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Synthesis and lyotropic phase behavior of novel nonionic surfactants for the crystallization of integral membrane proteins

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Abstract—A series of new sugar-based nonionic surfactants have been synthesized and their lyotropic liquid crystalline properties characterized. When in contact with water, these surfactants formed a range of lyotropic liquid crystalline phases, including cubic, hexagonal, and lamellar, as well as a separate micellar phase. These are features that have promise for the crystallization of integral membrane proteins.

Up to 30% of the genome of eukaryotic organisms is comprised of integral membrane proteins. These proteins perform some of the most fundamental cellular processes yet only around 150 integral membrane proteins have been crystallized and their structures determined.[1] The term 'integral membrane protein' (IMP) is a practical one defined by the protein's relationship with surfactants—an IMP requires detergent for membrane extraction and protein crystallization. The precise nature of this interaction between the protein and detergent is critical for the study of IMPs, but not well understood. Greater insight into this interaction should allow the rational choice or design of detergents with properties appropriate for the crystallization of particular IMP systems. Only a few classes of detergent have been of general utility in the crystallization of IMPs; alkyl polyoxyethylenes, zwitterionic surfactants, glucosides, and maltosides.[2] These detergents possess a range of properties but there is no clear indication of why these compounds in particular have proved to be successful.[3] An important physical property is the formation of lyotropic liquid crystalline phases, which are known to facilitate the crystallization of IMPs and their subsequent structure determination.[4] Both bicontinuous cubic (V_1) and lamellar (L_α) lyotropic liquid crystalline phases have been employed to crystallize IMPs; lamellar phases are especially good for the reconstitution and stabilization of IMPs as they more closely mimic the natural bilayer environment than micellar systems.[5,6]

Our aim was to synthesize new classes of nonionic surfactants for the stabilization and crystallization of integral membrane proteins. Nonionic surfactants are known as mild detergents and have minimal influence on protein conformation. We also wished to avoid amidic protons that can participate in hydrogen bonding with the amide backbone of an IMP, which can lead to protein denaturation. The surfactant should also have a low critical micelle concentration (CMC) to promote interaction with the IMP at low surfactant concentrations. Moreover, we aimed to avoid surfactants that incorporated bulky alkyl chains, such as monoolein, as we felt that large alkyl chains prevent close approach of the hydrophilic headgroups of the IMP, which ultimately determine the crystal packing of the crystallized IMP/surfactant mixture.[3] Gemini surfactants fulfill the latter two criteria particularly well, as the combination of two relatively short alkyl chains can keep the CMC low whilst maintaining a compact size. Drawing inspiration from gemini surfactants and established saccharide-based detergents, we decided to use a sugar core as the headgroup of our surfactants appended with two short lipidic chains. The alkylation of either a D-mannitol or a D-glucose core should leave enough hydroxyl groups exposed to maintain sufficient hydrophilicity to give water solubility (Schemes 1 and 2). Compounds like these are available in a few simple steps from commercially available starting materials, enabling

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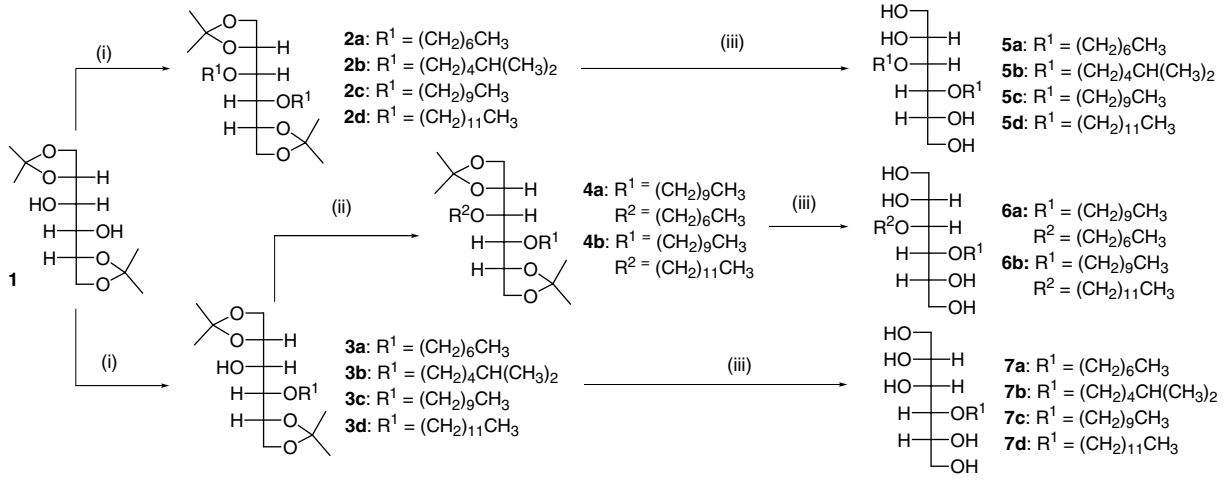
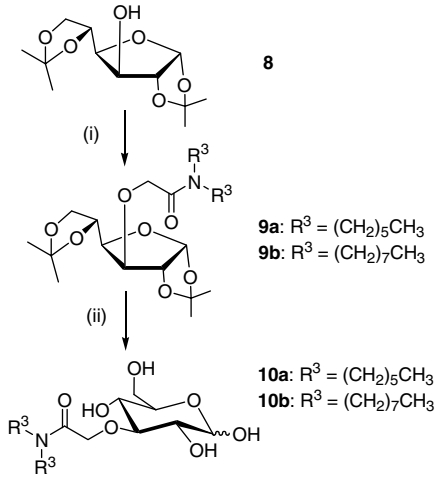


Scheme 1. Reagents and conditions: (i) KOH, R^1–Br, DMSO/toluene 4:1; (ii) KOH, R^2–Br, DMSO/toluene 4:1; (iii) Amberlyst® 15, EtOH–H_2O 95:5.



Scheme 2. Reagents and conditions: (i) $BrCH_2C(O)N(R^3)_2$, KOH, 4:1 toluene/DMSO; (ii) 90% CF_3CO_2H, H_2O.

rapid synthesis and screening of a range of surfactants with different physical properties. Similar compounds are known to display some of the properties important for the crystallization of IMPs; 3,4-*O*-diheptyl-D-mannitol (**5a**) has a CMC value of 63 μmol/L, while 3,4-*O*-dinonyl-D-mannitol and 3-*O*-nonyl-D-mannitol are known to have similarly low CMC values.[7]

The D-mannitol range of surfactants were synthesized from commercially available di-*O*-isopropylidene protected D-mannitol, which was deprotonated with potassium hydroxide, then stirred overnight with a slight excess (2.2 equiv) of either the heptyl, 5-methylhexyl, decyl, or dodecyl bromides in mixed DMSO/toluene. The chain lengths of these alkyl bromides were selected so all of the resultant lipids should have low CMC values. This initial alkylation step yields both the mono- and dialkylated species, each of which was isolated and purified by column chromatography. The isolation of the monoalkylated species made it possible to make surfactants with mixed chains by treating these monoalkylated mannitols with a different alkyl bromide in a subsequent alkylation step (Scheme 1). It was hoped that mixing alkyl chains of different lengths within the same mannitol lipids would disrupt packing within the crystalline phase, increasing solubility in water yet maintaining low CMC values. Deprotection of these intermediate mono- and dialkylated compounds with wet acidic Amberlyst® resin required stirring for an extended period of time at elevated temperatures (2 days at 60 °C), as it was found that incompletely deprotected mixtures were difficult to purify. Thus, provided this deprotection step was monitored closely over the 2 days, compounds **5a**–**7d** could all be obtained as pure white solids simply by removing the solvent under reduced pressure.

The solubility properties of the mono- and dialkylated D-mannitol surfactants were determined in distilled water. Despite the presence of four hydroxyl groups, dialkylated compounds **5a**–**d** and **6a**,**b** were insoluble in water at room temperature even at 0.1% wt/wt. However, the monoalkylated compounds **7a**–**d** had much improved solubility properties; **7a** and **7b** were soluble at 1% wt/wt and the longer chain analogues soluble between 1% and 0.1% wt/wt. Through surface tension measurements, the CMC values for **7a**–**d** were estimated as 50, 17, 1, and 1 mmol/L, respectively, though accurate determinations were limited by compound availability (**7a** and **7b**) or solubility (**7c** and **7d**). The lyotropic phase behavior with water was then determined for the D-mannitol surfactants using the contact preparation method.[8] Dialkylated compounds **5a**–**d** and **6a**,**b** were observed through crossed polarizers on a hot-stage microscope as the temperature was slowly increased. Initially the compounds were subjected to a heating and cooling cycle in the absence of water to ascertain their thermotropic liquid crystalline behavior. A drop of distilled water was then added and the heating/cooling cycle repeated to observe the lyotropic liquid crystalline behavior. Compounds **5a**–**d** and **6a**,**b** showed sharp melting points upon heating and some melting point depression upon cooling (Table 1), but no liquid crystallinity. Interestingly, the mixed heptyl/decyl man-

Table 1. Melting behavior observed for surfactants **5a**–**10b**

Compound	Melting temperature (neat)/°C	Crystallization temperature (neat)/°C	Melting temperature (with H_2O)/°C	Solubility at 25 °C (wt/wt in water)
5a	88	78	65	<0.1%
5b	89	<50	67	<0.1%
5c	94	87	82	<0.1%
5d	97	89	79	<0.1%
6a	75	<35	53	<0.1%
6b	66	63	61	<0.1%
7a	84	75	—[b]	>1%
7b	75	—[a]	—[b]	>1%
7c	—[b]	—[b]	—[b]	> 0.1%, <1%
7d	—[b]	—[b]	—[b]	> 0.1%, <1%
10a	—[c]	—[c]	57–65	> 0.1%, <1%
10b	—[c]	—[c]	88	> 0.1%, <1%

[a] Not determined.
[b] Liquid crystalline phases observed.
[c] An oil at room temperature.

nitol (**6a**) melts and recrystallizes at a lower temperature than either the diheptyl (**5a**) or the didecyl (**5c**) mannitol compounds, whilst the mixed decyl/dodecyl mannitol (**6b**) melts and recrystallizes at a lower temperature than either of its symmetric analogues **5c** or **5d**. This decrease in melting point substantiates our original hypothesis that mixing chains of different lengths would reduce crystallinity in these compounds. When heated in contact with water, these compounds melted and formed an immiscible liquid phase that recrystallized upon cooling. In contrast to the dialkylated derivatives, the single alkyl chain compounds **7a**–**d** formed thermotropic and lyotropic liquid crystalline phases. Neat **7c** and **7d** formed lamellar phases when heated, whilst the smaller derivatives **7a** and **7b** simply melted. When in contact with water, lyotropic phases were formed by all monoalkylated mannitols (Fig. 1a and b), Table 2). Compounds **7a**–**d** all gave concentrated micellar solutions (L_1 phases) at the high water concentrations found close to the contacting edge with water; the Krafft temperatures were estimated as 20, <10, 48, and 51 °C for **7a**–**d**, respectively. At lower water concentrations closer to the surfactant bulk, the monoheptyl mannitol **7a** gave a hexagonal H_1 phase (between 28 and 61 °C), whilst the branched isomer 5-methylhexyl mannitol **7b** also gave a hexagonal phase, but at lower temperatures

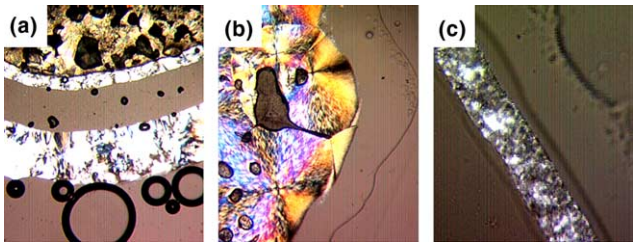


Figure 1. Microscopy images of lyotropic liquid crystalline phases viewed through crossed polarizers, for (a) monodecyl-D-mannitol surfactant **7c** at 78 °C (from top, solid/L_α/V_1/H_1/L_1); (b) monododecyl-D-mannitol surfactant **7d** at 58 °C (from left, solid/V_1/L_1); (c) 3-*O*-(*N*,*N*-dihexylacetamido)-D-glucose surfactant **10a** at 31 °C (from left, L_2/L_α/(L_2 or L_3)/L_1).

Table 2. Phase behavior observed for surfactants **7a**–**d** and **10a**

Compound	Lamellar phase neat/°C	Hexagonal phase with H_2O/°C	Bicontinuous cubic phase with H_2O/°C	Lamellar phase with H_2O/°C
7a	—[a]	28–61	—[a]	—[a]
7b	—[a]	<10–39	—[a]	—[a]
7c	95–150	51–99	59–>100	72–>100
7d	97–164	—[a]	57–>100	66–>100
10a	—[a]	—[a]	—[a]	<10–44

[a] Not observed.

(from below room temperature to 39 °C). The longer chain compounds **7c** (decyl) and **7d** (dodecyl) display additional lyotropic phases; they both form bicontinuous cubic phases and lamellar phases when in contact with water, whilst a separate L_1 phase also appears for **7d** at 51 °C. In fact, four lyotropic phases (L_1, H_1, V_1, and L_α) can be simultaneously observed for the decyl mannitol (**7c**) between the temperatures of 72 and 99 °C (Fig. 1a). The observation of L_α phases in the monoalkyl mannitols **7c** and **7d** suggests that reducing the number of alkyl groups in the mannitol surfactants has made the width of the hydrophilic and hydrophobic portions more comparable, promoting the formation of the lamellar phase. The occurrence of the separate L_1 phase for **7d** is unusual, and is similar to the behavior reported for *n*-decyl β-glucoside.[9] This indicates weak attractions between micelles, which will promote protein crystallization. The formation of cubic and lamellar phases for both **7c** and **7d** is also promising for membrane protein crystallization experiments. Since both these compounds display low solubility in water at 25 °C, they may need to be mixed with more conventional surfactants such as dodecyl maltoside in order to be utilized for membrane protein crystallization experiments.

To obtain compounds with improved water solubility, yet maintain the dual short alkyl chain motif which gives low CMC values, D-glucose surfactants **10a** and **10b** were designed (Scheme 2). In these compounds, in addition to the four hydroxyl groups, there is an aprotic

amide group that should improve the solubility without destabilizing internal protein hydrogen bonding. Furthermore, a range of related compounds, the *N*-D-gluco-*N*-methylalkanamides, are known to be good detergents for integral membrane proteins.[10] Our D-glucose range of surfactants were synthesized from commercially available 1,2:5,6-di-*O*-isopropylidene-α-D-glucofuranose. The unprotected C3 hydroxyl was deprotonated with KOH, then stirred with either 2-bromo-*N*,*N*-dihexylacetamide or 2-bromo-*N*,*N*-dioctylacetamide overnight.[11] The resultant alkylated sugar was mixed with 90% trifluoroacetic acid in water, which gave complete deprotection of the acetonide groups. The products were both obtained as hygroscopic oils, and the ^{1}H NMR spectra in CD_3OD showed that the deprotected sugars had reverted to the glucopyranose form, largely as the β anomers, but with significant amounts (15–35%) of the α anomer present.[12] The relative orientation of hydroxyl groups in different anomers can favor either intra- or intermolecular hydrogen bonding to give distinct liquid crystalline properties, but the behavior of the anomeric mixture cannot be predicted.[13] As anticipated, these D-glucose surfactants displayed improved solubility in distilled water at room temperature; both compounds were soluble at 0.1% wt/wt, and **10a** was soluble at 1% wt/wt. By measuring the surface tension, the CMC values for **10a** and **10b** were found to be 2 mmol/L and 33 μmol/L, respectively. The phase behavior of both compounds was determined as previously by visualization through crossed polarizers on a hot stage microscope (Table 2). Upon contact of neat *N*,*N*-dihexylacetamido-D-glucose **10a** with water at 25 °C, **10a** formed three phases; an isotropic L_2 phase for neat **10a**, a lamellar L_α phase, and then at higher water concentrations close to the water interface, another fluid isotropic phase, which may be an inverse micellar L_2 or sponge L_3 phase. When heated, the lamellar phase started to melt at 41 °C and had completely disappeared by 65 °C. After cooling the lamellar phase was regained at 45 °C, and continued to increase in size as the temperature was lowered to 10 °C. Neat *N*,*N*-dioctylacetamido-D-glucose **10b** was subjected to the same treatment as **10a**, but after the addition of a drop of water no liquid crystalline phases were observed. As the sample was heated, the compound simply absorbed water to form a separate liquid phase which melted at 88 °C. The lyotropic phase behavior of **10a** is particularly encouraging, as it displays a separated liquid phase at the water-rich side of a lamellar liquid crystalline phase which is almost certainly a micellar solution. This implies that there are attractive interactions between the surfactant micelles, a feature that will assist in the formation of membrane protein crystals. Combined with its high solubility, this makes **10a** an attractive candidate for further study.

We have synthesized several new nonionic surfactants as detergents for integral membrane crystallization, including a new class of D-glucose-based surfactants. The dialkyl D-mannitol range of surfactants displayed high crystallinity and low solubility, characteristics which were alleviated by removing an alkyl chain to increase hydrophilicity. The resultant monoalkyl D-mannitol derivatives have higher solubility and exhibit lyotropic liquid crystalline phases, such as lamellar, micellar, and cubic that have great potential for the crystallization of membrane proteins. Dialkylamido-D-glucose based surfactants also proved to have high water solubility at room temperature. Dihexylamido-D-glucose surfactant **10a** displayed excellent solubility and formed a lamellar liquid crystalline phase and a micellar solution at room temperature; both potentially useful for the crystallization of integral membrane proteins. Surfactants **7a–d** and **10a** are currently being tested for crystallization potential on the outer membrane proteins from *Neisseria meningitidis*; one of which rOpcA, is known to crystallize from two other detergent/amphiphile systems.[14]

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Supplementary data

A supplementary data section is provided, which includes full experimental details, for all reactions and analytical data for all compounds. Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.tetlet.2005.11.103.

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