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Trehalose-cored amphiphiles for membrane protein stabilization: importance of the detergent micelle size in GPCR stability†

Manabendra Das, ‡[a] Yang Du, [b] Jonas S. Mortensen, [c] Manuel Ramos,[d] Lubna Ghani, [a] Ho Jin Lee, [a] Hyoung Eun Bae, [a] Bernadette Byrne, [e] Lan Guan, [d] Claus J. Loland, [c] Brian K. Kobilka [b] and Pil Seok Chae *[a]

Despite their importance in biology and medicinal chemistry, structural and functional studies of membrane proteins present major challenges. To study diverse membrane proteins, it is crucial to have the correct detergent to efficiently extract and stabilize the proteins from the native membranes for biochemical/biophysical downstream analyses. But many membrane proteins, particularly eukaryotic ones, are recalcitrant to stabilization and/or crystallization with currently available detergents and thus there are major efforts to develop novel detergents with enhanced properties. Here, a novel class of trehalose-cored amphiphiles are introduced, with multiple alkyl chains and carbohydrates projecting from the trehalose core unit are introduced. A few members displayed enhanced protein stabilization behavior compared to the benchmark conventional detergent, *n*-dodecyl-β-D-maltoside (DDM), for multiple tested membrane proteins: (i) a bacterial leucine transporter (LeuT), (ii) the *R. capsulatus* photosynthetic superassembly, and (iii) the human β_2 adrenergic receptor (β_2AR). Due to synthetic convenience and their favourable behaviors for a range of membrane proteins, these agents have potential for membrane protein research. In addition, the detergent property–efficacy relationship discussed here will guide future design of novel detergents.

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Introduction

Integral membrane proteins are involved in all aspects of vital cellular activities including inter- or intra-cellular material transfer, signal transduction, photosynthesis, cell adhesion, cytoskeletal organization, protein trafficking, *etc*. Additionally, membrane proteins account for up to one third of the human proteome[1] and make up approximately half of all presently marketed therapeutic targets.[2] Thus, in depth information on the structures and functions of these biomacromolecules is of key importance for biology and rational drug design.[3] Unfortunately, membrane proteins are inherently challenging to study *in vitro* because of their low natural abundance, problematic overexpression, and tendency to misfold/denature once extracted from the native membranes into aqueous buffer. These bio-macromolecules are insoluble in aqueous solutions due to the presence of their hydrophobic transmembrane domains (TMs). To circumvent these issues, conventional detergents are used for membrane protein extraction and stabilization. Detergents shield the protein hydrophobic exterior from the polar aqueous environment. However, detergent micellar assemblies do not often adequately mimic some of the hallmarks of the native membranes. For example, *n*-octyl-β-D-glucoside (OG), *n*-nonyl-β-D-glucoside (NG) and lauryldimethylamine-*N*-oxide (LDAO) are widely used for membrane protein crystallization mainly due to the fact that they form small protein-detergent complexes (PDCs).[4] However, many membrane proteins are not stable in these detergents, resulting in irreversible nonspecific aggregation due to exposure of the hydrophobic TMs to a polar medium or penetration of detergent molecules between TM helices.[5] On the other hand, *n*-dodecyl-β-D-maltoside (DDM) is generally a mild detergent preferred for maintaining the native states

[a]*Department of Bionanotechnology, Hanyang University, Ansan, 155-88, Korea. E-mail: pchae@hanyang.ac.kr*
[b]*Molecular and Cellular Physiology, Stanford University, Stanford, CA 94305, USA. E-mail: kobilka@stanford.edu*
[c]*Department of Neuroscience, University of Copenhagen, DK-2200, Copenhagen, Denmark. E-mail: cllo@sund.ku.dk*
[d]*Department of Cell Physiology and Molecular Biophysics, Center for Membrane Protein Research, School of Medicine, Texas Tech University Health Sciences Center, Lubbock, TX 79430, USA. E-mail: lan.guan@ttuhsc.edu*
[e]*Department of Life Sciences, Imperial College London, London, SW7 2AZ UK. E-mail: b.byrne@imperial.ac.uk*
†Electronic supplementary information (ESI) available. See DOI: 10.1039/c8ob03153c
‡Current address: Molecular Biophysics, Technische Universität Kaiserslautern, Erwin-Schrödinger -Str. 13, 67663, Kaiserslautern, Germany.

of proteins in solution, but this maltoside tends to form relatively large PDCs unfavorable for protein crystallization and NMR studies.[6] In addition, many membrane proteins solubilized in a commercially available detergent are not stable enough for the downstream protein structure determination. Thus, the development of novel membrane-mimetic systems with improved efficacy is of importance for structural and functional characterization of membrane proteins.

Notable examples of several membrane-mimetics such as amphipols (Apols),[7] nanodiscs (NDs),[8] lipopeptide detergents (LPDs)[9] and β-peptides (BPs)[10] have been developed. These agents have been demonstrated to be effective at stabilizing multiple membrane proteins but form large PDCs or are ineffective at protein extraction. More importantly, these systems have had limited success in producing protein crystals. With carbohydrate-based head groups and structurally modifiable hydrophobic segments, several small amphipathic agents have also been invented, as exemplified by neopentyl glycol (NG) class amphiphiles (MNGs/GNGs),[11] facial amphiphiles (FAs),[12] mannitol-based amphiphiles (MNAs),[13] neopentyl glycol triglucosides (NDTs),[14] resorcinarene-based glucosides (RGAs),[15] penta-saccharide amphiphiles (PSEs)[16] and vitamin E-based glucosides (VEGs).[17] Some small detergents such as butane-1,2,3,4-tetraol-based maltosides (BTMs)[18a] and norbornane-based maltosides (NBMs)[18b] exploit molecular chirality to modulate detergent efficacy for protein stabilization. Of these small amphipathic agents, GNG-3 and MNG-3 have contributed to the determination of more than 35 new membrane protein crystal structures including the β_2 adrenergic, acetylcholine and opioid G-protein coupled receptors (GPCRs) in the last several years.[19] Using FA-5 or FA-7 alone or mixed with lipids (DMPC), Lee and co-workers obtained crystals of the ATP-binding cassette transporter (MsbA) and the GPCR-like bacteriorhodopsin, respectively.[12b] Herein, we introduced new glucoside and maltoside amphiphiles with trehalose as a conformationally restricted core unit, designated TCGs and TCMs, respectively (Fig. 1b). Three sets of trehalose-cored amphiphiles with structural variation were prepared to evaluate their stabilization efficacy for different membrane proteins including a G-protein coupled receptor (GPCR). We found that some of these agents conferred markedly enhanced stability to target proteins compared to DDM.

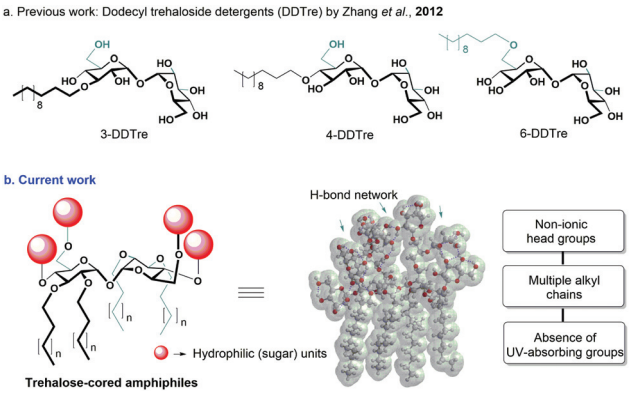

Fig. 1 Background for this study. Schematic chemical structures of previously reported dodecyl trehaloside detergents (DDTre)[20] (a) and new trehalose-cored amphiphiles (TCGs/TCMs) (b). DDTre has a trehalose unit used as the head group while TCGs/TCMs used this disaccharide unit as the central core of the amphiphiles. (b, *right*) Side view of the structure of a trehalose-cored amphiphile optimized by DFT calculations (B3LYP/6-31G*) in which the alkyl chains are segregated from the hydrophilic backbone in contact with water. These non-ionic trehalose-cored detergents do not interfere with optical spectroscopy in the far-UV range, and contain a dense hydrophobic group for effective protein stabilization.

Results and discussion

Detergent structures and physical characterization

First, we prepared two sets of trehalose-cored detergents with either a glucoside (TCG) or maltoside (TCM) hydrophilic group directly attached to the trehalose core (Fig. 2a). Along with four glucose/maltose units, both TCGs and TCMs share four alkyl chains as the hydrophobic group, but the alkyl chain length varies from C_5 to C_7 for the glucosides and from C_8 to C_{10} for the maltosides, as indicated in the detergent designation. For an additional set of amphiphiles, eight glucose units were attached to the central trehalose unit using a glycerol linker (TCG-Ls). Introduction of the glycerol linker allowed us to prepare water-soluble TCG-Ls with the alkyl chain length extended to C_{12}. This chain extension is important as the alkyl chain lengths of the TCGs with no linker are likely too short to stabilise membrane proteins. Of note, an optimal alkyl chain length for protein stability is variable within the range of C_{10} to C_{13} depending on the target protein. A good match in hydrophobic dimensions between detergent molecules and membrane proteins is necessary to maintain protein stability. Using glucoside and maltoside head groups, along with the glycerol linker, we varied the detergent alkyl chain length from C_5 to C_{12}. Furthermore, the head group architecture of these amphiphiles varied from tetra-glucoside to tetra-maltoside to octa-glucosides. We hypothesized that these TCGs/TCMs/TCG-Ls with their large range of structural variations may have different efficacies for membrane protein stabilization (Fig. 2b), providing insights into detergent structure property–efficacy relationships. Trehalose is widely used in life sciences as an additive for stabilizing proteins with a mechanism attributed to vitrification, water replacement and water entrapment.[21] Trehalose and its derivatives have found use in biomedical applications,[22,23] DNA amplification[24] and the pharmaceutical industries.[25] This trehalose unit has also been used as an amphiphile scaffold for membrane protein studies (Fig. 1a),[20] but a previous study utilized the disaccharide unit as the head group rather than the central core unit connecting the head and tail groups. Thus, this study is the first example of a disaccharide unit used as the detergent core for membrane protein studies. Due to the presence of two central pyranose rings, the new detergents have reduced conformational flexibility of the head and tail groups in aqueous solution. At

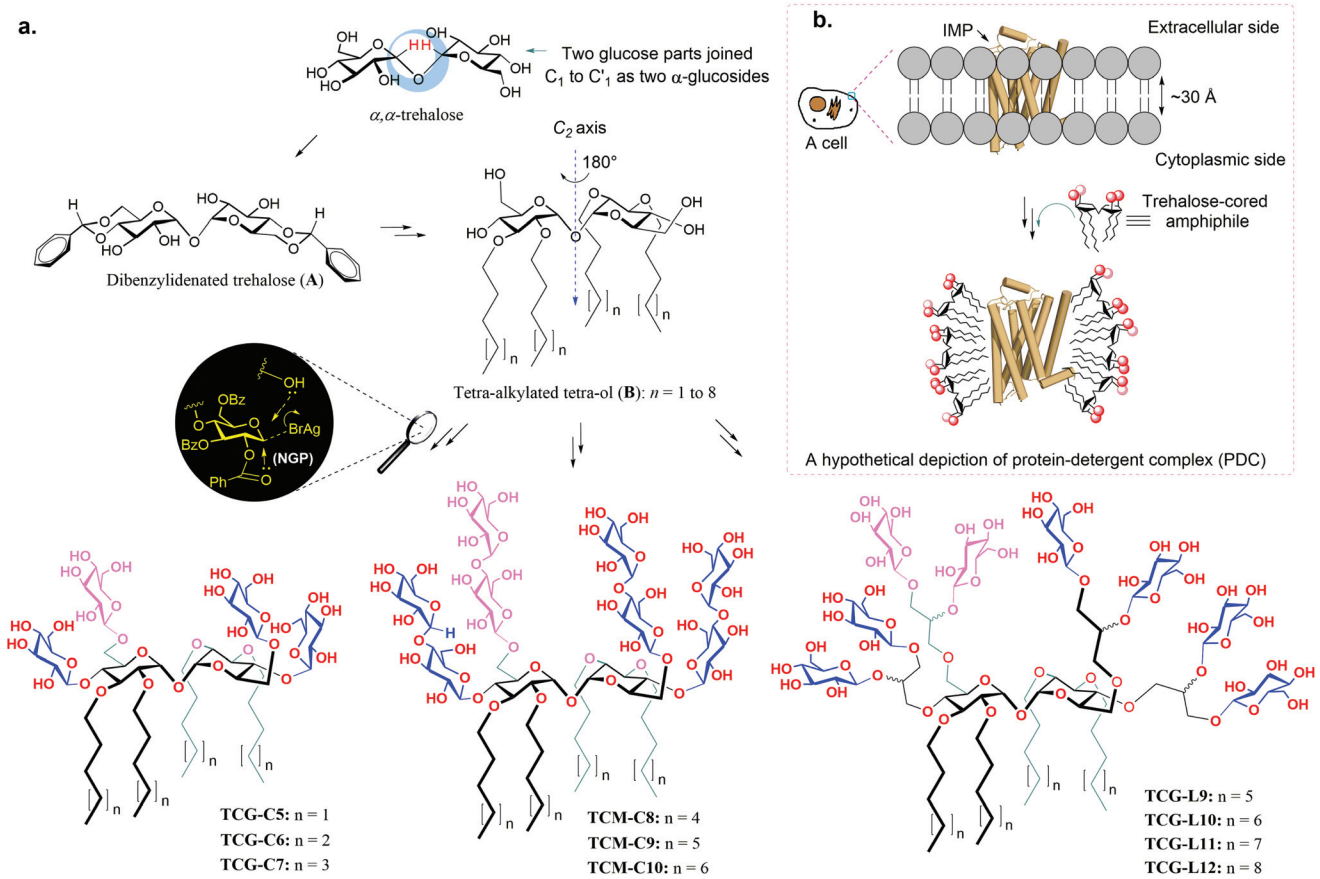

Fig. 2 (a) Synthetic scheme and chemical structures of newly prepared trehalose-cored amphiphiles (TCGs/TCMs/TCG-Ls). Novel amphiphiles were derived from α,α-trehalose *via* dibenzylidenated trehalose (**A**) and tetra-alkylated tetra-ol intermediates (**B**). The tetra-alkylated tetra-ol intermediates and TCGs/TCMs/TCG-Ls contain a C_2 axis passing through the central part of the molecules, indicated by the blue dotted line on the chemical structures of the tetra-ol intermediate. The inset within circle (black) illustrates a known mechanism of β-selective glycosylation involving neighboring group participation (NGP). (*b*) Schematic representation of a membrane protein interacting with one of the new detergents following extraction from the membrane.

the same time, due to the presence of multiple flexible alkyl chains, the detergent molecules should readily adopt an optimal conformation for protein stability when associated with membrane proteins. Thus, the new agents differ from both conventional detergents which contain either a very flexible alkyl chain (*e.g.* DDM) or facial amphiphiles (FAs) with conformationally locked hydrophobic groups.[12] In addition, as the current trehalose-cored amphiphiles contain no aromatic group, membrane proteins can be studied *via* UV spectroscopic methods, in comparison with aromatic group-containing detergents such as calix[4]arene based detergents (C4Cn),[26] ganglio-tripod amphiphiles (TPAs),[27] Chae's glycotritons (CGTs),[28] xylene-linked maltoside amphiphiles (XMAs)[29] and vitamin E-based glycosides (VEGs).[17] Energy-minimized conformations of the new agents obtained by density functional theory (DFT) calculation show that the four alkyl chains are well segregated from the carbohydrate-based head groups (Fig. 1b & Fig. S1). The hydrophobic length in these energy-minimized conformations increases from 7.7 to 11.0 to 13.2 Å when the detergent alkyl chain is extended from C7 to C10 to C12. In addition, a high hydrophilic density appeared to be attained by an intramolecular hydrogen bond network within the head groups (Fig. 1b, *right*).

Both TCG and TCM sets were prepared from D-(+)-trehalose according to a protocol comprising straightforward synthetic steps (Fig. 2a): acid-catalyzed formation of 4,6,4′,6′-dibenzylidenated trehalose (**A**), tetra-alkylation and benzylidene acetal removal (**B**), stereo-selective glycosylation and global deprotection (see Scheme S1† for details). In the case of TCG-Ls containing the glycerol linker, two additional steps of allylation and *syn*-dihydroxylation using OsO_4 were necessary to synthesize tetra-alkylated octa-ol intermediates (see Scheme S2† for details). High anomeric purity of all the new detergents was attained by stereo-selective β-glycosylation *via* neighboring group participation (*i.e.* anchimeric assistance) of the benzoyl group (inset within the circle in Fig. 2a).[30] The high purity of each new detergent was confirmed by their individual ^{1}H NMR spectra (Fig. S2–S5†). For example, when measured in CD_3OD, the axial anomeric protons of TCG-C5 attached to the anomeric carbons, designated H_a and $H_{a'}$ (Fig. 3a & S2†), produced

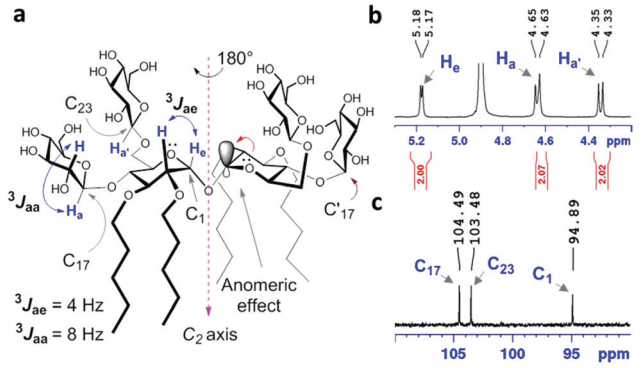

Fig. 3 (a) The chemical structure of TCG-C5 is given to illustrate the axial anomeric protons (H_a and $H_{a'}$) and equatorial anomeric protons (H_e) and their couplings with the neighboring protons (H in blue color). (b) Partial ^{1}H NMR spectrum in the anomeric region for TCG-C5 showing its high anomeric purity. The NMR spectrum of TCG-C5 gave two doublets at 4.64 and 4.34 ppm, along with a coupling constant ($^3J_{aa}$) of 8.0 Hz, typical peak characteristics of β-anomeric protons. TCG-C5 also contains α-anomeric proton (H_e), giving doublets at 5.18 ppm with a reduced coupling constant ($^3J_{ae}$ = 4.0 Hz). (c) A partial ^{13}C NMR spectrum of TCG-C5. Only anomeric carbon signals for TCG-C5 are assigned.

two separated peaks at 4.64 and 4.34 ppm as doublets, respectively. In addition, the vicinal coupling constants ($^3J_{aa}$) for these anomeric protons (H_a and $H_{a'}$) were 8.0 Hz, typical of a β-anomer, representing an exclusive formation of β-glycosidic bonds in the glycosylation step. Note that the α-anomeric proton (H_e) present in the central part of the trehalose moiety in TCG-C5 produced a peak that is downfield shifted to 5.18 ppm with a relatively small coupling constant ($^3J_{ae}$ = 4.0 Hz) (Fig. 3a and b). As for the TCMs, the ^{1}H NMR spectra were measured in DMSO-d_6 due to their solubility issues in CD_3OD and gave no clear indication of the stereochemistry of the newly formed glycosidic bonds because of peak overlap (Fig. S3†). In the cases of TCG-Ls with the good solubility in CD_3OD, the anomeric peaks were broader and more complex than the TCGs. This is because the OsO_4-catalyzed dihydroxylation produced epimeric isomers at the asymmetric carbons in the glycerol linker (see supplementary Scheme S2†) and there are multiple β-anomeric protons with dissimilar chemical environments in these linker-bearing detergents, as observed by the individual spectra (Fig. S4 & S5†). Peak appearance in the range of 4.3 to 4.7 ppm with an integration value of eight in the spectra, however, indicates that all newly formed glycosidic bonds have β-stereochemistry.

The identity of the new detergents was further confirmed by their ^{13}C NMR spectra. Note that the TCGs and TCMs are optically active because of a lack of a symmetry plane yet they contain a molecular symmetry axis (*i.e.* C_2 axis) vertically passing through the central part of the molecules. Consistent with this molecular symmetry, TCG-C5 showed three ^{13}C NMR peaks at 94.9, 104.5, and 103.5 ppm corresponding to the anomeric carbons (C1, C17 and C23), respectively (Fig. 3a and c). The upfield chemical shift of the anomeric carbon C1 (δ = 94.9 ppm) with respect to the other anomeric carbons (C17 and C23) is probably due to the shielding effect (also termed an anomeric effect) of a lone pair on the nearby oxygen atom donating electron density into the σ* molecular orbital of the C–O bond as indicated by a red arrow in Fig. 3a.

Most of the new agents were water-soluble up to more than 2.5 wt%, with no observed precipitation over a month at room temperature (Table 1). With moderate water-solubility (~2.0 wt%), TCG-C7, TCM-C10 and TCG-L12 required a brief sonication for complete dissolution. Critical micelle concentrations (CMCs) were determined by monitoring solubilization of a hydrophobic fluorescent dye (*i.e.*, diphenylhexatriene (DPH))[31] and the hydrodynamic radii (R_h) of the self-assemblies were estimated through dynamic light scattering (DLS) measurements. The results obtained for the novel agents are summarized in Table 1, along with DDM. Of the three sets, the TCGs with relatively short alkyl chains gave the highest CMCs. TCG-C5 and TCG-C6 gave CMCs (0.20 and 0.15 mM, respectively) comparable to that of DDM (0.17 mM), while that of TCG-C7 was about six times lower than that of DDM (0.03 *vs.* 0.17 mM). The CMCs of the TCMs and TCG-Ls (from 0.004 to 0.020 mM) were much lower than that of DDM, which indicates a stronger tendency of these agents to self-assemble compared to DDM. As expected from the increased hydrophobicity,

Table 1 Molecular weights (MWs), critical micelle concentrations (CMCs), water-solubility of novel agents (TCGs, TCMs and TCG-Ls) along with a conventional detergent (DDM) and hydrodynamic radii (R_h; n = 4) of their micelles in water at room temperature

Detergent	MW[a]	CMC (mM)	CMC (wt%)	R_h[b] (nm)	Solubility (wt%)
TCG-C5	1271.4	~0.20	~0.025	28 ± 1.0	~5.0
TCG-C6	1327.5	~0.15	~0.020	35 ± 1.0	~2.5
TCG-C7	1383.6	~0.03	~0.0042	39 ± 1.1	~2.0
TCM-C8	2088.3	~0.02	~0.0042	20 ± 1.0	~2.5
TCM-C9	2144.4	~0.009	~0.0019	22 ± 1.0	~2.5
TCM-C10	2200.5	~0.005	~0.0011	25 ± 1.0	~2.0
TCG-L9	2440.7	~0.01	~0.0024	2.9 ± 0.0	~10
TCG-L10	2496.8	~0.008	~0.0020	3.0 ± 1.0	~10
TCG-L11	2552.9	~0.006	~0.0015	3.1 ± 0.1	~5.0
TCG-L12	2609.0	~0.004	~0.0010	3.2 ± 0.0	~2.5
DDM	510.1	0.170	0.0087	3.4 ± 0.0	>10

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

within a set of detergents, CMC values were decreased with increasing alkyl chain length. For instance, the CMCs of the TCMs reduced from 0.02 to 0.01 to 0.005 with the alkyl chain length increasing from C8 to C9 to C10. A comparison of detergents with the same chain length revealed only minor differences in the CMCs. For example, the CMC of TCM-C9 was marginally lower than that of TCG-L9 (~0.009 *vs.* ~0.01 mM). This result indicates that the hydrophilic group has little effect on the CMC. Overall the new detergents, particularly TCMs and TCG-Ls, gave lower CMCs than detergents (0.01 to 20 mM) popularly used for membrane protein crystallization.[32] Thus, the new agents can be used in relatively low concentrations for membrane protein studies, but are likely to form strong interactions with the protein and thus maybe difficult to exchange.

The TCGs with a tetra-glucoside head group formed rather large self-assemblies ranging from 28 to 39 nm. Head group conversion from a tetra-glucoside (TCGs) to a tetra-maltoside (TCMs) resulted in a substantial reduction in the sizes of detergent self-assemblies to the range of 20 to 25 nm although these were still significantly larger than the size of DDM self-assemblies (3.4 nm). When a bulky octa-glucoside was introduced as a head group (TCG-Ls), a marked decrease in the self-assembly size was observed. The micelle sizes of the TCG-Ls were even smaller than that of DDM (2.9 to 3.2 nm *vs.* 3.4 nm). This change in the self-assembly size dependent on the detergent head group is likely to be due to a change in the geometry (*i.e.*, change in the relative volume ratio of the head and tail groups).[33] The TCGs with the smallest head group gave the largest self-assemblies while the TCG-Ls with the largest head group formed the smallest self-assemblies. Thus, the current study illustrated a way to systematically vary the self-assembly size by introducing stepwise changes in the head group volume. The sizes of self-assemblies formed by the individual sets of detergents also tended to vary with the alkyl chain length although the size variations were relatively small. For instance, the self-assembly size was increased from 20 to 22 to 25 nm when alkyl chain lengths of the TCMs were increased from C8 to C9 to C10. Similar trends were observed for the TCGs and TCG-Ls. When we investigated the size distribution of detergent assemblies, all new agents showed only one set of micellar populations, indicative of high homogeneity (Fig. S6†).

TCG-L10 micelles were further characterized by transmission electron microscopy (TEM) analysis. The TEM image reveals that TCG-L10 forms spherical micelles with an estimated size of ~7 nm (Fig. 4b) in diameter, roughly consistent with DLS data (D_h = 6.0 nm) (Fig. 4a). Similar trends were observed from the TEM images of TCG-C7 and TCM-C10 (Fig. S7†). Wet and dry samples were used for DLS and TEM measurements, respectively, which would be a reason for such a minor deviation in the micelle size between these two data.

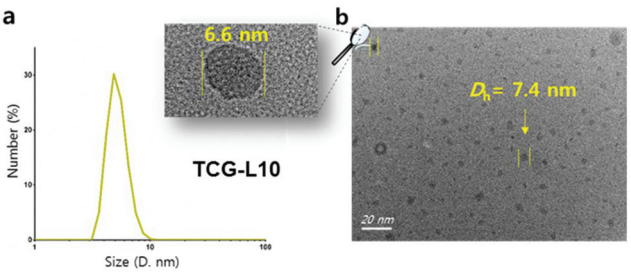

Fig. 4 (a) DLS profile and (b) TEM image of TCG-L10. Size distribution of TCG-L10 micelles was determined by DLS. The detergent was used at 1.0 wt% for DLS and TEM experiments. A minor discrepancy in the micelle size of TCG-L10 observed between DLS and TEM data could be an artifact originating from different sampling conditions (wet *vs.* dry). DLS: dynamic light scattering; TEM: transmission electron microscope.

Detergent evaluation with membrane proteins

The novel agents were evaluated using the photosynthetic *Rhodobacter (R.) capsulatus* super-assembly, comprising the light-harvesting complex I and the reaction centre complex (LHI-RC).[34,35] This LHI–RC super-assembly contains a large number of protein subunits, and is thus highly susceptible to protein denaturation. In addition, the complex contains multiple cofactors such as chlorophylls and carotenoids in the interior. In an intact structure, the LHI-RC complex gives rise to a strong absorbance at 875 nm (A_{875}), which decreases dramatically with protein denaturation.[27b,c] Thus, we utilized optical spectroscopy to assess the complex stability over time. Here, the super-assembly was extracted from the native membrane with 1.0 wt% DDM and purified in 0.0087 wt% of the same detergent (*i.e. 1x*CMC) using Ni^{2+}-NTA affinity chromatography. The DDM-purified LHI-RC complexes were diluted into buffer solutions containing the individual detergents (TCGs, TCMs, and TCG-Ls) to give final detergent concentrations of CMCs + 0.05 wt%. OG and DDM were used as control agents as these are representative glucoside and maltoside detergents, respectively. The protein sample was incubated at 25 °C for the first 10 days and then incubated at 35 °C for the next 10 days. This temperature variation allowed us to investigate how sensitive the integrity of the LHI-RC complex is to an elevated temperature (Fig. 5a, S7†).

Utilizing the strong absorbance of the native complex at 875 nm (A_{875}), we monitored the complex integrity at regular intervals during the 20-day incubation at room temperature. As can be seen in Fig. 5a, the LHI-RC complex in OG underwent rapid degradation, while DDM-solubilized complexes showed a gradual decrease in A_{875} over time. The rate of protein degradation was faster when the incubation temperature was increased to 35 °C. In DDM, less than 5% complex integrity was retained at the end of the incubation (day 20). The TCGs were better at maintaining the native structure of the complexes than DDM (Fig. S8a†) but the most stable complex was obtained using the TCMs and TCG-Ls. These agents retained 75–80% complex integrity at the end of the incubation period. An increase in detergent concentration from CMCs + 0.05 wt% to CMCs + 0.2 wt% resulted in a less stable complex in both DDM and OG (Fig. 5b) but little effect was observed in the case of the new agents (Fig. 5b & S8b†). It

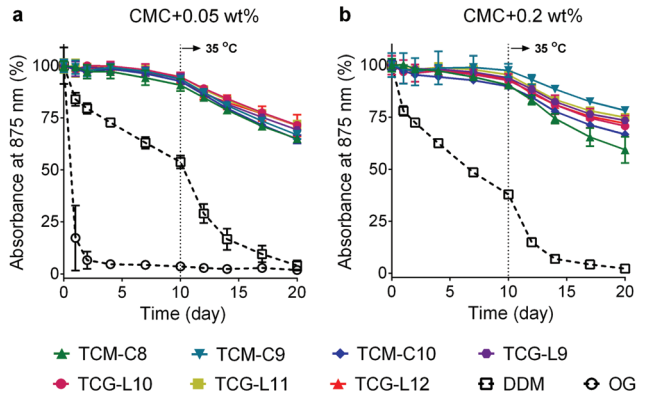

Fig. 5 Long-term stability of *R. capsulatus* superassembly comprising light harvesting complex I and reaction centre (LHI-RC) solubilized in individual detergents (DDM, OG, TCMs and TCG-Ls). Detergents were used at two different concentrations (CMC + 0.05 wt% (a) and CMC + 0.2 wt% (b)). Protein integrity was assessed by measuring the absorbance value at 875 nm (A_{875}) over the course of a 20-day incubation. The samples were incubated at 25 °C for the first 10-days, followed by another 10-day incubation at an elevated temperature of 35 °C, as indicated by the vertical dotted lines. The results are expressed as percentages of an absorbance value measured at the 0-day time point. Error bars, SEM, n = 2.

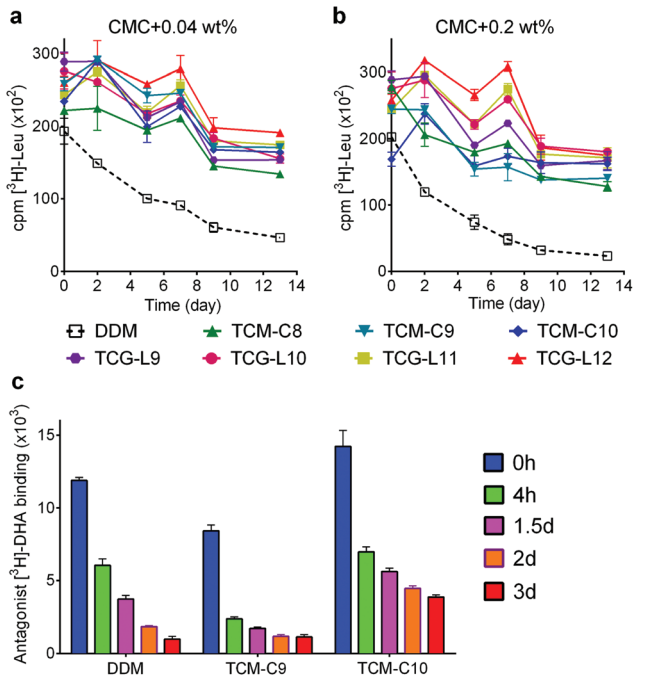

Fig. 6 Time course stability of LeuT (a and b) and β_2AR (c) solubilized in the novel detergents. A conventional detergent (DDM) was used as a control. For LeuT stability, TCMs and TCG-Ls were tested at CMCs + 0.04 wt% (a) and CMCs + 0.2 wt% (b) while two selected TCMs (TCM-C9 and TCM-C10) at CMCs + 0.2 wt% were used for β_2AR stability analysis (c). LeuT stability was assessed by monitoring the ability of the transporter to bind a radiolabeled substrate ([^{3}H]-leucine (Leu)) at regular intervals over a 13-day incubation at room temperature. Error bars, SEM, n = 2–3. β_2AR stability was assessed by measuring the ability of the receptor to bind a radio-labeled ligand ([^{3}H]-dihydroalprenolol (DHA)) at regular intervals over a 3-day incubation at room temperature. Error bars, SEM, n = 3.

is noteworthy that all the TCMs and TCG-Ls were far more effective than DDM and the TCGs at stabilizing the LHI–RC long-term.

These novel agents were further evaluated with the leucine transporter (LeuT), a prokaryotic homologue of the mammalian neurotransmitter/sodium symporter (NSS) family from *Aquifex aeolicus*.[36] The transporter was initially extracted from membranes with 1.0 wt% DDM and subsequently purified in 0.05 wt% of the same detergent. The DDM-purified LeuT was diluted into buffer solutions containing individual agents (TCGs/TCMs/TCG-Ls) or DDM to give final detergent concentrations of CMCs + 0.04 or CMCs + 0.2 wt%. We assessed protein stability by monitoring radiolabeled leucine ([^{3}H]-Leu) binding *via* scintillation proximity assay (SPA)[37] at regular intervals over a 13-day incubation at room temperature. At both detergent concentrations, the DDM-solubilized LeuT underwent a gradual loss in substrate binding ability over the incubation period (Fig. 6a and b). The TCG-solubilized transporters were substantially worse than DDM at maintaining transporter activity (Fig. S9†). The lipophilic length of this set (C5 to C7) seems to be too short to stabilize the transporter. In contrast, all the TCMs and TCG-Ls were markedly better than DDM at maintaining transporter stability long-term. There was no noticeable difference in detergent efficacy for protein stabilization between the TCMs and TCG-Ls under the conditions tested, but detergent efficacy tended to improve with increasing alkyl chain length: the best detergent was TCG-L12 and the worst detergent was TCM-C8. A similar detergent efficacy order was obtained when detergent concentrations were increased to CMCs + 0.2 wt%. Again, the TCMs and TCG-Ls were superior to DDM and the TCGs for LeuT stability over the course of the incubation. At this higher detergent concentration, however, the TCG-Ls appeared to be better than the TCMs, with the best performance observed for TCG-L12 (Fig. 6b). Although unclear in the TCM cases, detergent efficacy of the TCG-Ls for transporter stability tended to increase with increasing alkyl chain length at this high detergent concentration. Of the new agents, TCG-L12 with the longest alkyl chain was most effective at retaining the transporter activity at both concentrations (Fig. 6a and b).

We further evaluated the new agents using a G-protein coupled receptor (GPCR), the human β_2 adrenergic receptor (β_2AR).[38] The receptor was first extracted from the membranes using DDM and purified in the same detergent. The DDM-purified receptor was diluted in buffer solutions containing either the individual new agents or DDM. The final detergent concentrations were CMCs + 0.2 wt%. As a direct assessment of receptor stability in the individual agents, the ability of the receptor to bind the radioactive antagonist ([^{3}H]-dihydroalprenolol (DHA)) was measured.[39] Preliminary analysis obtained by measuring the initial ligand binding of the receptor following 30 min sample dilution indicated that only two compounds (TCM-C9 and TCM-C10) had detergent efficacy comparable to

that of DDM (Fig. S10†) with the rest of the amphiphiles showing reduced efficacy. Detergent efficacy for receptor stability tended to increase with increasing alkyl chain length, as observed for LeuT. For the two selected amphiphiles (TCM-C9 and TCM-C10), detergent efficacy was further investigated by monitoring ligand binding activity of the receptor at regular intervals over a 3-day incubation at room temperature (Fig. 6c). The DDM-solubilized receptor showed high initial activity, but a rapid loss in receptor activity was observed, giving only ~10% residual activity at the end of the incubation (day 3). TCM-C9 was a little worse than DDM at preserving receptor activity long term. However, TCM-C10 showed a more gradual decrease in receptor activity than DDM, retaining nearly 30% activity at the end of the incubation (Fig. 6c). Collectively, the detergent efficacy order for receptor stability was TCMs > TCG-Ls ≈ TCGs, with the best performance observed for TCM-C10.

Discussion

In this study, we introduced trehalose-cored glucosides (TCGs/TCG-Ls) or maltosides (TCMs) with four alkyl chains and multiple carbohydrate units. When evaluated with multiple membrane proteins, we found a large variation in detergent efficacy for protein stabilization depending on the target protein tested here. All three sets of the new agents were significantly better than DDM at stabilizing the LHI-RC complex. A similar result was observed with the TCMs and TCG-Ls for LeuT stability, but the TCGs were worse than DDM for this transporter. When the new agents were evaluated with β_2AR, only one new compound (TCM-C10) was better than DDM at stabilizing the receptor. The protein-specific nature of detergent efficacy observed here is consistent with a general notion that there is no single solution to effective maintenance of membrane proteins in aqueous solution. This is due to large variations in dimensions and properties of membrane proteins. For example, LHI-RC is known to be denaturation-sensitive while LeuT and β_2AR are prone to aggregation.[40] Despite the protein-specificity, it is notable that TCM-C10 displayed favorable behaviors for all three membrane proteins (LHI-RC, LeuT and β_2AR) tested here compared to DDM, a gold standard conventional detergent. TCG-L12 was very effective at stabilizing two of three membrane proteins (LHI-RC and LeuT). Detergent efficacy for protein stabilization tended to be strongly dependent on the alkyl chain length (Fig. S1†). In the case of LeuT, a detergent with a long alkyl chain was generally better than one with a short chain. The set of TCG-Ls with the relatively long alkyl chains (C11 to C12) was the best for LeuT stability, followed by the set of TCMs with an intermediate chain length (C8 to C10). The TCGs with the short alkyl chain (C5 to C7) were the least effective. Within each set of TCGs/TCMs/TCG-Ls, in addition, detergent efficacy for protein stabilization tended to improve with increasing alkyl chain length. A similar trend was found in detergent evaluation with β_2AR. Within individual detergent sets, particularly for the TCG-Ls, detergent efficacy for receptor stabilization was enhanced with increasing alkyl chain length. Due to a strong binding to the protein surface, a long alkyl chain detergent would be generally most suitable for membrane protein stability, especially for aggregation-susceptible proteins. This is consistent with the popular use of DDM, a C12 alkyl chain conventional detergent, compared to *n*-decyl-β-D-maltoside (DM) and OG for membrane protein research. However, this long alkyl chain preference for protein stability could not explain the inferior behavior of TCG-L11/L12 to TCM-C9/C10 for β_2AR stability observed here. This is because the detergent head group also plays an important role in detergent efficacy for protein stabilization. For β_2AR stability, the tetra-maltoside TCMs appeared to be better than the octa-glucoside TCG-Ls, as exemplified by the comparison of the TCM-C9/C10 *with* TCG-L9/L10. Despite having the same alkyl chain length, TCM-C9/C10 induced greater β_2AR stability than the TCG-L analogs. In the case of LeuT, the octa-glucoside detergents (TCG-Ls) appeared to be best for transporter stability, followed by tetra-maltoside TCMs and tetra-glucoside TCGs. Therefore, both the detergent alkyl chain length and head group identity are key players in stabilizing membrane proteins.

Most previous studies have demonstrated enhanced β_2AR stability in long alkyl chain detergents.[15,18,29] Thus, we expected that the TCG-Ls, particularly TCG-L11 and TCG-L12, would be better than the TCM-C9/C10 for β_2AR stability. This was our reasoning for the preparation of this third set of amphiphiles. However, the TCG-Ls were inferior to the TCMs for receptor stability, indicating the presence of an additional factor critical for detergent efficacy with respect to GPCR stability. An additional feature previously reported as favourable for β_2AR stability was a small CMC.[18,29] As the TCG-Ls and TCMs have very similar CMCs, this is unlikely to be the reason for the different receptor stabilities obtained. Notably, due to the large volume of the head group (*i.e.*, octa-glucoside), the TCG-Ls formed small micelles even in the case of the C12 alkyl chain detergent (TCG-L12; 3.2 nm), significantly deviating from the tetra-maltoside TCMs which formed substantially larger self-assemblies (20–25 nm). Thus, the small micelle size is likely to be responsible for the poor receptor stability seen in the TCG-Ls. When micelle sizes of the TCG-Ls were increased from 2.9 (TCG-L9) to 3.0 (TCG-L10) to 3.1 (TCG-L11) to 3.2 nm (TCG-L12), these linker-bearing agents showed rapidly enhanced receptor stability, further supporting the importance of the micelle size in receptor stability. As small micelles are less able to shield the hydrophobic surfaces of membrane proteins, a small micelle-forming detergent might be suboptimal at stabilising membrane proteins, particularly aggregation-prone ones. Small LDAO and OG micelles are typically far less effective than relatively large DDM micelles at stabilising membrane proteins. Thus, one way to improve TCG-L efficacy for β_2AR stabilization is to use a branched alkyl chain instead of a straight chain, which will increase the volume of the detergent hydrophobic group, thereby resulting in enlargement of the TCG-L micelles. Based on our analysis of the current results, at least four key factors (*i.e.*, alkyl chain length,

head group identity, CMC and micelle size) must be considered when designing a novel detergent for membrane protein study. Because multiple factors need to be incorporated into detergent structures, it is challenging to develop a novel detergent with optimal behaviour for multiple membrane proteins.

Conclusions

We have prepared UV-transparent trehalose-cored glucosides and maltosides by taking advantage of a modular synthetic approach. By systematic variation of the head group, we prepared new detergents with carbon chain lengths varying from C5 to C12. Of the novel amphiphiles, TCM-C10 and TCG-L12 in particular conferred enhanced stability to multiple membrane proteins, suggesting that these agents could be alternatives to conventional detergents for studying membrane proteins. In addition, the systematic structural variation allowed us to pinpoint multiple detergent characteristics necessary for stabilizing many different membrane proteins with diverse characteristics. The new chemical tools and detergent design principles introduced here will advance membrane protein research.

Conflicts of interest

The authors declare the following competing financial interest (s): P. S. C. and M. D. are inventors on a patent application (number: 10-2018-0155778) that covers the TCGs, TCMs and TCG-Ls.

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Notes and references

1 E. Wallin and G. von Heijne, *Protein Sci.*, 1998, **7**, 1029–1038.
2 (*a*) J. Drews, *Science*, 2000, **287**, 1960–1964; (*b*) D. S. Wishart, C. Knox, A. C. Guo, S. Shrivastava, M. Hassanali, P. Stothard, Z. Chang and J. Woolsey, *Nucleic Acids Res.*, 2006, **34**, D668–D672.
3 (*a*) C. R. Sanders and J. K. Myers, *Annu. Rev. Biophys. Biomol. Struct.*, 2004, **33**, 25–51; (*b*) J. P. Overington, B. Al-Lazikani and A. L. Hopkins, *Nat. Rev. Drug Discovery*, 2006, **5**, 993–996.
4 (*a*) H. Michel, in *Crystallization of Membrane Proteins*, ed. H. Michel, CRC Press, Boca Raton, Florida, 1991, ch. 3, pp. 73–88; (*b*) H. Michel, Crystallization of membrane proteins, in *International Tables for Crystallography Volume F: Crystallography of Biological Macromolecules*, ed. M. Rossmann and E. Arnold, Kluwer Academic Publishers, Dordrecht, 2001, pp. 94–100.
5 (*a*) R. M. Garavito and S. Ferguson-Miller, *J. Biol. Chem.*, 2001, **276**, 32403–32406; (*b*) C. K. Engel, L. Chen and G. G. Privé, *Biochim. Biophys. Acta*, 2002, **1564**, 47–56; (*c*) S. Lee, A. Mao, S. Bhattacharya, N. Robertson, R. Grisshammer, C. G. Tate and N. Vaidehi, *J. Am. Chem. Soc.*, 2016, **138**, 15425–15433.
6 M. le Maire, P. Champeil and J. V. Moller, *Biochim. Biophys. Acta*, 2000, **1508**, 86–111.
7 C. Tribet, R. Audebert and J. L. Popot, *Proc. Natl. Acad. Sci. U. S. A.*, 1996, **93**, 15047–15050.
8 D. J. K. Swainsbury, S. Scheidelaar, R. van Grondelle, J. A. Killian and M. R. Jones, *Angew. Chem., Int. Ed.*, 2014, **53**, 11803–11807.
9 C.-L. McGregor, L. Chen, N. C. Pomroy, P. Hwang, S. Go, A. Chakrabartty and G. G. Prive, *Nat. Biotechnol.*, 2003, **21**, 171–176.
10 H. Tao, S. C. Lee, A. Moeller, R. S. Roy, F. Y. Siu, J. Zimmermann, R. C. Stevens, C. S. Potter, B. Carragher and Q. Zhang, *Nat. Methods*, 2013, **10**, 759–761.
11 (*a*) P. S. Chae, *et al.*, *Nat. Methods*, 2010, **7**, 1003–1008; (*b*) P. S. Chae, R. R. Rana, K. Gotfryd, S. G. F. Rasmussen, A. C. Kruse, K. H. Cho, S. Capaldi, E. Carlsson, B. Kobilka, C. J. Loland, U. Gether, S. Banerjee, B. Byrne, J. K. Lee and S. H. Gellman, *Chem. Commun.*, 2013, **49**, 2287–2289; (*c*) K. H. Cho, H. E. Bae, M. Das, S. H. Gellman and P. S. Chae, *Chem. – Asian J.*, 2014, **9**, 632–638.
12 (*a*) Q. Zhang, X. Ma, A. Ward, W.-X. Hong, V.-P. Jaakola, R. C. Stevens, M. G. Finn and G. Chang, *Angew. Chem., Int. Ed.*, 2007, **46**, 7023–7025; (*b*) S. C. Lee, B. C. Bennett, W.-X. Hong, Y. Fu, K. A. Baker, J. Marcoux, C. V. Robinson, A. B. Ward, J. R. Halpert, R. C. Stevens, C. D. Stout, M. J. Yeager and Q. Zhang, *Proc. Natl. Acad. Sci. U. S. A.*, 2013, **110**, E1203–E1211.
13 H. Hussain, Y. Du, N. J. Scull, J. S. Mortensen, J. Tarrasch, H. E. Bae, C. J. Loland, B. Byrne, B. K. Kobilka and P. S. Chae, *Chem. – Eur. J.*, 2016, **22**, 7068–7073.
14 A. Sadaf, J. S. Mortensen, S. Capaldi, E. Tikhonova, P. Hariharan, O. Ribeiro, C. J. Loland, L. Guan, B. Byrne and P. S. Chae, *Chem. Sci.*, 2016, **7**, 1933–1939.
15 H. Hussain, Y. Du, E. Tikhonova, J. S. Mortensen, O. Ribeiro, C. Santillan, M. Das, M. Ehsan, C. J. Loland, L. Guan, B. K. Kobilka, B. Byrne and P. S. Chae, *Chem. – Eur. J.*, 2017, **23**, 6724–6729.
16 M. Ehsan, Y. Du, N. J. Scull, E. Tikhonova, J. Tarrasch, J. S. Mortensen, C. J. Loland, G. Skiniotis, L. Guan, B. Byrne, B. K. Kobilka and P. S. Chae, *J. Am. Chem. Soc.*, 2016, **138**, 3789–3796.
17 M. Ehsan, Y. Du, I. Molist, A. B. Seven, P. Hariharan, J. S. Mortensen, L. Ghani, C. J. Loland, G. Skiniotis, L. Guan, B. Byrne, B. K. Kobilka and P. S. Chae, *Org. Biomol. Chem.*, 2018, **16**, 2489–2498.
18 (*a*) M. Das, Y. Du, J. S. Mortensen, O. Ribeiro, P. Hariharan, L. Guan, C. J. Loland, B. K. Kobilka, B. Byrne and

P. S. Chae, *Chem. Sci.*, 2017, **8**, 1169–1177; (*b*) M. Das, Y. Du, O. Ribeiro, P. Hariharan, J. S. Mortensen, D. Patra, G. Skiniotis, C. J. Loland, L. Guan, B. K. Kobilka, B. Byrne and P. S. Chae, *J. Am. Chem. Soc.*, 2017, **139**, 3072–3081.

19 (*a*) D. M. Rosenbaum, C. Zhang, J. Lyons, R. Holl, D. Aragao, D. H. Arlow, S. G. F. Rasmussen, H.-J. Choi, B. T. DeVree, R. K. Sunahara, P. S. Chae, S. H. Gellman, R. O. Dror, D. E. Shaw, W. I. Weis, M. Caffrey, P. Gmeiner and B. K. Kobilka, *Nature*, 2011, **469**, 236–240; (*b*) K. Haga, A. C. Kruse, H. Asada, T. Yurugi-Kobayashi, M. Shiroishi, C. Zhang, W. I. Weis, T. Okada, B. K. Kobilka, T. Haga and T. Kobayashi, *Nature*, 2012, **482**, 547–551; (*c*) J. F. White, N. Noinaj, Y. Shibata, J. Love, B. Kloss, F. Xu, J. Gvozdenovic-Jeremic, P. Shah, J. Shiloach, C. G. Tate and R. Grisshammer, *Nature*, 2012, **490**, 508–513; (*d*) A. C. Kruse, A. M. Ring, A. Manglik, J. Hu, K. Hu, K. Eitel, H. Hubner, E. Pardon, C. Valant, P. M. Sexton, A. Christopoulos, C. C. Felder, P. Gmeiner, J. Steyaert, W. I. Weis, K. C. Garcia, J. Wess and B. K. Kobilka, *Nature*, 2013, **504**, 101–106; (*e*) P. S. Miller and A. R. Aricescu, *Nature*, 2014, **512**, 270–275; (*f*) E. Karakas and H. Furukawa, *Science*, 2014, **344**, 992–997; (*g*) V. Kane Dickson, L. Pedi and S. B. Long, *Nature*, 2014, **516**, 213–218; (*h*) A. K. Shukla, G. H. Westfield, K. Xiao, R. I. Reis, L.-Y. Huang, P. TripathiShukla, J. Qian, S. Li, A. Blanc, A. N. Oleskie, *et al.*, *Nature*, 2014, **512**, 218–222.

20 H. Tao, Y. Fu, A. Thompson, S. C. Lee, N. Mahoney, R. C. Stevens and Q. Zhang, *Langmuir*, 2012, **28**, 11173–11181.

21 (*a*) R. D. Lins, C. S. Pereira and P. H. Hunenberger, *Proteins: Struct., Funct., Genet.*, 2004, **55**, 177–186; (*b*) M. Sakurai, Biological Functions of Trehalose as a Substitute for Water, in *Water and Biomolecules*, ed. K. Kuwajima, Y. Goto, F. Hirata, M. Kataoka and M. Terazima, Springer, Berlin, Heidelberg, 2009, pp. 219–240.

22 K. M. O'Neill, P. F. Brent, O. D. Claire, W. J. Peter and M. S. Benjamin, *Pure Appl. Chem.*, 2017, **89**, 1223–1249.

23 L. Simeone, D. Milano, L. De Napoli, C. Irace, A. Di Pascale, M. Boccalon, P. Tecilla and D. Montesarchio, *Chem. – Eur. J.*, 2011, **17**, 13854–13865.

24 Y. Mizuno, P. Carninci, Y. Okazaki, M. Tateno, J. Kawai, H. Amanuma, M. Muramatsu and Y. Hayashizaki, *Nucleic Acids Res.*, 1999, **27**, 1345–1349.

25 S. Ohtake and Y. Wang, *J. Pharm. Sci.*, 2011, **100**, 2020–2053.

26 R. Matar-Merheb, M. Rhimi, A. Leydier, F. Huché, C. Galián, E. Desuzinges-Mandon, D. Ficheux, D. Flot, N. Aghajari, R. Kahn, A. D. Pietro, J.-M. Jault, A. W. Coleman and P. Falson, *PLoS One*, 2011, **6**, e18036.

27 (*a*) D. T. McQuade, M. A. Quinn, S. M. Yu, A. S. Polans, M. P. Krebs and S. H. Gellman, *Angew. Chem., Int. Ed.*, 2000, **39**, 758–761; (*b*) P. S. Chae, M. J. Wander, A. P. Bowling, P. D. Laible and S. H. Gellman, *ChemBioChem*, 2008, **9**, 1706–1709; (*c*) P. S. Chae, A. C. Kruse, K. Gotfryd, R. R. Rana, K. H. Cho, S. G. F. Rasmussen, H. E. Bae, R. Chandra, U. Gether, L. Guan, B. K. Kobilka, C. J. Loland, B. Byrne and S. H. Gellman, *Chem. – Eur. J.*, 2013, **19**, 15645–15651.

28 P. S. Chae, M. J. Wander, K. H. Cho, P. D. Laible and S. H. Gellman, *Mol. BioSyst.*, 2013, **9**, 626–629.

29 K. H. Cho, Y. Du, N. J. Scull, P. Hariharan, K. Gotfryd, C. J. Loland, L. Guan, B. Byrne, B. K. Kobilka and P. S. Chae, *Chem. – Eur. J.*, 2015, **21**, 10008–10013.

30 G. H. Veeneman, S. H. Leettwen and J. H. Boom, *Tetrahedron Lett.*, 1990, **31**, 1131–1134.

31 A. Chattopadhyay and E. London, *Anal. Biochem.*, 1984, **139**, 408–412.

32 A. Stetsenko and A. Guskov, *Crystals*, 2017, **7**, 197.

33 R. C. Oliver, J. Lipfert, D. A. Fox, R. H. Lo, S. Doniach and L. Columbu, *PLoS One*, 2013, **8**, e62488.

34 P. D. Laible, C. Kirmaier, C. S. Udawatte, S. J. Hofman, D. Holten and D. K. Hanson, *Biochemistry*, 2003, **42**, 1718–1730.

35 D. C. Youvan, S. Ismail and E. J. Bylina, *Gene*, 1985, **33**, 19–30.

36 (*a*) G. Deckert, P. V. Warren, T. Gaasterland, W. G. Young, A. L. Lenox, D. E. Graham, R. Overbeek, M. A. Snead, M. Keller, M. Aujay, R. Huber, R. A. Feldman, J. M. Short, G. J. Olsen and R. V. Swanson, *Nature*, 1998, **392**, 353–358; (*b*) A. Yamashita, S. K. Singh, T. Kawate, Y. Jin and E. Gouaux, *Nature*, 2005, **437**, 215–223.

37 M. Quick and J. A. Javitch, *Proc. Natl. Acad. Sci. U. S. A.*, 2007, **104**, 3603–3608.

38 D. M. Rosenbaum, V. Cherezov, M. A. Hanson, S. G. Rasmussen, F. S. Thian, T. S. Kobilka, H. J. Choi, X. J. Yao, W. I. Weis, R. C. Stevens and B. K. Bobilka, *Science*, 2007, **318**, 1266–1273.

39 (*a*) S. E. Mansoor, H. S. McHaourab and D. L. Farrens, *Biochemistry*, 2002, **41**, 2475–2484; (*b*) X. Yao, C. Parnot, X. Deupi, V. R. P. Ratnala, G. Swaminath and D. Farrens, *Nat. Chem. Biol.*, 2006, **2**, 417–422; (*c*) G. Swaminath, J. Steenhuis, B. Kobilka and T. W. Lee, *Mol. Pharmacol.*, 2002, **61**, 65–72.

40 K. H. Cho, M. Husri, A. Amin, K. Gotfryd, H. J. Lee, J. Go, J. W. Kim, C. J. Loland, L. Guan, B. Byrne and P. S. Chae, *Analyst*, 2015, **140**, 3157–3163.