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PAPER

Tethered non-ionic micelles: a matrix for enhanced solubilization of lipophilic compounds

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A specific mechanism for tethering micelles composed of non-ionic detergents is presented. The mechanism does not require any precipitant, high ionic strength or temperature alterations. Rather, it relies on complexes between hydrophobic chelators embedded within the micelle and appropriate metal cations in the aqueous phase, serving as mediators. The approach was applied to: (i) four non-ionic detergents (tetraethylene glycol monooctyl ether (C8E4), *n*-dodecyl-β-D-maltoside (DDM), octyl β-D-1-thioglucopyranoside (OTG), and *n*-octyl-β-D-glucopyranoside (OG)), (ii) two hydrophobic chelators (bathophenanthroline and *N*-(1,10-phenanthrolin-5-yl)decanamide, Phen-C10) and (iii) five transition metals (Fe^{2+}, Ni^{2+}, Zn^{2+}, Cd^{2+}, and Mn^{2+}). The mandatory requirement for a hydrophobic chelator and transition metals, capable of binding two (or more) chelators simultaneously, was demonstrated. The potential generality of the mechanism presented derives from the observation that different combinations of [detergent : chelator : metal] are able to induce specific micellar clustering. The greater solubilization capacity of tethered-micelles in comparison with untethered micelles was demonstrated when the water insoluble aromatic molecule fluorenone (8 mM = 1.44 mg mL^{-1}) and two highly lipophilic antibiotics: chloramphenicol (5 mM = 1.62 mg mL^{-1}) and tetracycline (1.5 mM = 0.66 mg mL^{-1}) were solubilized – only when the micelles were tethered.

Introduction

Detergents are amphipathic molecules which are driven by the hydrophobic effect to assemble spontaneously into non-covalent macro-assemblies (*micelles*) when the concentration exceeds a broad threshold called the critical micelle concentration (cmc).[1–4] Further addition of detergent above the cmc ideally increases only the micelle concentration while keeping the free detergent concentration constant.[5] The dynamic character of micelles[1,2,4,6,7] is emphasized by their ability to undergo major structural alterations, *e.g.* from a spherical shape to ellipsoidal or rodlike assemblies,[8,9] in particular in the presence of an additional detergent, lipid or protein.[10] Dilute aqueous micellar suspensions can be thought of as ideal solutions where the micelles do not interact with one another.[11]

However, global physical and chemical modifications in the micellar environment can induce their interaction. Such changes may include: (a) inclusion of polymeric precipitants (*e.g.* polyethylene glycols (PEGs)) or salts (*e.g.* ammonium sulfate (AS)), (b) increase in the ionic strength or (c) temperature alterations.[1,11,12] Under these conditions, initially isotropic and transparent solutions can become turbid and a transient state called the "*cloud-point*",[9,13] representing clusters of micelles, is reached. *Cloud points* are generated due to an increase in micellar size, intermolecular attraction or both.[8,14–19] Further micellar aggregation results in phase separation and formation of two distinct phases: a detergent rich phase and a detergent poor phase.[1,11,20] Inclusion of nonpolar molecules (*e.g.* aliphatic hydrocarbons) will generally increase the temperature at which the *cloud-point* is reached, whereas salts will have the opposite effect.[4] Still, most non-ionic detergents will reach the *cloud-point* at temperatures above 50 °C.[21]

Thus it is evident that micellar aggregation processes currently in use are governed by major physical and/or chemical modifications of the system. For some biological applications, *e.g.* membrane protein purification,[22,23] it has been demonstrated that room temperature phase separation of surfactant systems is desirable. However, the only suitable non-ionic surfactant is Triton X-114 due to its low *cloud-point* temperature (22–23 °C).[24] Other popular non-ionic detergents cannot be used due to their high *cloud-point* temperatures which would denature the native conformation of the membrane protein. We therefore sought to develop a specific approach for tethering detergent micelles composed of non-ionic detergents. This approach is based on the formation of strong complexes between hydrophobic chelators embedded within the micelle and transition metals in the aqueous phase, serving as mediators (Fig. 1). We speculated that

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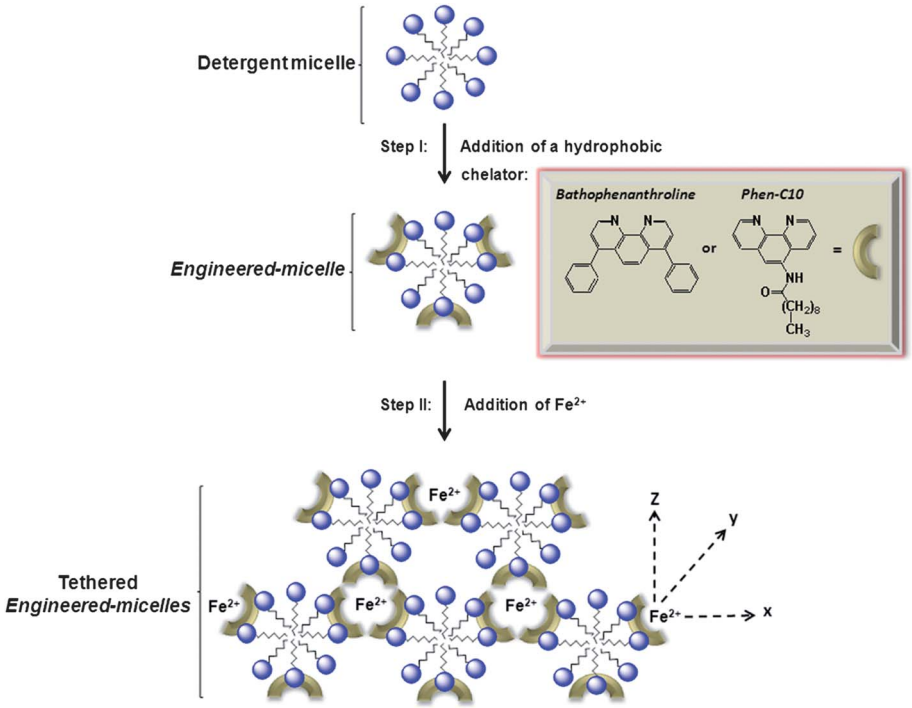

Fig. 1 Scheme of the mechanism for transforming micelles into tethered *Engineered-micelles*. Step I: detergent micelles are transformed into the corresponding *Engineered-micelles* by the addition of a hydrophobic chelator (*e.g. bathophenanthroline or Phen-C10*). Step II: introduction of Fe^{2+} induces specific tethering of *Engineered-micelles* into 3D micellar aggregates.

hydrophobic chelators containing the 1,10-phenanthroline moiety would partition into the micelle, thereby transforming it into what we term an *Engineered-micelle.* This in turn, would cluster with other *Engineered-micelles* only in the presence of appropriate transition metals (*e.g.* Fe^{2+}) capable of binding two or more 1,10-phenanthrolines, simultaneously.[25] Here we present our study of the parameters which affect the tethering mechanism specificity and efficiency in the presence of several different non-ionic detergents and diverse [hydrophobic chelator : metal] complexes. We find that the tethering of *Engineered-micelles* constitutes a general and robust mechanism for micellar aggregation.

Results and discussion

Light microscopy

To demonstrate the generality of the proposed tethering strategy we initiated our study with *Engineered-micelles* composed of bathophenanthroline and four distinct non-ionic detergents. Bathophenanthroline seemed to fit with the requirements of the presented tethering mechanism since it (i) is lipophilic,[26] (ii) binds to diverse metal cations, including Fe^{2+} at high affinity in a 3 : 1 stoichiometric ratio, respectively,[25] and (iii) was shown to embed itself at the membrane interface.[27] Indeed, incubation of bathophenanthroline with aqueous micellar suspensions containing either tetraethylene glycol monooctyl ether (C8E4), *n*-dodecyl-β-D-maltoside (DDM), octyl β-D-1-thioglucopyranoside (OTG) or *n*-octyl-β-D-glucopyranoside (OG) led to the spontaneous formation of red oily globules only in the presence of Fe^{2+} ions (Fig. 2, +chelator) but not in their absence (Fig. 2, −chelator). These results provided direct evidence for the participation of bathophenanthroline in the tethering mechanism. Oily globules appeared within seconds-to-minutes and possessed different sizes and shapes depending on the identity of the detergent used, but all were red colored (Fig. 2). The red color derives from the [(bathophenanthroline)$_3$: Fe^{2+}] complex which served as a convenient indicator for the presence of the complex in the interior of the detergent aggregate. However, the use of a negatively charged detergent, sodium dodecyl sulfate (SDS) or a zwitterionic detergent, *N*,*N*-dimethyldodecylamine *N*-oxide (LDAO), did not result in any micellar aggregation under identical conditions (not shown) presumably due to charge repulsion between the head groups.

The dependence of the tethering mechanism on Fe^{2+} was demonstrated by repeating the experiment in the presence of Mg^{2+} ions. The latter form weak complexes with the phenanthroline moiety at a stoichiometric ratio of 1 : 1 and thus were not expected to promote formation of micellar aggregates.[25] Consistent with this notion, we found that Mg^{2+} did not induce clustering of *Engineered-micelles* composed of OG (*i.e.* OG-*Engineered-micelles*) but rather the formation of micro-crystals (Fig. 3A) that were found to be comprised of bathophenanthroline as determined by electron spray ionization (ESI) analysis (Fig. 3B). It was therefore concluded that micellar tethering should entail a metal cation capable of binding two or more bathophenanthrolines, simultaneously.

Bathophenanthroline could not be replaced by a less hydrophobic chelator. The results shown in Fig. 3C indicate that the high lipophilicity of bathophenanthroline is essential as 1,10-phenanthroline failed to induce micellar aggregation even after days of incubation (not shown). Thus the additional two phenyl groups in bathophenanthroline (which are lacking in

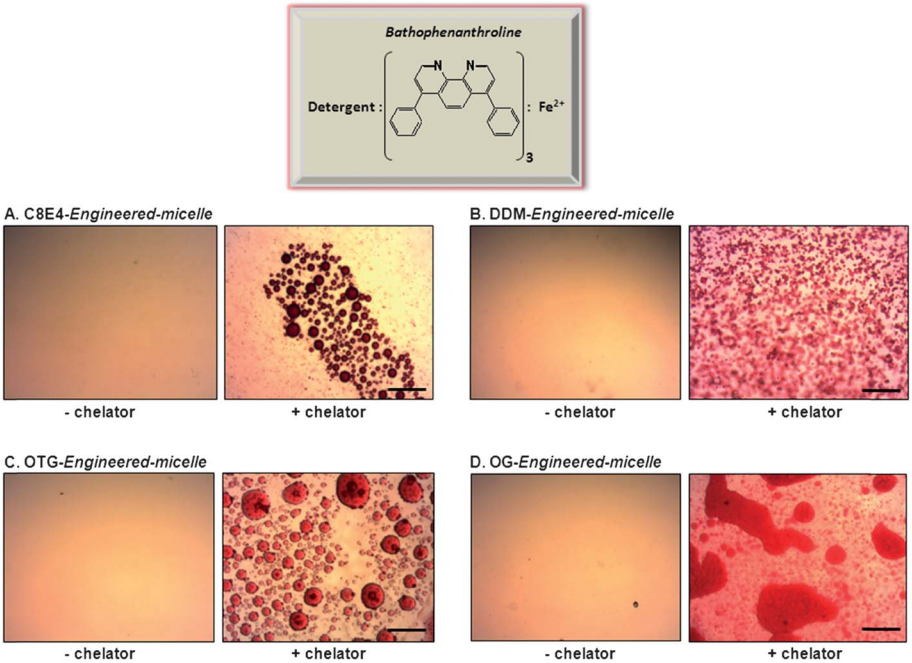

Fig. 2 The dependence of the tethering mechanism on bathophenanthroline. Light microscope images of *Engineered-micelles* composed of non-ionic detergents in the presence or absence of the hydrophobic chelator bathophenanthroline. All samples contain identical concentrations of NaCl and Fe^{2+}. Scale bars in (A), (C), (D) represent 0.1 mm and in (B) 0.04 mm.

1,10-phenanthroline) seem to promote efficient chelator partitioning into the micelles and hence, their transformation into the corresponding *Engineered-micelles*. In addition, experiments with detergent concentrations below the cmc failed to show the desired phase separation. For example, when OG was present at 5 mM, far below its known cmc (18–19 mM),[28] dark precipitates

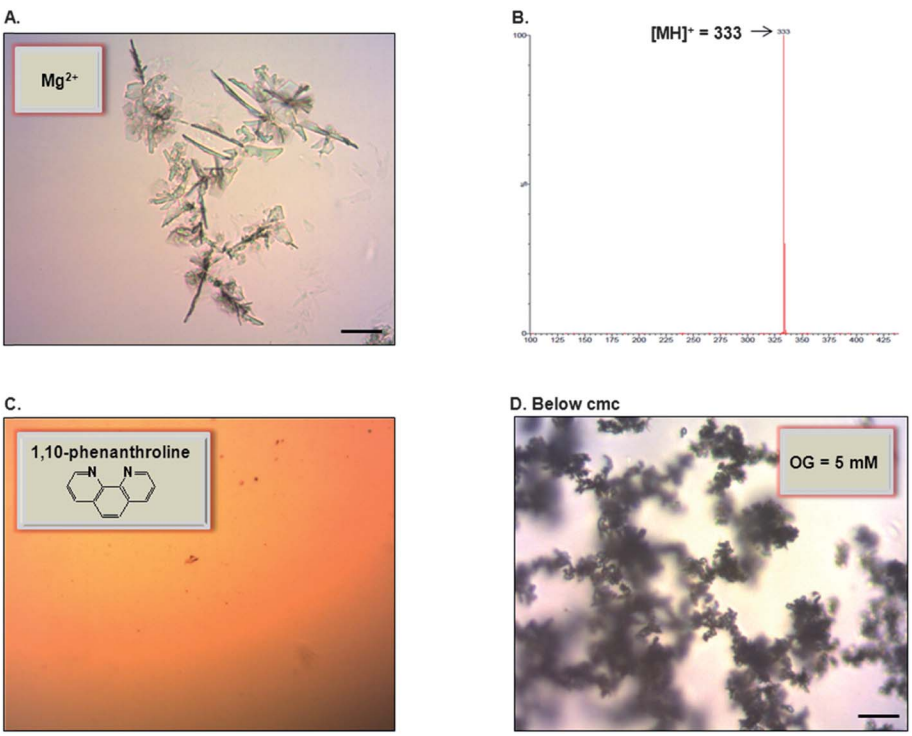

Fig. 3 Parameters affecting micellar tethering. Light microscope images of: (A) OG-*Engineered-micelles* in the presence of $MgCl_2$. (B) Electron spray ionization (ESI) analysis of extensively washed micro-crystals obtained in (A). (C) OG-*Engineered-micelles* in the presence of Fe^{2+} and 1,10-phenanthroline. (D) As in (C) but with OG concentration of 5 mM. Scale bars in (A) and (D) represent 0.04 mm.

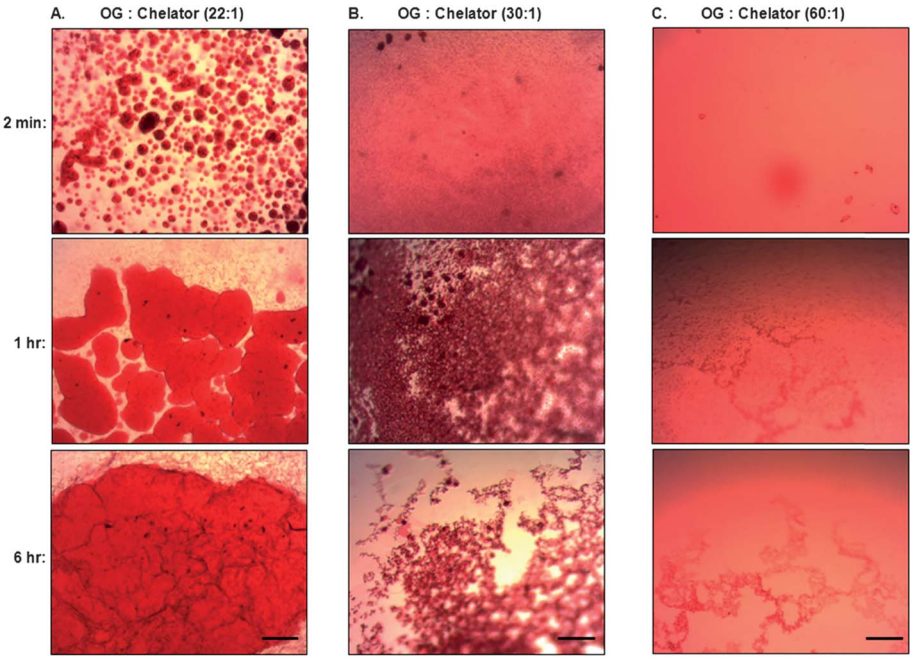

Fig. 4 Effect of [detergent : chelator] ratio on process efficiency. Light microscope images of a time course experiment in the presence of tethered OG-*Engineered-micelles* containing bathophenanthroline at the concentrations indicated for detergent and chelator respectively. Scale bars represent 0.1 mm.

were generated (Fig. 3D) rather than the red oily droplets which appeared at 22 mM (Fig. 2D). Similar results were obtained with all other detergents (not shown).

We found that the stoichiometric ratio between the detergent and hydrophobic chelator affects process efficiency. As the detergent : chelator ratio increases, tethering efficiency decreases. For example, high tethering efficiency of OG-*Engineered-micelles* was observed at stoichiometric ratios of 22 : 1, 30 : 1 (Fig. 4A and B) and 40 : 1 (not shown) but not at 60 : 1 (Fig. 4C). These findings are in agreement with the known aggregation number of OG (being between 70 and 87)[28–30] and imply that, a minimum of two hydrophobic chelators per micelle are required to induce efficient clustering.

Since numerous detergents contain aliphatic chains (rather than aromatic residues as in bathophenanthroline), it was necessary to test the tethering strategy on a phenanthroline derivative covalently linked to a long hydrocarbon tail. We therefore studied the tethering capabilities of the synthesized (*N*-(1,10-phenanthrolin-5-yl)decanamide, *Phen-C10*) under conditions identical to those applied on bathophenanthroline. Similar tethering efficiency was found with this analog as well, but the size of the resulting micellar aggregates differed. For example, tethered DDM-*Engineered-micelles* or OTG-*Engineered-micelles* containing *Phen-C10* were significantly larger than those which contained bathophenanthroline (compare Fig. 5A and C with Fig. 2B and C). Conversely, tethered OG-*Engineered-micelles* obtained with *Phen-C10* were smaller than those observed with bathophenanthroline (compare Fig. 5B with Fig. 2D). Moreover, C8E4-*Engineered-micelles* containing *Phen-C10* did not generate any oily phase rather a red precipitate (not shown). These findings imply that, the chemical structure of the hydrophobic anchor has a significant role in determining the size and shape of the resulting detergent aggregate.

We found that the tethering mechanism is not limited to the use of Fe^{2+} ions and could be induced with other transition metals. Therefore, a series of four cations (Ni^{2+}, Zn^{2+}, Cd^{2+}, and Mn^{2+}) capable of binding three phenanthroline moieties simultaneously at different binding affinities[25] were tested. Phase separation occurred in all combinations whether bathophenanthroline (Fig. 6A–D) or *Phen-C10* (Fig. 6E–H) was present, but not in their absence (not shown). And, consistent with our previous observations, the identity of the hydrophobic anchor (two phenyl groups *vs.* an aliphatic chain) dictated the size and shape of the resulting aggregates. In three out of the four metals tested with OTG-*Engineered-micelles*, those that contained *Phen-C10* (Fig. 6E, F and H) were significantly larger than those obtained with bathophenanthroline (Fig. 6A, B and D). Interestingly, tethering in the presence of Cd^{2+} led to a precipitate in the presence of *Phen-C10* (Fig. 6G), rather than to oily globules with bathophenanthroline (Fig. 6C).

Dynamic light scattering

Additional characterization of the tethering mechanism at the micellar level was acquired with dynamic light scattering (DLS). Fig. 7 shows how OTG-micelles are unaltered under experimental conditions (a and b) whereas the addition of the [(bathophenanthroline)$_3$: Fe^{2+}] complex transforms them into large aggregates within minutes (c). The hydrodynamic radius found for the individual micelles 9 nm agrees with the literature values.[31] The aggregation process proceeds with time with the formation of even larger particles (*i.e.* hydrodynamic radii 107 nm and 1155 nm after 5 min of incubation (c); 204 nm and 1572 nm after 30 min (d)). Assuming that the micelles retain their identity upon aggregating to form large, globular particles, one may estimate that the two sizes of particles observed would

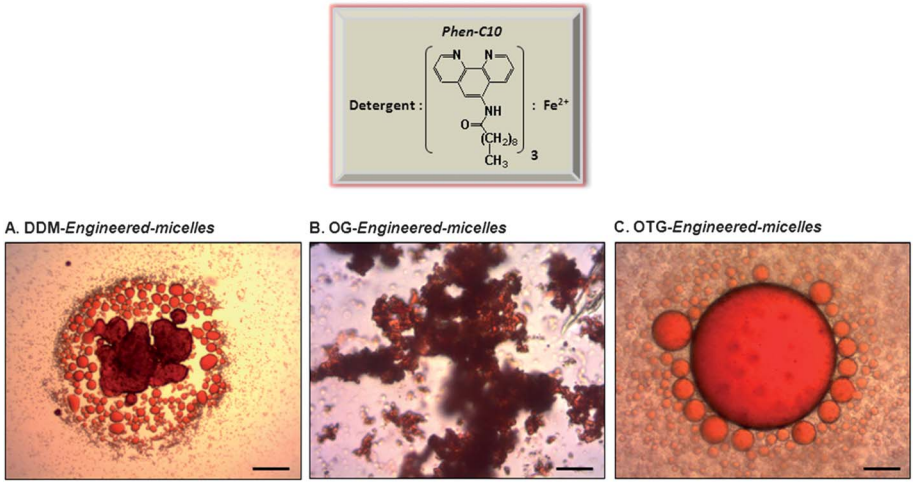

Fig. 5 Micellar tethering in the presence of *Phen-C10*. Light microscope images of tethered *Engineered-micelles* composed of the non-ionic detergents indicated with *Phen-C10* and Fe^{2+}. Scale bars in (A) and (C) represent 0.1 mm and in (B) 0.04 mm.

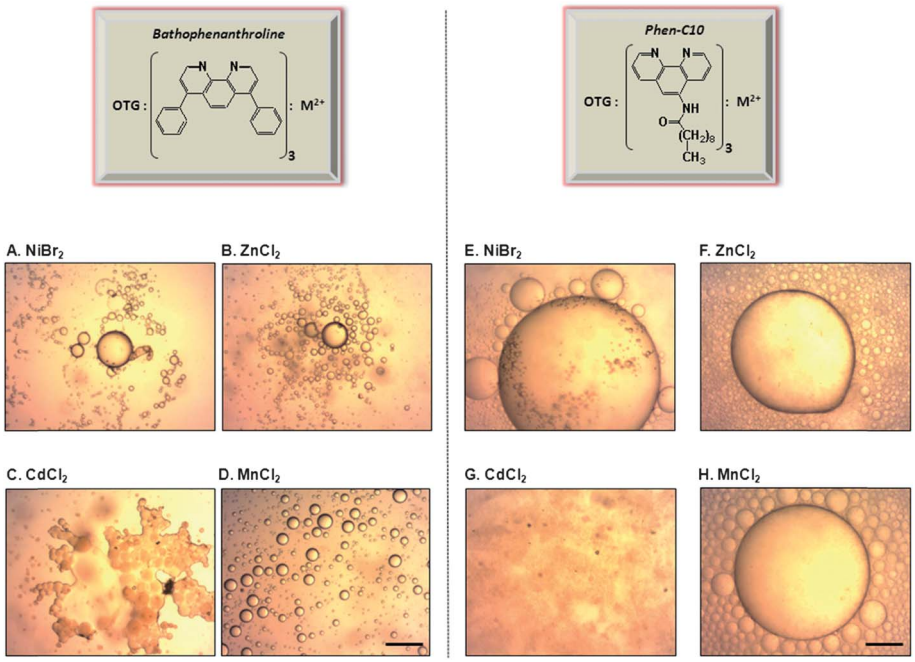

Fig. 6 Tethering *Engineered-micelles* with transition metals other than Fe^{2+}. (A–D) Light microscope images of tethered OTG-*Engineered-micelles*, containing bathophenanthroline and the transition metals indicated. (E–H) As in (A)–(D), but in the presence of *Phen-C10*. Scale bar represents 0.1 mm.

contain on the order of either 1000 or 10^6 close-packed OTG micelles. It should be emphasized that, addition of only the chelator or Fe^{2+} did not show a similar aggregation phenomenon/progression (not shown). The quantitative disappearance of the peak representing independent OTG micelles in parallel with the formation of tethered *Engineered-micelles* became apparent also on a macroscopic scale: precipitation occurred within a few hours. Similar behavior was observed with tethered C8E4-*Engineered-micelles* and OG-*Engineered-micelles*, but not with tethered DDM-*Engineered-micelles* (Fig. 7B). We propose that the significantly smaller diameter of the tethered DDM-*Engineered-micelles* (Fig. 2B) may be the cause for this difference in precipitation behavior. Although Fig. 1 depicts the micellar aggregate as being composed of tethered micelles which preserve their structure, it is clear that the data currently available cannot exclude a different scenario in which, upon tethering, the micelles undergo major structural alterations which may lead to fusion.

Solubilization of hydrophobic guest molecules

The greater solubilization capability of tethered *Engineered-micelles* in comparison with untethered micelles was demonstrated with the water insoluble molecule: fluorenone. Whereas incubation of fluorenone (8 mM) with common OG micelles (22 mM) led to the formation of crystals within an hour (Fig. 8A,

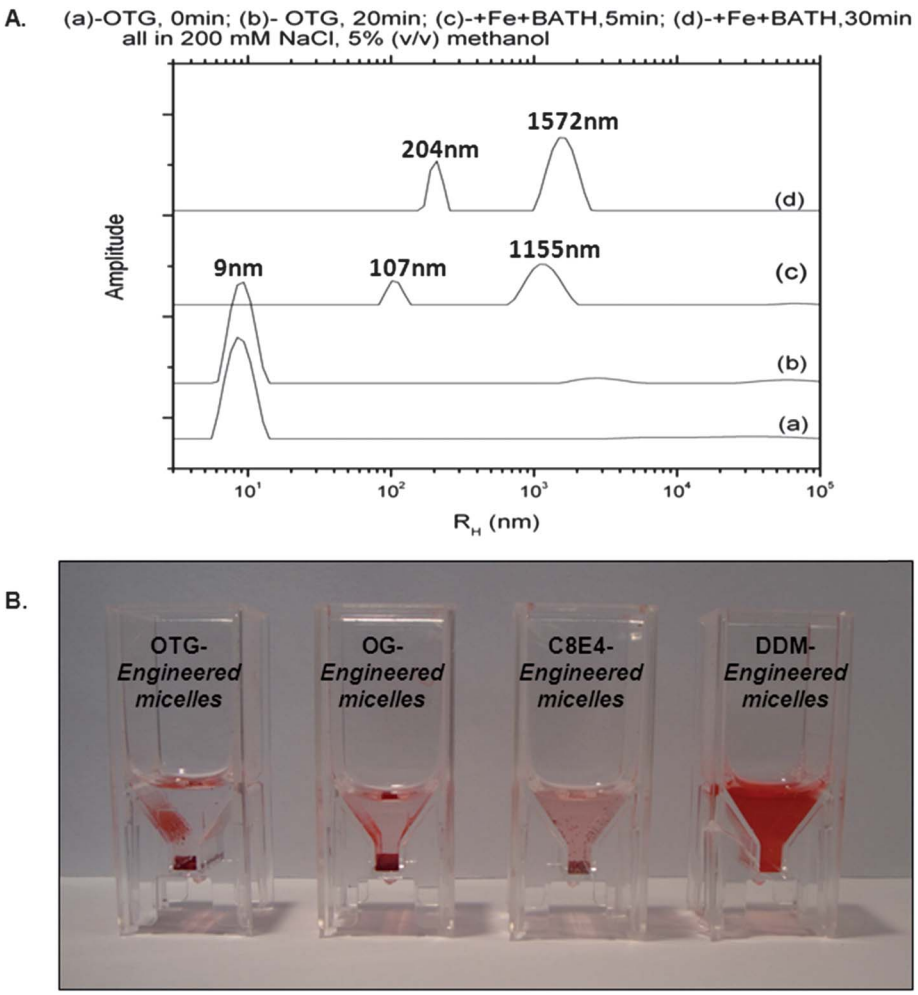

Fig. 7 DLS analysis of the tethering process. (A) DLS size distribution of OTG particles in the absence (a and b) and presence (c and d) of the [(bathophenanthroline)$_3$: Fe^{2+}] complex at the times indicated. (B) Cuvettes containing tethered *Engineered-micelles* composed of the detergents indicated, following a few hours incubation at room temperature.

left), its incubation with tethered OG-*Engineered-micelles* resulted in numerous red oily droplets and suppression of crystallization (Fig. 8A, right). This phenomenon repeated itself with other hydrophobic molecules such as the two antibiotics chloramphenicol (50 mM) (Fig. 8B) and tetracycline (15 mM) (Fig. 8C). These results imply that tethered micelles may represent a novel solubilization matrix for other highly lipophilic therapeutics and allow their administration in the body in addition to the current use of common micelles, soluble polymers and liposomes.[32] However, such an application would of course require additional experimental evidence demonstrating the biocompatibility of the metal, chelator and detergent intended for use in biologically relevant systems. In this regard it is interesting to note that tethered micelles are stable under acidic conditions as well. When experiments with OG or OTG, Fe^{2+} and bathophenanthroline were conducted in the presence of sodium citrate (60 mM) at different pH (5, 4, and 2.8) micellar aggregates similar to those observed at pH 7 were generated (not shown). These results are consistent with the wide pH values in which bathophenanthroline is known to function as a chelator for Fe^{2+}.[33]

Experimental

Materials

Bathophenanthroline, 1,10-phenanthroline, 1,10-phenanthroline-5-amine, tetraethylene glycol monooctyl ether (C8E4), *n*-dodecyl β-D-maltoside (DDM), octyl β-D-glucopyranoside (OG), octyl β-D-1-thioglucopyranoside (OTG), *N*,*N*-dimethyldodecylamine *N*-oxide (LDAO), sodium dodecyl sulfate (SDS), fluorenone, tetracycline, chloramphenicol, NaCl, $FeSO_4$ and $MgCl_2$ were obtained from Sigma-Aldrich (St Louis, MO).

Synthesis of *N*-(1,10-phenanthrolin-5-yl)decanamide (Phen-C10)

Decanoyl chloride (0.5 mL, 2.5 mmol) was added slowly for 30 min to a vigorously stirred solution of 1,10-phenanthroline-5-amine (400 mg, 2.05 mmol) in saturated aqueous sodium hydrogen carbonate solution (18 mL) at 20 °C. The mixture was stirred vigorously till the starting material disappeared (TLC, 3 h) and then extracted with ethyl acetate (3 × 10 mL). A few drops of pyridine were added to the organic layer which was then washed successively with 5% hydrochloric acid (10 mL), 5%

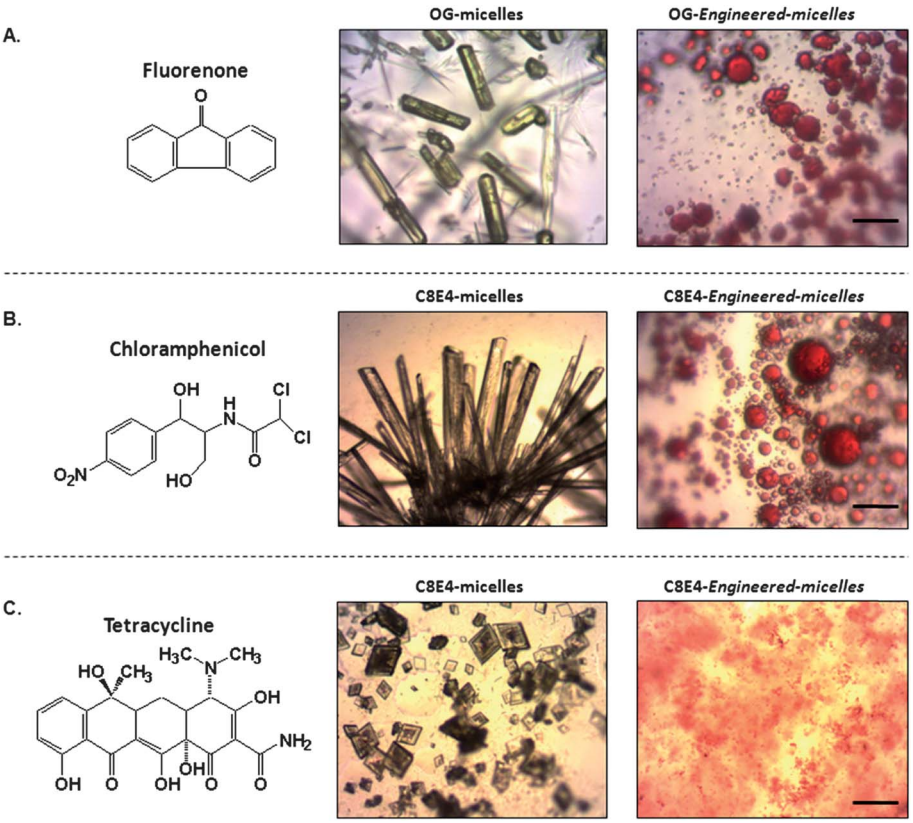

Fig. 8 Solubilization capability of tethered *Engineered micelles*. (A) Fluorenone in the presence of OG-micelles (left) or tethered OG-*Engineered-micelles* (right) one hour after addition of fluorenone. (B) As in (A), chloramphenicol in the presence of C8E4-micelles (left) or tethered C8E4-*Engineered-micelles* (right). (C) As in (B), but in the presence of tetracycline. Scale bar represents 0.1 mm.

sodium hydrogen carbonate solution (10 mL), and water until neutral. The organic layer was dried over anhydrous Na_2SO_4, then concentrated *in vacuo* and the residue was purified by silica gel column chromatography to afford an orange yellow solid. Yield 82%, 590 mg, m.p. 78–80 °C; IR (KBr, cm^{-1}) 2928 (s), 2855 (m), 1663 (m), 1536 (s), 1422 (m), 1217 (m), 758 (vs); ^{1}H NMR (MeOH-d_4, 400 MHz) 0.90 (t, J = 7.0 Hz, 3H), 1.25–1.55 (m, 12H), 1.81 (quint, J = 7.4 Hz, 2H), 2.60 (t, J = 7.4 Hz, 2H), 4.64 (br s, 1H), 7.70 (dd, J = 8.0, 4.4 Hz, 1H), 7.75 (dd, J = 8.4, 4.4 Hz, 1H), 7.98–7.90 (unresolved m, 1H), 8.32 (dd, J = 6.7, 1.5 Hz, 1H), 8.49 (dd, J = 8.4, 1.5 Hz, 1H), 9.00 (dd, J = 2.9, 1.5 Hz, 1H), 9.07 (dd, J = 2.9, 1.5 Hz, 1H); ^{13}C NMR (MeOH-d_4,100 MHz) 14.57, 23.80, 27.03, 30.49, 30.52, 30.58, 30.70, 33.09, 37.58, 122.66, 124.24, 124.86, 126.41, 129.54, 132.64, 132.85, 137.47, 144.97, 146.78, 150.48, 150.81, 176.06; MS (ES^+, Ar) *m*/*z* (rel. intensity) 350 (MH^+, 100), 271 (8), 193 (27); HRMS (ES^+, Ar) calculated for $C_{22}H_{28}N_3O$ 350.2232, found 350.2246.

General protocol for preparation of tethered *Engineered-micelles*

Engineered-micelles composed of non-ionic detergents were prepared by the addition of a freshly prepared 20 mM bathophenanthroline/methanol into aqueous solutions containing 10 mM DDM, 20 mM C8E4, 44 mM OG or 60 mM OTG, with vigorous vortexing. Final bathophenanthroline concentrations were 1 mM, 1 mM, 2 mM and 2 mM respectively. Aliquots (4 μL) of *Engineered-micelles* were placed immediately on siliconized cover slides (Hampton Research, Aliso Viejo, CA) and mixed with an equal volume of an aqueous solution containing 6 mM $FeSO_4$ in 400 mM NaCl. The combined drops were incubated at 20 °C over a reservoir containing 200 mM NaCl in VDX™ crystallization plates (from Hampton Research).

The effect of Mg^{2+}, chelator hydrophobicity and low detergent concentration on the tethering process

The general protocol described above was modified by replacing: (i) 6 mM $FeSO_4$ with 6 mM $MgCl_2$, (ii) 20 mM bathophenanthroline with 20 mM 1,10-phenanthroline or (iii) 44 mM OG with 10 mM OG. All other constituents were unchanged.

DLS measurements of OTG

Samples (100 μL) for DLS measurements were prepared according to the general protocol. However, the concentration of OTG was doubled to allow clear identification of individual micelles.

The effect of the [detergent : chelator] stoichiometric ratio on the efficiency of the tethering process

OG-*Engineered-micelles* were prepared according to the general protocol while increasing only the concentration of OG. All other constituents were unchanged.

Engineered-micelles containing *Phen-C10*

Engineered-micelles containing *Phen-C10* were prepared by replacing only the 20 mM bathophenanthroline solution in the general protocol with a 20 mM *Phen-C10*/methanol solution.

Tethering *Engineered-micelles* with other transition metals

Aliquots (4 μL) of *OTG-Engineered-micelles* containing either bathophenanthroline or *Phen-C10* were incubated with an equal volume of an aqueous solution containing 6 mM $NiBr_4$, 6 mM $ZnCl_2$, $CdCl_2$ or $MnCl_2$, all in 400 mM NaCl.

Solubilization of fluorenone, chloramphenicol and tetracycline with tethered OG-*Engineered micelles*

To 9 μL of freshly prepared tethered OG-*Engineered micelles* (see general protocol above), 1 μL of 80 mM fluorenone in methanol was added, followed by several rounds of gentle aspiration. The 10 μl drop was incubated at 20 °C over a reservoir containing 400 mM NaCl in VDX™ crystallization plates (from Hampton Research). The same protocol was used for the solubilization of chloramphenicol (50 mM in methanol) and tetracycline (15 mM in 25% DMSO : 75% methanol) in the presence of C8E4-*Engineered micelles* (see general protocol above). Control experiments were conducted in the absence of bathophenanthroline and Fe^{2+} ions.

Methods

Light microscopy

Images of hanging drops were obtained using an Olympus CX-40 light microscope equipped with an Olympus U-TV1X-2 digital camera. Particle analysis using ImageJ (NIH) software showed that the size distributions were too broad and irregular for any meaningful statistical analysis to be performed.

Dynamic light scattering

Dynamic light-scattering studies of the aggregational state of the *Engineered-micelles* were carried out using a Viscotek 802 dynamic light-scattering instrument (Malvern Inc., England). OmniSIZE software (Viscotek) was used to calculate the particle size distributions. The sizes reported were obtained by averaging the results of ten scans of 30 seconds each.

Crystal analysis by mass spectrometry

Electron spray ionization (ESI) of extensively washed crystals was performed using a Q-Tof mass spectrometer (Waters, UK).

Conclusion

Engineered-micelles composed of non-ionic detergents and hydrophobic chelators containing the phenanthroline moiety can be tethered specifically with appropriate metal cations. This allows control over the micellar aggregation process and hence, circumvents the need for precipitants, high ionic strength or elevated temperatures. The potential generality of the mechanism presented here derives from its demonstration with two chelators, four non-ionic detergents and five transition metals. It is possible that these engineered detergent aggregates may find use in facilitating membrane protein purification processes, as carriers for hydrophobic therapeutics or in other procedures which require controlled detergent phase separation.

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