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Cryo-TEM structural analysis of conjugated nonionic *engineered-micelles*

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Abstract

Conjugated *engineered-micelles*, *i.e.* micelles that are composed of nonionic detergents and hydrophobic chelators and subsequently conjugated in the presence of divalent metal ions, have been shown to be remarkably suited to the task of membrane protein purification, maintaining these proteins in their native state. They also efficiently solubilize highly hydrophobic antibiotics. To date, however, the morphological changes induced in the initially spherical or ellipsoidal micelles by conjugation have not been explored. In this study, the very rapid sample-vitrification protocol of cryogenic transmission electron microscopy (cryo-TEM) has been used to capture structural transformations that *engineered-micelles* undergo immediately following conjugation with the [(bathophenanthroline)$_3$:Fe^{2+}] hydrophobic complex. We found that condensed thread-like aggregates are formed when the detergents used are: octyl β-D-glucopyranoside (OG), octyl β-D-thioglucopyranoside (OTG) or pentaethylene glycol monododecyl ether ($C_{12}E_5$). However, with β-D-maltoside (DM), *n*-dodecyl β-D-maltoside (DDM) or β-D-glucopyranoside (DDG), lamellar structures, some of which appear as stacked lamellae or multilamellar vesicles (MLV's), were observed. Such architectural changes occur under very mild conditions *i.e.* low detergent concentration, no temperature or pH alterations and without the presence of any precipitants such as PEG or ammonium sulfate.

Introduction

Spontaneous self-association of surfactants and detergents into micellar assemblies occurs when the concentration passes a threshold called the critical micelle concentration (cmc).[1-4] Within the micelle, the molecules interact via non-covalent bonds that allow the formation of diverse micellar shapes (*e.g.* spherical, ellipsoidal, rod-like, disc-like) depending on molecular properties and environmental parameters such as concentration, temperature, and ionic strength.[5-6] These structures exhibit a dynamic nature.[1,2,4,7,8] Further global physical and chemical modifications in the micellar environment can also induce their interaction. Such modifications may include inclusion of polymeric precipitants (*e.g.* polyethylene glycol (PEG) or salts (*e.g.* ammonium sulfate (AS)); increase in ionic strength; or temperature alterations. [1,9,10] In nonionic systems, under these conditions, initially isotropic and transparent solutions can become turbid and a transient state called the *cloud-point* [6,11] representing clusters of micelles, may be reached. Further micellar aggregation results in phase separation and formation of two distinct phases: a detergent rich phase and a detergent poor phase.[1,9,12] Still, most nonionic detergents will reach the *cloud-point* at temperatures above 50˚C.[13]

In a previous study we described a potentially general conjugation mechanism producing micellar interactions under mild conditions, and demonstrated its applicability with several nonionic detergents.[14] Conjugated micelles were shown to be remarkably suited to the task of membrane protein purification, maintaining these proteins in their native state.[15] The membrane proteins preferentially partition into the micellar aggregate while hydrophilic proteins remain in the surrounding aqueous medium. We also reported that conjugated micelles are capable of solubilizing highly hydrophobic antibiotics.[14] Micellar conjugation relies on the prior formation of *engineered-micelles, i.e.* micelles composed of a nonionic detergent and containing a hydrophobic metal chelator which was shown to be embedded at the water/surfactant interface.[16] Such *engineered-micelles* are conjugated in the presence of divalent metal cations (*e.g.* Fe^{2+}) capable of binding several chelators simultaneously (Figure 1, left). Although micellar conjugation was shown to be specific and totally dependent on the presence of both the lipophilic chelator and the metal ion, no description of the architecture of conjugated *engineered-micelles* has heretofore been provided.

Therefore, in this report we use cryo-TEM to study the association of *engineered-micelles* immediately following conjugation with the [(bathophenanthroline)$_3$:Fe^{2+}] complex ([batho:Fe^{2+}] complex). The cryo-TEM technique has the distinct advantage that sample immobilization through cooling takes place in a sufficiently short time that self-assembled aggregates in solution remain unchanged and no further sample treatment is required. A number of different surfactants were studied: octyl β-D-glucopyranoside (OG), octyl β-D-thioglucopyranoside (OTG), pentaethylene glycol monododecyl ether ($C_{12}E_5$), β-D-maltoside (DM), n-dodecyl β-D-maltoside (DDM) and β-D-glucopyranoside (DDG), and the structural transformations which they undergo were characterized (Figure 1).

Results

Cryo-TEM imaging of conjugated *engineered-micelles*

1. *octyl β-D-glucopyranoside (OG), octyl β-D-thioglucopyranoside (OTG), pentaethylene glycol* monododecyl *ether ($C_{12}E_5$)*

Engineered-micelles composed of OG, OTG or $C_{12}E_5$ were prepared and conjugated as described in the Experimental section. The micellar aggregates of OG and OTG consisted of densely packed, entangled thread-like micelles with a defined boundary between the micellar aggregate and the aqueous phase (Figure 2A, 2B, respectively). These micellar aggregates generally covered large areas on the microscope grid and their sizes were on the micron scale (not shown). On the macro-scale, light microscopy images show the spontaneous formation of red oily droplets generated in the presence of the above detergents and the red-colored [batho:Fe^{2+}] complex (Figure 2A, B). Importantly, control experiments in the absence of the metal, absence of chelator, or both, did not result in the formation of either red or colorless, oily droplets.[14] These results are in agreement with cryo-TEM data that did not show any micellar aggregates in the absence of the [batho:Fe^{2+}] complex. Rather, only single, independent micelles were observed (data not shown). Similar to OG and OTG, micelles of $C_{12}E_5$ led to condensed aggregates containing entangled thread-like micelles (Figure 2C).

2. *n-decyl-β-D-maltoside (DM)*

Condensed thread-like micellar aggregates were also formed from conjugated *engineered-micelles* composed of DM (Figure 3A). These aggregates were fused to small membranes at their boundary with the aqueous phase (see arrows in Figure 3A). Although micellar aggregates were the predominant product, a few isolated multilamellar vesicles (MLV's) could also be identified (see inset in Figure 3A).

3. n-dodecyl-β-D-maltoside (DDM)

Interestingly, conjugation of *engineered-micelles* composed of DDM resulted in the formation of aggregates that included densely packed MLV's and membranes (Figure 4A). The outer diameter of the MLV's was determined to be on the order of 100 nm, similar to that observed for MLV's composed of DM (inset, Figure 3A; Table 1) and,

in some cases, the particles were partially merged. The membranes protruded from the aggregate and overlapped the carbon grid (see black arrows in Figure 4A). In addition to the merged MLV's, individual MLV's were also detected (Figure 4B), and these were generally associated to some degree with a membrane (Figure 4C) and could be partially (Figure 4D) or totally surrounded by it (Figure 4E). At lower magnification, micron-sized stacks of membranes could be observed (Figure 4F). Three control experiments provided evidence that the [batho:Fe^{2+}] complex is required for the production of the detergent architectures described above. In the absence of this complex, only individual DDM micelles were observed (Figure 4G). Furthermore, no MLV's were found in the absence of either the metal or the chelator alone (data not shown). The center-to-center distance between concentric bilayers in the MLV's is estimated to be consistent with the length of the DDM molecule, being ~25 Á in its fully extended chain conformation, plus interbilayer water.

4. *n-dodecyl-β-D-glucoside (DDG)*

Two distinct detergent architectures were produced with DDG. These were (i) condensed aggregates several microns in size with clearly defined boundaries (Figure 5A), and MLV's (Figure 5B). The MLV's were significantly larger (~400-500 nm) than those obtained with DDM (Table 1).

Discussion

The lack of morphological data for nonionic detergent micelles specifically conjugated *via* the [batho:Fe^{2+}] complex[14] and previously shown to be effective both in the purification of membrane proteins[15] and in the solubilization of hydrophobic antibiotics[14], has motivated a comprehensive cryo-TEM analysis of the resulting detergent aggregates. Nonionic detergents containing alkyl chains of various lengths and either sugar (*e.g.* glucose, maltose) or non-sugar (*e.g.* ethylene glycol) headgroups were studied immediately following addition of the metal. Some of the results described here were unanticipated.

Engineered-micelles composed of OG, OTG or $C_{12}E_5$ do not produce lamellar structures upon conjugation

Using cryo-TEM we found that conjugation of micellar suspensions containing either OG or OTG, each with a short eight-carbon alkyl chain, at concentrations above the cmc, leads to the formation of detergent aggregates only in the presence of the [$(batho)_3$:Fe^{2+}] complex (Figure 2A,B). Although both detergents produce similar aggregation forms, *i.e.* entangled thread-like micelles, no membranes or MLV's were detected in either case. Conjugated aggregates formed from OG or OTG were densely packed and characterized by clearly defined boundaries with the aqueous phase. The packing density within the micellar aggregates was greater in the case of OG than for OTG (Figure 2A, B). OG and OTG differ only with respect to a single atom, *i.e.* an oxygen atom in OG vs. a sulfur atom in OTG, both of which are covalently linked to the anomeric carbon with the same β-configuration. It is possible that the observed condensed packing is due to the presence of the [batho:Fe^{2+}] complex where Fe^{2+} ions can bind to the chelator with high affinity ($K_d \sim 10^{-21}$ M).[15]

Results similar to those observed with OG were obtained with $C_{12}E_5$. Even though these detergents differ in the length of their aliphatic chains (*i.e.* 8 vs. 12 carbons) and the chemical structure of their headgroups (glucose *vs.* ethylene glycol moieties), $C_{12}E_5$ produced condensed entangled thread-like micelles upon conjugation (Figure 2C), at a concentration and temperature where $C_{12}E_5$ alone forms only small spheroidal micelles.

Engineered-micelles composed of DM, DDM and DDG produce lamellar structures upon conjugation

Cryo-TEM micrographs of conjugated *engineered-micelles* composed of DM revealed new architectures, not observed with OG, OTG or $C_{12}E_5$. Though condensed micellar aggregates containing thread-like micelles were still dominant, membranes (Figure 3) and MLV's (Figure 3, inset) were observed as well. However, MLV's were rare and the membranes generated were relatively small and could only sometimes be detected at the boundary between the aggregate and the aqueous phase (Figure 3). Nevertheless, these new architectures, though constituting a minor population, suggested that lamellar formation is promoted by: (a) increased length of the alkyl chain and/or (b) the presence of a disaccharide maltose headgroup; the headgroups of OG and OTG contain the monosaccharides, glucose or thioglucose, respectively. Therefore, conjugation experiments were first repeated with DDM, a longer chain analog of DM, so as to preserve the chemical identity of the headgroup while allowing assessment of the effect of the alkyl tail length on promoting lamellar structures. The prevailing architecture found with conjugated *engineered-micelles* composed of DDM were membranes, though thread-like micelles and MLV's, both partially merged and independent, were also observed (Figure 4). Thus, the fraction of lamellar structures and the size of the membranes both increased when the alkyl chain was increased by two carbons. The role of the maltose headgroup in promoting lamellar structures was studied by comparing the results obtained for DDG with those for DDM. The hydrocarbon chains of both detergents contain 12 carbons; only the chemical compositions of their headgroups differ. Analysis of conjugated *engineered-micelles* composed of DDG revealed two major architectures: densely packed micellar aggregates (Figure 5, A) and numerous MLV's with outer diameters in the range of ~400-500 nm (Figure 5, B). A minor population of small membranes, fused to the MLV's, was also observed (data not shown).

It should be noted that the current structural heterogeneity of the systems, as described above, did not allow further useful sample characterization by scattering techniques, *e.g.* small angle X-ray scattering (SAXS) or small angle neutron scattering (SANS). However the identification of MLV's comprising DM, DDM or DDG is based on measurements of the particle outer diameter, the number of constituent, concentric layers, their continuity and strong curvature (Table 1). Although the diameter of classical MLV's may reach the micron range, smaller sizes, similar to ours, are also observed.[17] The interbilayer spacing, the accuracy of which may be

somewhat limited by the TEM imaging conditions, is determined by the length of the surfactant molecules comprising the MLV and also by the thickness of the water layer between the polar head groups. On the basis of the known structures of the individual surfactants, the values obtained here (~5-8 nm) appear to be quite reasonable.

Modeling

Previously reported SAXS analysis for OG, DM and DDM micelles shows that all three detergents form oblate ellipsoidal micelles with similar ellipticity.[18] For OG the axial ratio of the hydrophobic core is ~11/21; for DM, 13.6/23; and for DDM 16/28. The thickness of the polar headgroup region is 2.9-3.5 Å for the glucoside and 5.4-5.8 Å for the maltoside. The aggregation number N_a increases with increase in the length of the alkyl chain: 86-103 for DM, 135-149 for DDM, but only 70-90 for OG. The cmc is also strongly dependent on the chain length, *e.g.* 1.8mM for DM and 0.17 mM for DDM.[18] The fact that, upon micellar conjugation, three different alkyl saccharide detergents (DDM, DM and DDG) generated membranes and MLV's - to differing extents - implies that other detergents containing sugar headgroups may lead to similar results. Yet, OG did not form MLV's, while DDG did, so the hydrophobicity (chain length) must also be taken into account.[19] It was expected that the observation that membranes (Figure 4A, C, D, E) and stacks of membranes (Figure 4E) were ubiquitous in all DDM samples tested must also be related to the packing parameter $P=V_o/l_oA_e$ where V_o is the surfactant tail volume, l_o is the tail length and A_e is the equilibrium area per molecule at the aggregate surface.[20] Whereas packing parameters with values lower than 1/3 favor the formation of spherical micelles, values ~1 favor the production of lamellar phases; cylindrical micelles are characterized by intermediate values of the packing parameter. In general, as the length of the carbon chain increases, the packing parameter value increases as well. However, DDM and DM have very similar values of P- *i.e.* 0.38-0.4 while $C_{12}E_5$ has only a marginally smaller packing parameter, *i.e.* 0.35 and all three values are closer to favoring cylindrical micelles than lamellae.[21] Therefore, the formation of the lamellar structures observed here (Figure 3, inset, Figure 4A-E, and Figure 3B) must be ascribed to the introduction of the [batho:Fe^{2+}] complex. This complex, in principle, can induce profound changes in the structure of the original micelle as well as of the resulting aggregates. These structural changes may derive from several parameters: (i) the rigid and bulky aromatic chelator embedded within

the micelle; (ii) the strong binding affinity of the chelator to Fe^{2+} [14] and (iii) the octahedral geometry of the resulting complex.

A potentially simple, practical method for obtaining MLV's?

All the features discussed above (*i.e.* chelator rigidity, the strength of the [Batho:Fe^{2+}] complex and its octahedral geometry), alone and/or in combination, may affect the micellar shape (prior to conjugation), and further promote micellar transformation into a lamellar phase upon conjugation with Fe^{2+} ions. Utilization of the [Batho:Fe^{2+}] complex in combination with DDM, DDG, or to lesser extent, DM, may potentially represent a simple and practical method for obtaining MLV's. Although the micrographs presented here reveal strongly heterogeneous aggregational forms, it is likely that varying the individual concentrations and ratios of the detergent / chelator / metal components will influence the resulting size, morphology and abundance of MLV's. If this supposition will be borne out by subsequent experimentation, comparison of the method presented in this study with standard MLV's production protocols in the literature points to several fundamental advantages: (a) no need for the initial presence of membranes; (b) no utilization of organic solvents followed by their evaporation at elevated temperatures; (c) employment of only a single non-ionic detergent rather than mixtures of detergents and lipids (*e.g.* lecithin, cholesterol); (d) a simple two-step protocol, performed under mild conditions, not requiring mechanical manipulation (*e.g.* sonication, agitation, pressure filtration, evaporation, vortexing)

Experimental

Materials

Bathophenanthroline, pentaethylene glycol monododecyl ether ($C_{12}E_5$), decyl β-D-maltoside (DM), *n*-dodecyl β-D-maltoside (DDM), octyl β-D-glucopyranoside (OG), *n*-dodecyl β-D-glucopyranoside (DDG), octyl β-D-thioglucopyranoside (OTG), $FeSO_4$ were obtained from Sigma-Aldrich (St. Louis, MO).

Protocols for preparation of conjugated *engineered-micelles*

Conjugated *engineered-micelles* composed of non-ionic detergents (except DDG) were prepared using the following two-step protocol: **Step I:** Freshly prepared 20mM bathophenanthroline\methanol (5 µl) was added to an aqueous solution containing one of the following detergents: 25mM DDM (10 µl); 40mM DM (10 µl); 25mM $C_{12}E_5$ (5 µl). In the case of OG or OTG, 10 µl of the chelator were added to either 200mM OG (22 µl) or 200mM OTG (30 µl). Addition of the chelator was performed with continuous, vigorous vortexing (5-10 seconds) and was followed immediately by addition of double distilled water (DDW) to a final volume of 100 µl. **Step II:** Aliquots (50µl) of the product of Step 1 were immediately vortexed with an equal volume of an aqueous solution containing 6mM $FeSO_4$ in 400mM NaCl. Conjugated *engineered-micelles* composed of OG, OTG or DDM were immediately prepared for analysis by cryo-TEM, since the complexation occurred very rapidly, whereas conjugated *engineered-micelles* comprised of DM or $C_{12}E_5$ were prepared for cryo-TEM analysis only after 2 and 5 minutes, respectively, because of the lower complexation rate. Though all manipulations were carried out at room temperature, the $FeSO_4$\NaCl solution was stored at 4 ºC.

Preparation and conjugation of *engineered-micelles* composed of DDG were the same as in **Step I** described above but with the following changes: 5 µl of 50mM DDG\methanol was added with constant vortexing to 5 µl of freshly prepared bathophenanthroline solution. This was followed immediately by addition of DDW to a final volume of 100 µl. Conjugation was performed as described in **Step II**. Samples were prepared for cryo-TEM analysis after 2 minutes of incubation at room temperature.

Methods

Cryo-TEM analysis of conjugated *engineered-micelles*

Samples (10 µl) for cryo-TEM were prepared in the controlled environment vitrification system (CEVS), equilibrated at 25 °C and at saturation. Vitrified specimens were examined in an FEI T12 G^2 TEM operating at 120 kV. Images were recorded under low dose conditions as described previously.[22,23] Measurements on the cryo-TEM images were made with ImageJ (imagej.nih.gov) software.

Light microscopy

Images were obtained using an Olympus CX-40 light microscope equipped with an Olympus U-TV1X-2 digital camera.

Conclusion

Cryo-TEM imaging reveals that conjugation *via* the [$(batho)_3$:Fe^{2+}] hydrophobic complex promotes major structural transformations of non-ionic detergent micelles from spherical or ellipsoidal shapes to densely packed thread-like micelles, membranes and MLV's. This change in morphology is apparently responsible for the utility of conjugated *engineered-micelles* in both the purification of membrane proteins and the solubilization of hydrophobic antibiotics. On the basis of the observations reported here, we further suggest that, by varying the concentrations and ratios of the detergent / chelator / metal components, nonionic surfactants such as DM, DDM and DDG, as well as others possessing long aliphatic chains (≥12 carbons) and sugar-containing headgroups, may produce homogeneous lamellar preparations, including MLV's, *via* this mild procedure. Nevertheless, for those applications which require the rapid formation of detergent aggregates, even though morphologically heterogeneous, in the absence of precipitants, salts, high detergent concentrations or pH alterations, and independent of the *cloud-point* temperature of the detergent used, the results of the current study may also prove to be of value.

Acknowledgments

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Graphical abstracts

Figure 1: Illustration depicting possible aggregation states of *engineered-micelles* following conjugation with Fe^{2+} ions. Micelles composed of non-ionic detergents (OG, OTG, $C_{12}E_5$, DM, DDM, DDG) are transformed into *engineered-micelles* upon the insertion of the hydrophobic metal chelator bathophenanthroline. These are then conjugated specifically in the presence of Fe^{2+}. Possible structural transformations occurring following conjugation with the [chelator:metal] complex are indicated.

Figure 2: Light microscopy and cryo-TEM images of conjugated *engineered-micelles* composed of OG, OTG ,and $C_{12}E_5$. Black arrows in **A-C** depict the boundary between micellar aggregates and the bulk solution. Top, middle and bottom scale bars, in the light microscopy images, represent 40 μm.

Figure 3: Light microscopy and cryo-TEM images of conjugated *engineered-micelles* composed of DM. The conjugation of *engineered-micelles* composed of DM results in the coexistence of micellar aggregates, membranes (see arrows) and MLV (see inset). Scale bar in the light microscopy image equals 40 μm.

Figure 4: Light microscopy and cryo-TEM images of conjugated *engineered-micelles* composed of DDM. The red color in the light microscopy image is derived from the [$(batho)_3$:Fe^{2+}] red complex. Panels A-F show: (A) Concentric, dense multilamellar vesicles (MLV's). (B) Isolated MLV. (C) MLV's with a protruding membrane. MLV's partially (D) or totally (E) surrounded by a membrane. (F). Stacked membranes, filling the carbon grid. (G) Control: independent DDM micelles in the absence of the [batho:Fe^{2+}] complex. Arrows show single micelles. Scale bar in the light microscopy image equals 40 μm.

Figure 5: Light microscopy and cryo-TEM images of conjugated *engineered-micelles* composed of dodecylglucoside (DDG) . The conjugation of *engineered-micelles* composed of DDG leads to the formation of: (A) condensed micellar aggregates with a defined boundary (see arrow) and to (B) MLV's. Scale bar in the light microscopy image equals 40 μm.

Figures

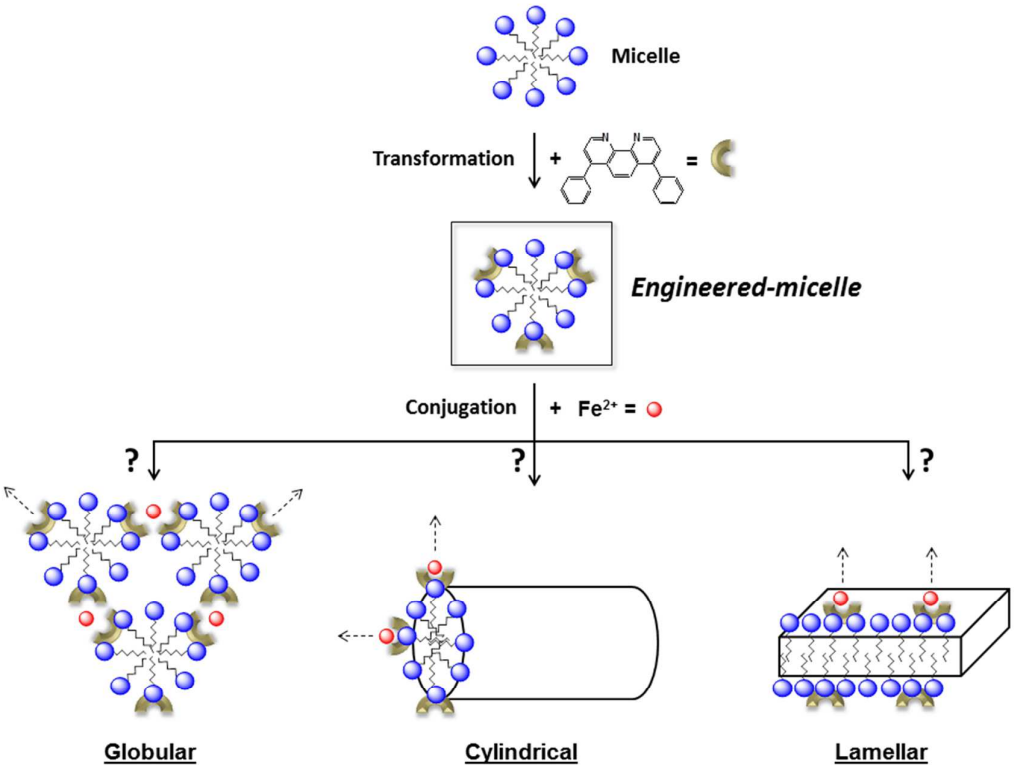

Figure 1

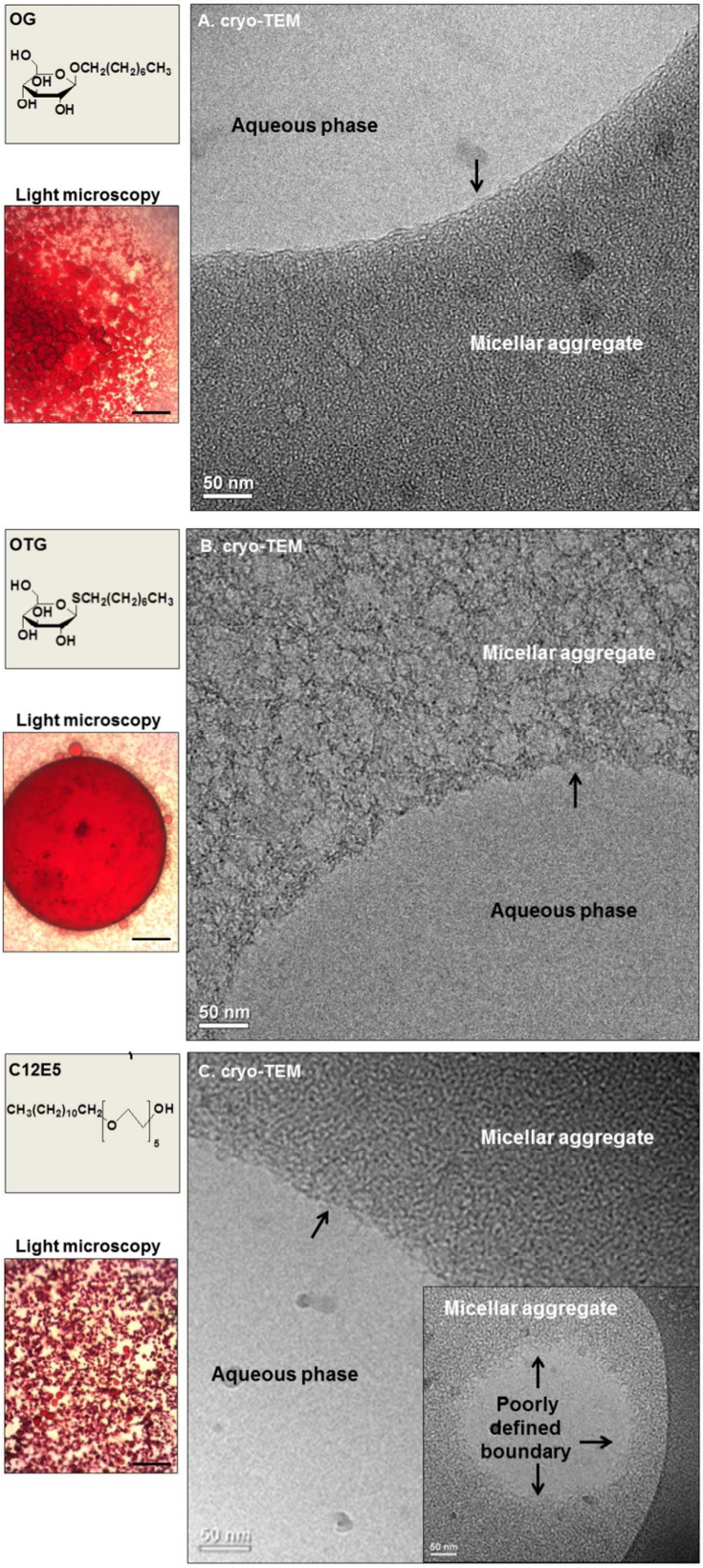

Figure 2

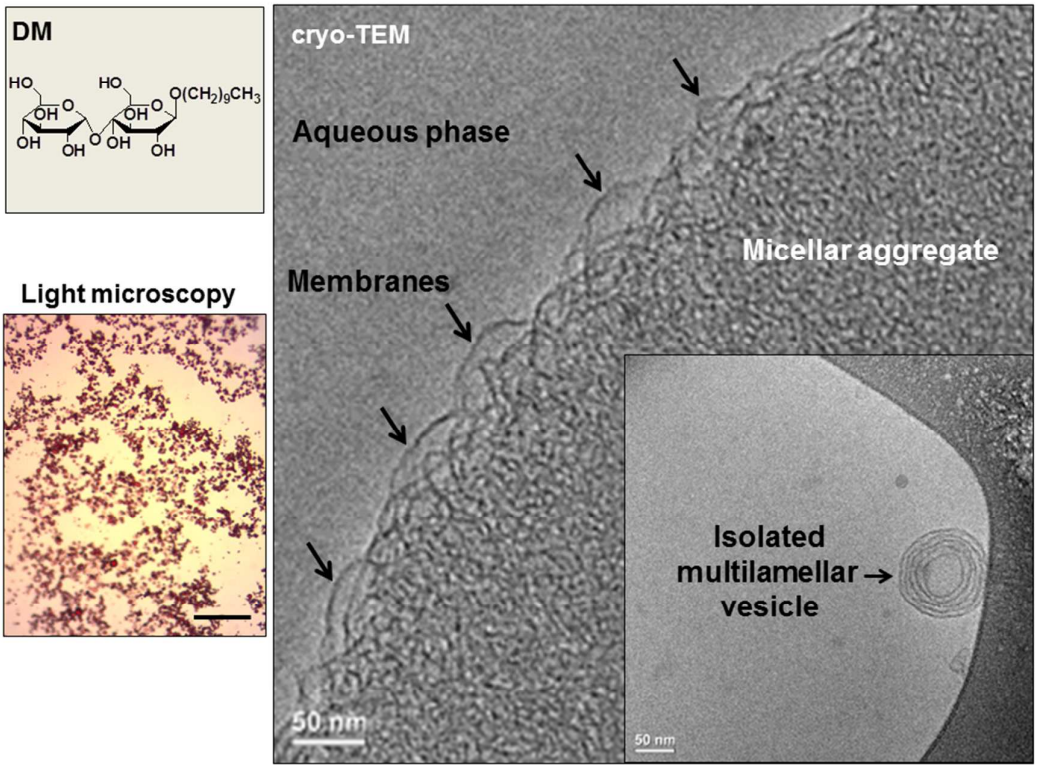

Figure 3

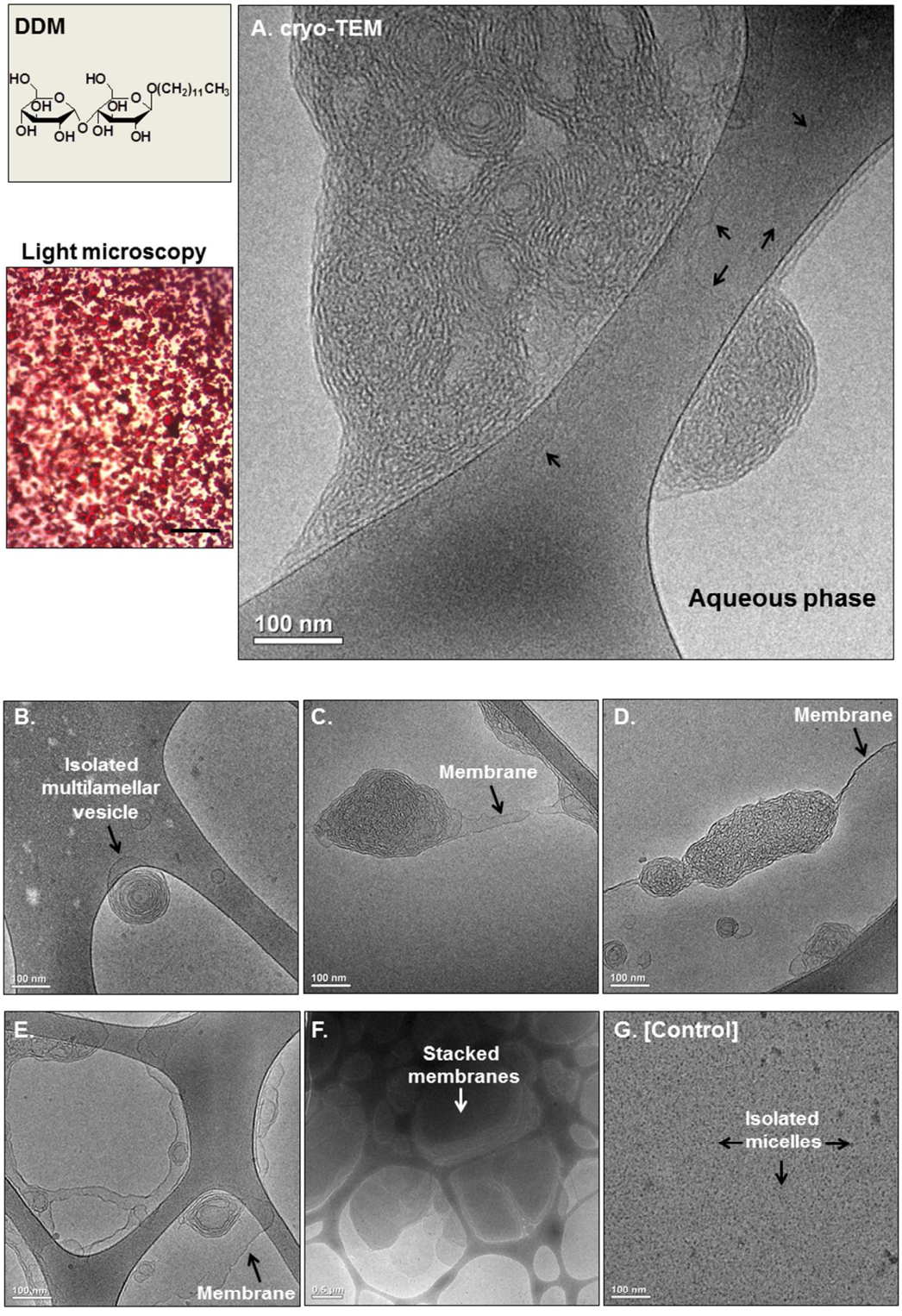

Figure 4

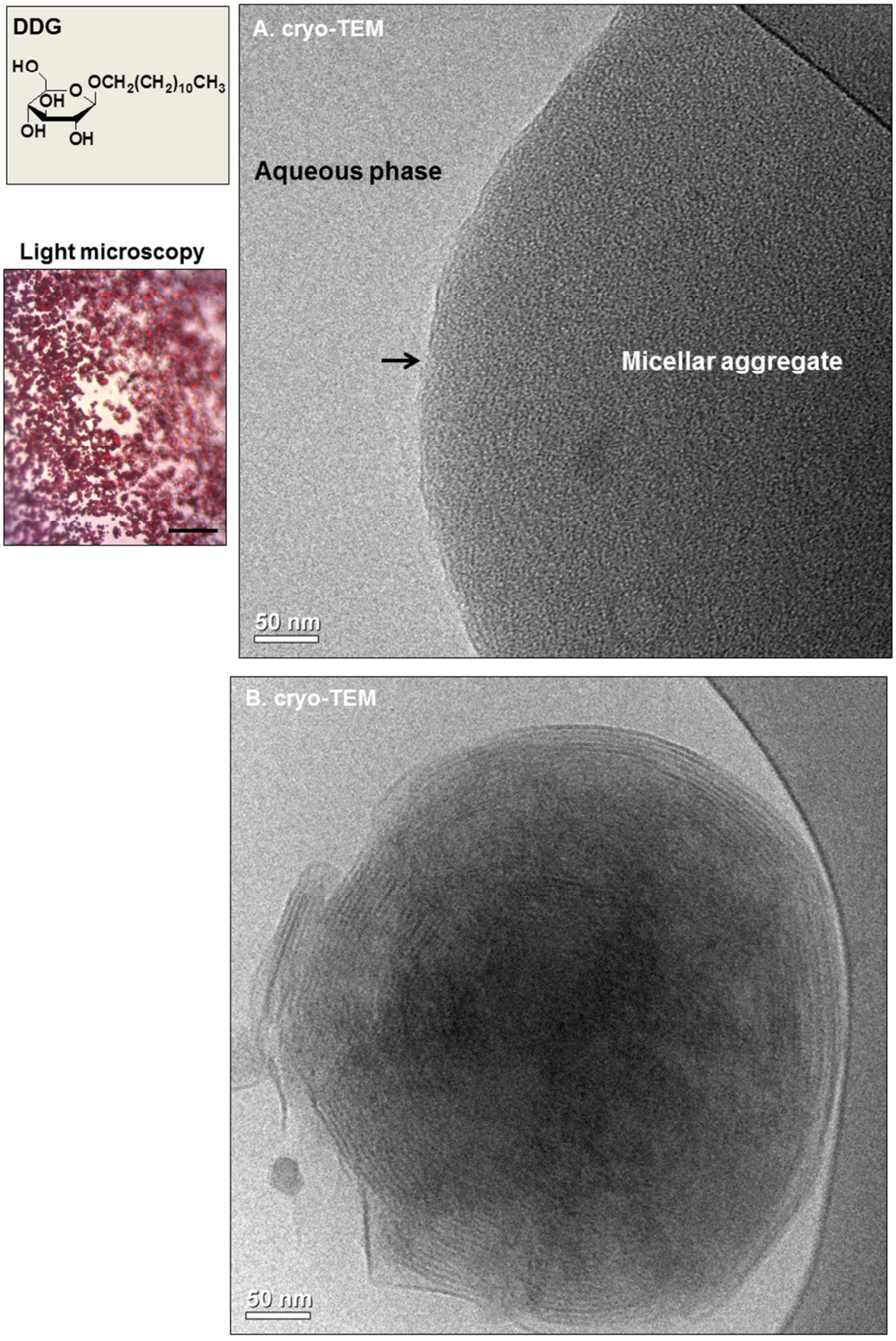

Figure 5

Table 1. Properties of MLV's observed in cryo-TEM images of engineered micelles of DDM, DDG and DM immediately following conjugation

Figure	Sample	MLV outer diameter (nm)	Layer Curvature (nm^{-1})	# of layers	Layer continuity	Average Interlayer spacing[c] (nm)
2B	DDM	155	>0.0128	9	Yes -[a]	7.6
2E	DDM	85	>0.0109	4	Yes -[a]	5.9
3B	DDG	438	>0.0046	>11	Too large[b]	4.8
4 inset	DM	116	>0.0172	5	Yes	8

[a]Continuity of the concentric layers can not be followed full circle.

[b]The MLV is too large and the image contrast insufficient to determine complete layer continuity.

[C]The accuracy of the interbilayer spacing is limited by the TEM resolution.

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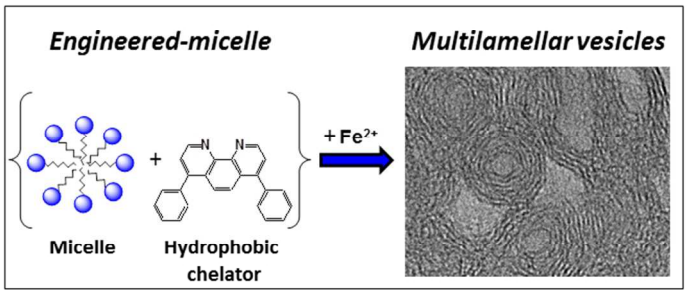
Keywords

Micelles, nonionic surfactants, detergents, engineered-micelles, multilamellar vesicles, cryo-TEM.



Following conjugation with the [(bathophenanthroline)3:Fe2+] complex, engineered-micelles composed of alkyl-saccharide detergents transform into membranes and multilamellar vesicles.

254x190mm (96 x 96 DPI)