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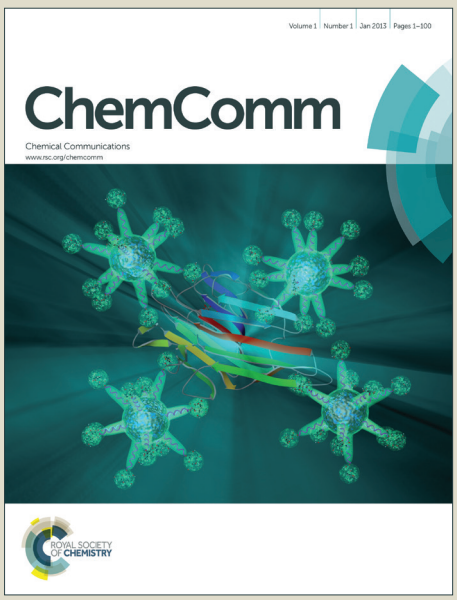

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Adamantane-based Amphiphiles (ADAs) for Membrane Protein Study: Importance of Detergent Hydrophobic Group in Membrane Protein Solubilisation

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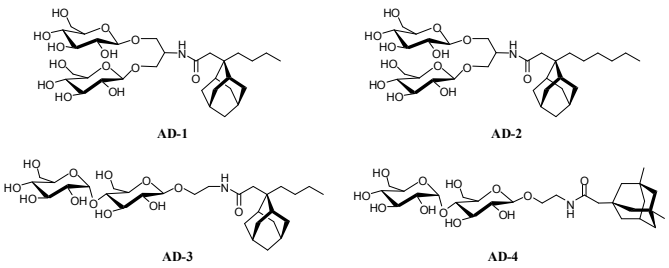
We prepared adamantane-containing amphiphiles and evaluated with a large membrane protein complex in terms of protein solubilisation and stabilization efficacy. These agents were superior to conventional detergents, especially in terms of the membrane protein solubilisation efficiency, implying a new detergent structure-property relationship.

Detergents are essential components in membrane protein research because these amphipathic agents have an ability to associate with membrane proteins to form protein-detergent complexes (PDCs).[1] These molecules tend to form self-assemblies called micelles in an aqueous medium and are responsible for PDC formation.[2] These agents are not only used to extract/solubilize membrane proteins from membranes, but also supply membrane-mimetic environments for membrane protein stabilization in the subsequent processes of protein purification and crystallization. Despite a large number of detergent studies, information regarding detergent structure-property relationship is seriously limited. By closely inspecting detergent results, however, we could extract some ideas thanks to the availability of a large number of statistical reports.[3] Despite the existence of many conventional detergents, it is notable that only a few have been successfully used for membrane protein structural studies as exemplified by LDAO (lauryldimethylamine–*N*–oxide), OG (*n*–octyl–β–D–glucopyranoside) and DDM (*n*–dodecyl– β–D–maltopyranoside).[4] LDAO and OG are known to be inferior to maltoside-bearing detergents (e.g., DDM) in membrane protein stabilization, but these agents are superior in terms of membrane protein solubilisation efficiency,[5] indicating that membrane protein solubilisation efficiency could be a critical detergent property, along with membrane protein stabilization efficacy, for successful membrane protein crystallization. A similar conclusion can be reached by analyzing the behaviors of novel amphiphiles. Many novel classes that have invented so far include amphipols,[6a-c] tripod amphiphiles (TPAs),[6d-g] hemifluorinated surfactants (HFSs),[6h,i] peptide-based agents (e.g., lipopeptide detergents (LPDs), β-peptides and short peptides),[6j-l] nano-assemblies (e.g., nanodiscs (NDs) and nanolipodisq),[6m,n] facial amphiphiles (FAs),[6o,p] rigid hydrophobic group-bearing amphiphiles (chobimalt and glycosylated diosgenin-based amphiphile (GDN)), [6q,r] glucose or maltose neopentyl glycols (GNGs or MNGs)[6s-v] and calixarene-based surfactants.[6w] In general, novel amphiphiles possessing favorable protein solubilisation efficiency (e.g., FAs, TPAs, MNGs and GNGs) were successful in membrane protein structural study.[6o,7a-o] Thus, here we focus on the membrane protein solubilisation along with the protein stabilization in the evaluation of new agents. First, we designed and prepared several adamantane (AD)-based amphiphiles with a carbohydrate hydrophilic group. Then, we evaluated their protein solubilisation and stabilization efficacy for a large, multi-subunit complex. In addition to a reasonably good protein stabilization efficacy, these new agents were particularly superior to their conventional counterparts in membrane protein solubilisation, indicating the favorable role of the adamantane group in detergent behaviors.



Scheme 1. Chemical structures of newly prepared adamantane-based amphiphiles (AD-1, AD-2, AD-3 and AD-4). AD-1 shares a branched diglucoside group with AD-2, and AD-3 shares a maltoside group with AD-4.

New amphiphiles with an adamantane ring were designated AD-1, AD-2, AD-3 and AD-4 according to variations in the hydrophilic and hydrophobic groups (**Scheme 1**). AD-1 and AD-2 share a branched diglucoside head group, but are different in their hydrophobic moieties, possessing butyl and hexyl chain appendages from the adamantane ring, respectively. The same hydrophobic group of AD-1 was introduced to generate AD-3, but with a maltoside head group. AD-4 contains a unique hydrophobic group:

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an adamantane ring with two additional methyl groups at the C3 and C5 positions. Thus, AD-3 and AD-4 are hydrophobic variants sharing a maltoside head group. The critical micelle concentrations (CMCs) and micelle size of the new detergents were estimated using a hydrophobic fluorescent dye, diphenylhexatriene (DPH),[8] and dynamic light scattering (DLS), respectively. The summarized data for the four AD agents and two conventional detergents (DDM and LDAO) are presented in **Table S1**. For further comparison, two previously reported MPA-2 variants, MPA-2(C12) and MPA-2 (C14) (**Fig. S1**), are also included in the table. The CMC values of the AD agents are unexpectedly high compared to their conventional counterparts with a similar number of carbon units. For example, AD-1 and MPA-2 (C14) share a branched diglucoside head group, but vary in their hydrophobic groups, having C15 and C14 units, respectively. By considering the higher number of carbon atoms in the lipophilic region, AD-1 is expected to have a lower CMC value than MPA-2 (C14). However, the CMC value of AD-1 was estimated to be ~40 times higher than that of MPA-2 (C14) (~6.4 mM *vs.* ~0.17 mM). This data indicates that the self-aggregation behavior of this AD agent is largely influenced by the presence of the adamantane ring; this large hydrophobic group cannot fit well into the congested region of detergent micelle interior.[9] Micelles formed by AD agents were variable in terms of their hydrodynamic radii (R_h), depending on the headgroup; branched diglucoside-bearing agents (AD-1 and AD-2) tend to form micelles as small as DDM micelles whereas maltoside-bearing agents (AD-3 and AD-4) form larger micelles than does DDM. The large micelle sizes of the latters originate from their molecular shapes; maltoside-bearing AD agents have a molecular geometry close to a cylinderical shape.[10]

For the evaluation of the new amphiphiles, we utilized a engineered photosynthetic superassembly from *Rhodobacter* (*R.*) *capsulatus*, comprised of reaction centers (RCs) and light-harvesting complex I (LHI).[11] As a start, *R. capsulatus* membranes enriched in LHI-RC complexes were treated with individual detergents at various concentrations depending on their CMCs; the low CMC detergents (AD-2 and AD-3) were used at 10xCMCs while the high CMC detergents (AD-1 and AD-4) were used at 5xCMC and 2xCMC, respectively. Two conventional detergents (DDM and LDAO) were also included for comparison and used at 50xCMC and 10xCMC, respectively. After detergent treatment, cellular debris and insolubilized membrane fragments were isolated as a pellet via ultracentrifugation. The pellets were suspended in an aqueous buffer and their spectra were taken in order to quantify the amounts of the insolubilized complexes (**Fig. S2**). The conformational states of the detergent-solubilized complexes were assessed by taking the UV-visible spectra of the supernatant solutions (**Fig. 1a**).[6e] In a previous study, DDM was found to be the most successful amongst dozens of conventional detergents in terms of its solubilisation efficiency and stabilization efficacy for the superassembly.[6e] As shown in **Fig. 1a**, AD-1 and AD-2 possessing a branched diglucoside group maintained the native conformation of the complex just as effectively as DDM in the course of protein solubilisation. In contrast, these agents showed a substantial difference in their membrane protein solubilisation efficiency. As seen in **Fig. S2**, AD-1 solubilized LHI-RC less efficiently than DDM (~70% *vs.* ~75%) but AD-2 demonstrated increased efficiency (~80%; **Table 1**). The increased hydrophobicity of AD-2 would be responsible for this enhanced behavior; AD-2 has an additional two-carbon unit when compared to AD-1. Of note, these new agents (AD-1 and AD-2) are the hydrophobic variants of previously reported MPA-2 analogs.[6e] When we compare these hydrophobic group variants in terms of their solubilisation efficiencies, the AD agents are clearly superior to the MPA-2 analogs (~70% to ~80% *vs.* ~30%).[6e] This intriguing result prompted us to evaluate two maltoside-bearing AD agents (AD-3 and AD-4). The use of AD-3, that is the hydrophilic group variant of AD-1, significantly increased the protein solubilisation yield (~90%), which is better than that of DDM in this context. When we turned to AD-4 containing two additional methyl groups at the C3 and C5 positions of the adamantane ring, we found that this agent could extract the superassembly almost quantitatively, with efficiency comparable to LDAO. While LDAO destroyed most LHI complexes, the AD-4-solubilized superassembly underwent only partial structural degradation in the course of protein solubilisation. These results show that AD-4 has both enhanced stabilization efficacy and solubilisation efficiency comparable to LDAO. However, these maltoside-bearing agents (AD-3 and AD-4) were inferior to DDM in the protein stabilization efficacy. These comparisons (AD-1/AD-2 *vs.* MPA-2s and AD-3/AD-4 *vs.* DDM) clearly demonstrate the favorable role of the adamantane rings for membrane protein solubilisation efficiency.

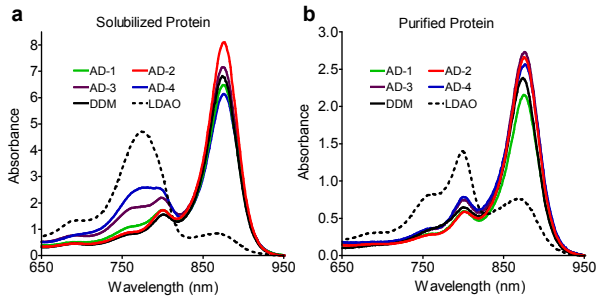

Figure 1. Absorbance spectra of *R. capsulatus* superassembly (a) solubilized and (b) purified in AD agents (AD-1, AD-2, AD-3 and AD-4) and two conventional detergents (DDM and LDAO). The detergents were used at different concentrations due to the large variation in their CMC values (50xCMC for DDM, 10xCMC for AD-2, AD-3 and LDAO, 5xCMC for AD-1 and 2xCMC for AD-4). Protein purification was performed via Ni-NTA affinity column chromatography by the use of an elution buffer including 1xCMC detergent and 1.0 M imidazole.

The individual detergent-solubilized complexes were subjected to the next step of protein purification. After purification via immobilized metal affinity chromatography (IMAC), we obtained the absorption spectra of these high purity samples to investigate detergent efficacy over the course of protein purification (**Fig. 1b**). Overall, the spectral shapes for these samples are similar to those of the detergent-solubilized protein samples, indicating the consistency in the relative order of detergent stabilization efficacy during protein solubilisation and purification. However, scrutinizing these spectra reveals that the extent of protein denaturation in the AD-3 and AD-4-purified samples is significantly reduced following protein purification. This is indicated by decreases in the peaks at both ~760 nm and ~800 nm in the spectra of the detergent-purified proteins. These spectral changes in the purified protein samples can be in part explained by the restoration of the native complex conformations over the course of protein purification. The reduction in detergent concentrations in the purification process is likely responsible for

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such conformational restoration; the use of excess detergent micelles is detrimental to membrane protein stability.[12]

It is noteworthy that only a small number of novel amphiphiles were shown to be superior to conventional detergents in terms of membrane protein solubilisation efficiency. One representative is TPA-class materials with three alkyl chains in their lipophilic region. The superior performance of this class was well supported by the comparative studies with their conventional counterparts. For instance, a TPA with an *N*-oxide head group (TRIPAO) was more efficient than LDAO in the solubilisation of bacteriorhodopsin (bR).[7a] Glyco-TPAs (TPA-2 and TPA-4) solubilized LHI-RC complexes more efficiently than their conventional counterparts (monopod analogs (MPA-2s; **Fig. S1**) and DDM, respectively).[6e] These data indicate the advantageous effect of TPA architecture over the conventional one on membrane protein solubilisation. Such favorable solubilisation behaviors were also observed for current AD agents whose architecture deviates significantly from that of TPAs. The AD-containing amphiphiles displayed enhanced solubilisation behaviors, to the point that their solubilisation efficiencies are comparable to those of TPAs. For example, the branched diglucoside-bearing AD agents (AD-1 and AD-2; ~70% and ~80%, respectively) were as efficient as TPA counterparts (TPA-2, TPA-2-S and TPA-8; ~50%, ~ 70%, and ~80%, respectively) in the solubilisation of LHI-RC complexes (**Fig. S3**).[6e,g] A similar result was observed for the maltoside-bearing agents; AD-3 and AD-4 were comparable to TPA-4 (~90% and ~100% *vs.* ~95%) (**Fig. S3**). It is impossible to know the precise reason for the enhanced behavior of AD agents, but the high hydrophobic density and/or a large protein-contacting area of an adamantine ring are believed to be responsible for this superiority (see supplementary information and **Fig. S4** for details). We could not find correlations between detergent solubilisation efficiency and detergent properties such as micelle size and CMC value (see supplementary information for details). Based on the comparative analyses described above, we suggest that the incorporation of an adamantane into detergent hydrophobic group is an alternative strategy to enhance detergent efficiency for membrane protein solubilisation. The detergent structure-property relationship found here provides a useful guideline in designing novel amphiphiles.

In conclusion, for a photosynthetic superassembly, four adamantane-based amphiphiles (AD-1, AD-2, AD-3 and AD-4) showed enhanced efficiencies in protein solubilisation relative to conventional detergents, along with reasonably good stabilization efficacy. Thus, these adamantane-bearing amphiphiles are likely to find use in membrane protein structural study. More importantly, the current study enabled us to suggest the key features of detergent hydrophobic groups for efficient protein solubilisation, which will help the rational design of novel amphiphiles in the future.

Notes and references

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