl

hemisuccinate (CHS). CHS is known to improve the stability of many GPCRs by specifically binding to the receptor surface.¹⁹ The DDM-purified β_2 AR was diluted into individual detergent-containing buffers to give a final detergent concentration of CMC +0.2 wt%. The receptor stability was assessed *via* radio-labelled ligand ($[^3\text{H}]$ -dihydroalprenolol (DHA)) binding assay.²⁰ Of the TNMs, TNM-C12L showed receptor activity closest to that seen for DDM (Fig. S5, ESI[†]); the other TNMs were inferior to DDM in this regard. Based on this preliminary result, this novel agent was selected for further evaluation in terms of its ability to stabilise the receptor in the long-term by measuring the ligand binding activity of the receptor at regular intervals during a 4 day incubation at room temperature. In this evaluation, the initial activity of the DDM-solubilised receptor was a little higher than that of the TNM-C12L-solubilised protein (Fig. 3c). However, the DDM-solubilised receptor underwent fast activity loss over time, whereas the TNM-C12L-solubilized protein displayed full retention of receptor activity over the course of the 4 day incubation. No other tested detergent in this or other studies has produced such a stable receptor,^{9e} indicating that this agent may hold significant potential for GPCR study.

Detergent efficacy is known to be protein-specific. In the current study, we found that the most stabilising TNM was different for each of the target proteins. Of the TNM-Ls and TNM-Ss, TNM-C9L and TNM-C11S were most effective at stabilizing LHI-RC complexes, respectively. As for UapA stability, the best detergents of these two classes were TNM-C12L and TNM-14S, respectively. When LeuT was used for detergent evaluation, TNM-C10L and TNM-C11S were the best, while for β_2 AR, TNM-C12L yielded the most stable protein. It is impossible to know precise reasons for the protein-specific effects of the detergents. Potential reasons could be a variation in the membrane protein propensity to aggregate/denature and/or a range of diversity in the dimensions of protein hydrophobic/hydrophilic surfaces. Consequently, an optimal value for detergent HLB will be different depending on the tendency of a target protein to denature/ aggregate and the ideal length for the detergent alkyl chain will vary depending on the hydrophobic surface dimensions of the target protein. In spite of such protein-specific behaviours of the detergents, it is noteworthy that we found some general trends with respect to membrane protein stabilisation. More specifically, TNM-C12L stabilizes all the target proteins studied here except LeuT; for this transporter, TNM-C10L was best. Similarly, TNM-C11S stabilised all the membrane proteins except β_2 AR. Notably, TNM-11S and TNM-C12L were highly effective at retaining the initial activity of LeuT and β_2 AR, respectively, in stark contrast to the rather rapid decrease in activities of these proteins when solubilised in DDM. Based on this study, we expect that a single agent cannot be a magic bullet for all or even most membrane proteins, but it is likely that a set of detergents (*e.g.*, TNM-C12L and TNM-C11S along with TNM-C10L) would serve as research tools suitable for stabilising multiple membrane proteins.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1.

Long-term stability of LHI-RC complexes solubilized in individual TNM-Ls (a) or TNM-Ss (b) at CMC + 0.04 wt%. Protein integrity was assessed *via* the A_{875} value obtained from the UV-Vis spectrum. The spectra were measured at regular intervals during a 10 day incubation at 15 °C for TNM-Ls or 20 °C for TNM-Ss, followed by a further next 10 day incubation at 32 °C. Error bars, SEM, $n = 2$.

Fig. 2. Thermal denaturation profile of UapA solubilised in individual novel agents (TNM-Ls (a) and TNM-Ss (b)) or DDM used at CMC +0.2 wt%. The relative amount of the folded protein was monitored using the CPM assay carried out at 40 °C for 120 min. The data is representative of three independent experiments.

Fig. 3.

Long-term substrate or ligand binding activity for LeuT (a and b) solubilised in individual TNMs and β_2 AR (c) solubilised in a single TNM (TNM-C12L). Detergents were used at CMC +0.04 wt% and CMC +0.2 wt% to measure the activities of LeuT and β_2 AR, respectively. LeuT activity was measured at regular intervals during a 10 day incubation at room temperature *via* scintillation proximity assay (SPA) using a radio-labelled substrate ($[^3\text{H}]$ -leucine). The β_2 AR ligand binding activity was assessed at regular intervals using the antagonist $[^3\text{H}]$ -dihydroalprenolol (DHA) over a 4 day incubation at room temperature. Error bars, SEM, $n = 3$.

Scheme 1.

Chemical structures of novel TNMs with a long (TNM-Ls) or short spacer (TNM-Ss) between the two neopentyl glycol units. The detergent alkyl chain length varied from C9 to C14 for TNM-Ls and from C11 and C14 for TNM-Ss. The carbon number in the alkyl chain was utilized for detergent designation.