Thermal and Structural Behavior of Dioctadecyldimethylammonium Bromide Dispersions Studied by Differential Scanning Calorimetry and X-Ray Scattering

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Abstract

Dioctadecyldimethylammonium bromide (DODAB) is a double chain cationic lipid, which assembles as bilayer structures in aqueous solution. The precise structures formed depend on, e.g., lipid concentration and temperature. We here combine differential scanning calorimetry (DSC) and X-ray scattering (SAXS and WAXS) to investigate the thermal and structural behavior of up to 120 mM DODAB in water within the temperature range 1–70°C. Below 1 mM, this system is dominated by unilamellar vesicles (ULVs). Between 1 and 65 mM, ULVs and multilamellar structures (MLSs) co-exist, while above 65 mM, the MLSs are the preferred structure. Depending on temperature, DSC and X-ray data show that the vesicles can be either in the subgel (SG), gel, or liquid crystalline (LC) state, while the MLSs (with lattice distance d = 36.7 Å) consist of interdigitated lamellae in the SG state, and ULVs in the LC state (no Bragg peak). Critical temperatures related to the thermal transitions of these bilayer structures obtained in the heating and cooling modes are reported, together with the corresponding transition enthalpies.

Introduction

In dilute aqueous solution, and above its gel-to-liquid crystalline temperature (T_m = 45°C), dioctadecyldimethylammonium bromide (DODAB) self-assembles as unilamellar vesicles (ULVs) [1–3], providing potential application, e.g., as a non-viral vector in gene therapy [4]. DODAB concentration and solvent composition, as well as mechanical constraints (e.g., sonication or extrusion), affect the vesicle structure [5–7]. Together, these and other factors allow tuning of vesicle properties for specific purposes and applications.

A wide range of methods has been used to investigate DODAB and other vesicle systems. Among these, differential scanning calorimetry (DSC) and X-ray scattering (SAXS and WAXS) offer particular opportunities for studying thermal and structural aspects of these systems. The profile of the DSC thermograms and X-ray scattering curves can be sensitively monitored as function of various factors, including lipid concentration, ionic strength, pH, equilibration time, or method of preparation, to reveal the structural organization of the lipid bilayers [5,6,8].

DODAB aqueous dispersions are usually prepared by the heating method at a temperature safely above its T_m typically 55–65°C [1,9]. On cooling, the bilayer structures are preserved even though the aggregate structure can change [10,11]. The dominating structures, however, are highly dependent on DODAB concentration [12–14]. The precise concentration range of single ULV or multilamellar structure (MLS) formation is still a subject of controversy [11–13].

Given the remaining uncertainties of the structures formed in this system, we here report on systematic combined investigations of structural and thermal properties in this system over a wide range of concentration and temperature, including systematic variations in sample history, to carefully map the structural and thermal transition in DODAB dispersions. In doing so, we demonstrate that ULV, ULV + MLS, and MLS are the dominating structures up to 1 mM, between 1 and 65 mM, and above 65 mM DODAB, respectively. Enthalpies and hysteresis are furthermore reported for the transition between these states at different DODAB concentrations.

Materials and Methods

DODAB (>98%, Lot # 0001406711) was supplied by Sigma-Aldrich (St. Louis, USA), and used without further purification. Milli-Q quality water was used throughout for sample preparations.

Preparation of DODAB Dispersions

DODAB dispersions were prepared by weighing the appropriate amount of DODAB, followed by water addition to obtain the desired concentration. DODAB/water mixtures were gently stirred magnetically during a few minutes at 65°C to obtain
optically homogeneous dispersions, which were then cooled to room temperature (25°C) and equilibrated for the desired time prior to DSC or X-ray experiments.

DSC Experiments

A VP-DSC Microcalorimeter (Microcal, Inc., Northampton, USA) was used to collect DSC data, and the Origin 7.0 software (supplied by the manufacturer) used to record and analyze the data. Details on the equipment setup can be found elsewhere [12]. Briefly, degassed sample and reference (water) were poured into the (Tantalum, 0.5 ml, non-capillary type) twin cells, and experiments performed by heating or cooling the sample at the desired scan rate (20°C/h, if not stated otherwise) in the temperature range 1–65°C. Thermograms were recorded as changes in heat capacity, at constant pressure, as a function of temperature.

In line with the conventional nomenclature in the area, subgel, gel, and liquid crystalline states refer to the ordered (“freezed”), semi-ordered (“tilty”), and disordered (“melted”) conformational state of the lipid chains in the bilayer structures, respectively. The critical temperatures $T_s$ ($T'_s$) and $T_m$ ($T'_m$) account for the gel-subgel and gel-liquid crystalline transition temperatures, respec-

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Figure 1. Bilayer structures and representative DSC thermograms in DODAB dispersions. Dominant DODAB bilayer structures in water at 1°C and representative heating/cooling thermograms for each structure at 1, 5, and 80 mM. On the thermograms, “#” identifies the endotherm and exotherm related to the MLSs. The remaining peaks are related to the ULVs. The horizontal arrows indicate heating or cooling scans.

![Figure 1](https://doi:10.1371/journal.pone.0044702.g001)

Figure 2. Thermograms of fresh and equilibrated DODAB dispersions. Sequence of (A) heating (curves 1, 3 and 5) and cooling (curves 2, 4 and 6) traces for fresh DODAB dispersion at 0.1 mM, obtained using null pre-scan time at 1 or 65°C. (B) Sequence of heating traces of four-month equilibrated DODAB dispersion at 1.0 mM obtained using null pre-scan time (exception for curve 7, 16 h). The offset between successive traces equals 3.6°C. The horizontal arrows indicate heating or cooling scans.

![Figure 2](https://doi:10.1371/journal.pone.0044702.g002)
tively of the lipids in the vesicle bilayers in the heating (cooling) process. Similarly, $T_p$ ($T'_p$) refer to the subgel-liquid crystalline transition temperature of the lipids in the multilamellar structures (MLS) in the heating (cooling) process. Coagel, finally refer to MLS below $T_p$.

Figure 3. Heating thermograms of a 10 mM DODAB dispersion. Sequence of selected heating thermograms for DODAB 10 mM in water, obtained for $T_i = 25$ C using null pre-scan time, or $T_i = 1$ C using pre-scan times 0, 1, 2, 3,..., 16 h, as indicated. Offset between two successive traces is 0.6 C. The horizontal arrow indicates heating scan.

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Figure 4. Effect of equilibration time on critical temperatures and enthalpy of DODAB dispersions. Effects of pre-scan time on $T_s$, $T_m$, and $T_p$ (A) and transition enthalpies (B) for DODAB 10 mM in water, obtained from the thermograms data analysis.

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X-ray Scattering (SAXS and WAXS)

Simultaneous SAXS and WAXS measurements on DODAB/water samples were performed using a MBraun instrument (Graz, Austria). The dispersions were placed in 2.0 mm diameter Mark-tubes made of glass (Hilgenberg GmbH, Malsfeld, Germany), which were then flame-sealed. A Kratky camera with line collimation was used. CuKα radiation (λ = 1.542 Å) was generated using a Seifert Iso-Debyeflex 3003 generator operating at 2 kW, 50 kV and 40 mA. The camera uses a multiplex to record both small (SAXS) and wide (WAXS) angle scattering. The diffraction pattern was recorded by a MBraun linear position sensitive detector PSD 50 M and stored digitally. The exposure time was 1 h for each sample. According to Bragg’s law, the scattering angle (2θ) is related to the scattering vector q by q = 2π sinθ/λ, and the lattice spacing d = 2π/qBragg, where qBragg is the q vector at the peak maximum [15]. The sample-detector distance was fixed at 300 mm.

Results and Discussion

DODAB Bilayer Structures

Up to 120 mM in the range 1–70°C, DODAB dispersions display the following thermal transitions: an exotherm at Tp=53°C and three endotherms at Tm=36°C, Tm=45°C, and Tm=53°C, on heating, and two exotherms at Tp'=40°C and Tm'=15°C, on cooling. As will be discussed in detail below, DSC and SAXS data demonstrate a progression from unilamellar vesicles (ULVs) at DODAB concentrations below 1 mM, to co-existence of ULVs and MLS species. Together, these findings suggest that the Tp-endotherm at 53°C on heating and the reverse LC-to-SG exotherm at Tp'=40°C on cooling (notice that Tp=Tm~40°C). Above Tp, the MLS revert to ULVs, demonstrated by the SAXS and WAXS curves (discussed below) displaying no Bragg peak.

Between 1 and 65 mM DODAB, the dispersions contain both ULVs and MLSs, the relative amount of MLSs increasing with DODAB concentration, and that of ULVs decreasing, to completely disappear around 65 mM. The thermal behavior of these co-existing structures is similar to those of the single ULV and MLS species.

DODAB Thermal Transitions

The Tc- and Tm-endotherms, as well as their Tp- and Tm reverses, are undoubtedly related to the ULVs [11,16], while the Tp-endotherm and its reverse Tp-exotherm are related to MLSs [11,13,16]. The physical meaning of the Tp-endotherm has not, however, been conclusively elucidated so far. In a previous study, we reported on the Tp-endotherm for DODAB at 5 mM, which was subsequently associated to the presence of dispersed lamellar structures [1,12]. More recently, Coppola et al. [13] related it to a structural transition from ULVs + lamellar fragments to MLVs (multilamellar vesicles) that takes place above 10 mM DODAB, while Wu et al. [11] referred to it as the coagel-to-LC transition, occurring above 7.5 mM DODAB.

As will be discussed below, the DSC data indicate that the Tp-transition accounts for the SG-to-LC transitions within the MLSs, whose reverse transition takes place at Tp=40°C. The SAXS and WAXS data, in turn, indicate that below and above Tp, the dominating structures are multilamellar and unilamellar, respectively. Together, these findings suggest that the Tp-endotherm for the MLSs has similar physical meaning as the Tm-endotherm for ULVs.

Unilamellar Vesicles (ULVs)

Figure 2A depicts a series of heating and cooling thermograms for a fresh aqueous dispersion of DODAB at 0.1 mM. Similar traces were also obtained for the same dispersion equilibrated for six months at 25°C (not shown), indicating considerable vesicle stability. As shown, all thermograms present small single gel-LC transitions at Tm=45.5°C and Tm=40.5°C in the heating and
cooling modes, respectively, with a thermal hysteresis $\Delta T_m = 5^\circ C$, characteristic of ULVs [3,16]. Since the $T_p$-endotherm is not discernible, ULVs are the preferred structure at 0.1 mM.

At 1.0 mM, the heating thermograms for a 4-month equilibrated dispersion displays the $T_s$- and $T_m$-endotherms and their reverse $T'_s$- and $T'_m$-exotherms in the cooling mode (Figure 2B), characteristic of vesicles, with hysteresis $\Delta T_c = 22^\circ C$ and $\Delta T_m = 5^\circ C$, respectively [16]. Thus, the $T_p$-endotherm, characteristic of the SG-to-LC transition of MLSs [10,11], starts appearing at 1.0 mM DODAB, which was consequently taken as the onset of the MLS formation (Figure 1). The intensity of this transition does not change with the longer equilibration time of 16 h at 1°C (curve 7), indicating co-existence of ULVs/MLSs and that the dispersion is dominated by ULVs (larger $T_m$- than $T_p$-endotherm). Similar thermograms were obtained for fresh 1 mM DODAB dispersion (not shown).

The enthalpies of these transitions are: $\Delta H_s = 39$ kJ/mol and $\Delta H_m = 46$ kJ/mol, $\Delta H'_s = 31$ kJ/mol and $\Delta H'_m = 51$ kJ/mol. One should here note that $\Delta H'_m > \Delta H_m$, which is due to a contribution to $\Delta H'_m$ from the $T'_p$-transition, indicating that the 40°C-exotherm is due to both the $T'_m$ and $T'_p$-exotherms.

**Figure 6. Pictures of a fresh and an equilibrated 10 mM DODAB dispersion and representative thermograms.** Top: pictures taken at 25°C of fresh (left) and equilibrated for three days (right) DODAB dispersion at 10 mM. Bottom: Respective heating and cooling DSC thermograms showing that the relative intensity of the $T_p$-endotherm is higher for the equilibrated than for the fresh sample. On the thermograms, “#” identifies the endotherm and exotherm related to the MLSs and the horizontal arrows indicate heating or cooling scans.

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with increasing DODAB concentration and equilibration time. Within this concentration range, the fraction of ULVs thus decreases while that of MLSs increases as DODAB concentration is raised. The effect of equilibration time furthermore illustrates the slow kinetics of the LC-to-SG transition, as reported also previously [10,11,16]. Overall, the height of the $T_p$-endotherm is larger for equilibrated than for fresh samples, while the opposite is true for the $T_s$- and $T_m$-endotherms, indicating the slow formation of MLSs. A sequence of heating/cooling cycles, however, tends to inhibit the $T_p$-endotherm and to increase the relative enthalpies of the $T_s$- and $T_m$-endotherms, as shown in Figures 3 and 4.

The thermograms for a fresh DODAB dispersion at 5 mM (not shown) resemble those for 1 mM (Figure 2B), indicating that the dispersion contains mainly ULVs. After equilibrating for 45 days at 25°C, however, the $T_p$-endotherm at 53°C is clearly discernible (Figure 5A), indicating the presence of MLSs which transform into ULVs on heating past $T_p$ owing to the SG-to-LC transition of the lipids in these structures. As discussed further below, this is indeed compatible with the SAXS data.

Further increase in DODAB concentration results in an increase in the $T_p$-endotherm and a corresponding decrease in the $T_s$- and $T_m$-endotherms. At 10 mM, for example, the height of the $T_p$-endotherm of a fresh dispersion is comparable to those for the $T_s$- or $T_m$-endotherms (Figure 5B). In subsequent scans, the enthalpy of the $T_p$-endotherm decreased, while those at $T_s$ and $T_m$ increased (Figure 5B), indicating that the amount of MLSs was

**Figure 7.** Effect of scan number and DODAB concentration on the transition enthalpies. (A) Effect of scan number on the $T_s$, $T_m$, and $T_p$-enthalpies of DODAB dispersion at 10 mM, and (B) effect of DODAB concentration on the $T_s$, $T_m$, and $T_p$-enthalpies.

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**Figure 8.** Effect of DODAB concentration on the thermogram profile. Effect of DODAB concentration on the heating (A) and cooling (B) thermograms. Pre-scan time 15 min for all scans. Numbers 20 to 80 besides the curves refer to DODAB concentration (mM). The horizontal arrows indicate heating or cooling scans.

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reduced after thermal cycling, again due to the slow LC-to-SG transition [11,16]. These data clearly indicate a higher fraction of MLSs at 10 than at 5 mM, and also in well equilibrated samples.

The effect of pre-scan time is shown in Figure 3 for a fresh 10 mM DODAB dispersion. As seen, there is a pronounced change in enthalpy after the first thermal cycle. Subsequently, the Tp-enthalpy decreases smoothly to a plateau after 5 h, while the Ts- and Tm-enthalpies increase quickly to a plateau. These data illustrate that an equilibration time larger than 16 h is necessary to complete the LC-to-SG transition.

Figure 6 shows two pictures of the same 10 mM DODAB dispersion taken at 25 °C, one freshly prepared (top left) and the other equilibrated for three days at 25 °C (top right), along with their characteristic heating and cooling thermograms. Together with the DSC and X-ray data, the transition from a clear to a milky system with equilibration indicates that the system is initially dominated by ULVs, which revert to MLSs after equilibration.

Figure 7A shows the effect of the number of thermal cycles on the enthalpy of the Ts-, Tm-, and Tp-endotherms for DODAB 10 mM. On increasing the number of cycles, the Tp-enthalpy decreases, while the Ts- and Tm-enthalpies increase, indicating that freshly cooled dispersions are rich in ULVs, while MLSs dominate in equilibrated dispersions, as shown also in Figure 6. The concentration obviously plays an important role for the structural organization of DODAB aggregates in this concentration range. Overall, as DODAB concentration increases, the Tp-enthalpy increases, while the Ts- and Tm-enthalpies decrease to disappear around 65 mM (Figure 7B). The decrease in Tp-enthalpy above 30 mM DODAB is an artifact due to the high intensity of the Tp-peak which appears truncated in the thermograms (Figure 8).

The Tm- and Ts-endotherms at 45 and 36 °C, respectively, vanish when DODAB concentration approaches 65 mM (Figure 8A), indicating that with increasing concentration, ULVs are progressively replaced by MLSs. In the cooling mode, both the Ts- and Tm-exotherms can be distinguished in the mixed region (Figure 8B). At 20 mM DODAB, the Ts-, Tm-, Tp-, T's- and T'm-enthalpies are 19, 23, 43, 13 and 35 kJ/mol, respectively. Again, the T'm-enthalpy is larger than the Tm-enthalpy, indicating that in...
the T<sub>m</sub>-exotherm there is also a contribution from the reverse T<sub>p</sub>-transition.

Multilamellar Structures (MLSs)

Beyond 65 mM, the dispersions are dominated by MLSs in the SG or LC state, respectively, below or above T<sub>p</sub>, in the heating, or below and above T<sub>p</sub>, in the cooling process. Upon heating from 1 to 65 °C, the thermograms of the MLS dispersions display a single T<sub>p</sub>-endotherm at 53 °C, characteristic of the SG-to-LC transition [11], in a similar way to samples from the mixed (MLV + MLS) region. Upon cooling, the thermograms display a single T<sub>p</sub>-exotherm at 40 °C, due to the LC-to-SG reverse transition. The SG-LC transitions for the MLSs thus present a thermal hysteresis ΔT<sub>p</sub> = 13 °C.

The lower boundary (65 mM) of the single MLS region (Figure 1) is lower than previously reported in literature [11,13], probably because the samples were submitted to equilibration times sufficiently long for the MLS LC-to-SG transition to be complete before starting the heating scans.

X-ray Scattering

Samples rich in MLSs were also investigated by SAXS and WAXS as a function of temperature (Figure 9). On increasing the temperature from 1 to 50 °C, the SAXS curves for 120 mM DODAB (Figure 9A) show a well-defined Bragg peak with peak maximum at 0.172 Å<sup>-1</sup>, independently of temperature, which gives a lattice distance d = 36.7 Å, in good agreement with previously reported data [11,16]. As the temperature approaches T<sub>p</sub> = 53 °C, the Bragg peak becomes sharper and more intense, indicating structural growth. No peak was obtained at 60 and 70 °C, indicating that above T<sub>p</sub> = 53 °C, the multilamellar structures in the LC state transform into unilamellar bilayers (probably ULVs).

On cooling, the Bragg peak starts appearing at the same position at 40 °C, in good accordance with the DSC data, presenting a single exotherm at T<sub>p</sub> = 40 °C (Figure 8). The intensity of the Bragg peaks obtained in the cooling is lower than those in the heating process. This also might be related to the slow kinetics of gel formation in the cooling process, as indicated by DSC (Figure 8) and also reported elsewhere [11,16]. Similar behavior was obtained for the SAXS and WAXS curves for 40 mM DODAB dispersion, within the ULV + MLS region, except for the occurrence of lower intensity peaks (Figure 10), indicating that the MLSs are similar in both the single MLS and mixed ULV/MLS dispersions.

Using synchrotron X-ray radiation, we previously observed a Bragg peak at a DODAB concentration of 5.0 mM, but not at 1.0 mM [14], indicating that the onset of MLS formation lies between 1 and 5 mM, in good agreement with the present DSC results.

The extended chain length of DODAB can be estimated from \( l_{\text{max}} = 1.3 + 1.265n \), where \( n \) is the number of hydrocarbons per lipid chain. This gives \( l_{\text{max}} = 24.8 \AA \), suggesting a bilayer thickness of 50 Å. Since \( d \) was found to be considerably smaller than this (37 Å), our SAXS data suggest that the DODAB molecules are interdigitated in the MLS bilayers. A similar conclusion was previously reached for dilute (5 mM) DODAB dispersions [14]. Because of this, the “melting” temperature (T<sub>m</sub>) of DODAB in MLSs is likely to be larger than that in ULVs (T<sub>m</sub>), as also reported for 1,2-dihexadecyl-sn-glycero-3-phosphatidylcholine bilayers [18].

Since there is no change in the Bragg peak position of DODAB MLSs up to 50 °C, the gel-to-LC (chain “melting”) temperature (T<sub>p</sub>) must be above 52 °C. This suggests that T<sub>p</sub> = 53 °C is actually the “melting” temperature of DODAB in MLSs, equivalently to T<sub>m</sub> ≈ 45 °C for ULVs.

The WAXS curves (Figure 9B) also support these findings. Up to 50 °C, there are four well-defined peaks characteristic of macroscopically ordered structures, such as MLSs (or coagel), while above 50 °C, there is no peak at all, characteristic of isotropic structures, such as ULVs. In the cooling process, a reverse thermal behavior was observed, in good accord with that obtained by SAXS (Figure 9A).

In Figure 10A and 10B we see similar SAXS and WAXS curve profiles for DODAB at 40 mM, in the ULV/MLS mixed dispersions, indicating identical structures to the single MLS dispersion.

Conclusions

Based on DSC and SAXS/WAXS, preferred structures in DODAB dispersions were found to depend on concentration, temperature, and sample history. Up to 1 mM, ULVs dominate, displaying two heating endotherms at T<sub>p</sub> = 36 °C and T<sub>m</sub> ≈ 45 °C, and their cooling reverses at T<sub>p</sub> = 14 °C and T<sub>m</sub> = 40 °C, respectively. Above 65 mM, the dispersion is dominated by MLSs and ULVs, respectively, below and above T<sub>p</sub> = 53 °C in the heating, or below and above T<sub>p</sub> = 40 °C in the cooling process. The subgel-liquid crystalline transitions in MLSs thus display hysteresis ΔT<sub>p</sub> = 13 °C. Interestingly, T<sub>p</sub> > T<sub>m</sub> while T<sub>p</sub> = T<sub>m</sub>. The larger T<sub>p</sub> value was related to an interdigitated conformation of DODAB chains in the MLS bilayers. ΔT<sub>p</sub> = ΔT<sub>m</sub> indicates the chain “freezing” (reverse to “melting”) is slower for MLSs than for ULVs, probably because of the chain interdigitation. In the intermediate 1–65 mM, MLSs and ULVs co-exist. It was also shown that sample thermal history plays important role for the structures formed in this system, demonstrated to be due the slow LC-to-SG transition. Finally, it was shown that T<sub>p</sub> = 53 °C is actually the “melting” temperature of DODAB in MLSs, equivalently to T<sub>m</sub> ≈ 45 °C for ULVs.

Supporting Information

Table S1 Summary of DODAB aggregate structures at different temperature and DODAB concentration. Here, CS refers to samples cooled from T<sub>f</sub> = 65 °C, WS to samples warmed from T<sub>i</sub> = 1 °C, LC to Liquid-crystalline, ULV to unilamellar vesicles, and MLS to multilemellar structures. (DOC)

Author Contributions

Conceived and designed the experiments: EF MM. Performed the experiments: RDA PH EF. Analyzed the data: RDA PH. Contributed reagents/materials/analysis tools: MM. Wrote the paper: EF MM.

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