Spectroscopy and Recognition Chemistry of Micelles from Monoalkyl Phosphoryl Nucleosides

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We describe the properties of aqueous micelles obtained from *n*-alkyl phosphoryl nucleosides, in particular *n*-hexadecylphosphoryladenosine (C16-AMP), uridine (C16-UMP), and -cytidine (C16-CMP). These compounds were obtained enzymatically. It is shown that each of these compounds form micelles spontaneously in water with a critical micelle concentration in the range of 20−35 μM and an aggregation number of 69, which indicates that the chemical structure of the bases has no significant influence on the aggregation behavior. UV-absorption and circular dichroic measurements suggest that the nucleoside is in an aqueous environment, as expected from the amphiphilic character of the compounds. UV absorption suggests a moderate self-stacking among the bases for each type of micelle. When we mixed micelles bearing complementary bases with each other (e.g., C16-AMP with C16-CMP), a weak hypochromic effect was observed, which can be taken as an indication of complementary base interaction. However, such electronic perturbation was observed also in noncomplementary bases, e.g., when C16-CMP micelles were mixed with C16-UMP micelles. These micelle data are compared with the corresponding data obtained with liposomes obtained from phosphatidyl nucleosides. All together, these data illustrate a novel type of polymeric nucleoside interaction with which no covalent bonds form among the monomers, and in which the nucleobases are distributed as a supramolecular spherical aggregate.

Introduction

During the past few years we and other research groups have become interested in lipids bearing a covalently attached nucleobase.[1−4] These compounds are of interest because they link the chemistry of lipids with the chemistry of nucleic acids, in addition to having therapeutical relevance.[5−7] Particularly attractive are liposomes from phosphatidyl nucleosides, obtained from complementary nucleobases, e.g., (1,2-dioleoyl-*sn*-glycero-(3)phosphatidyl)-adenosine and (1,2-dioleoyl-*sn*-glycero(3)-phosphatidyl)-uridine, because they permit one to question whether and to what extent they are able to display the complementary binding and recognition properties that are characteristic of the linear DNA macromolecules. The work describing these binding properties with liposomes was published elsewhere.[8] One problem, which became apparent during such studies, was that the recognition and binding between nucleobases requires several days for reaching equilibrium. This is because the dynamics of lipids within and among the liposomes is per se an extremely slow process.

Micelles, albeit not possessing the double layer of liposomes and the corresponding analogy with biological membranes, offer the advantage of a high dynamic and of a much greater structural simplicity.

In this article, we describe the preparation and properties of aqueous micelles obtained from *n*-hexadecylphosphoryladenosine (C16-AMP), *n*-hexadecylphosphorylcytidine (C16-CMP), and *n*-hexadecylphosphoryluridine (C16-UMP). In particular, we describe their aggregation properties as measured by UV-analytical ultracentrifugation, as well their UV-absorption and circular dichroism (CD) spectroscopic properties.

The mutual binding properties of complementary micelles (e.g., those obtained from C16-AMP with those obtained from C16-UMP) will be studied by UV spectroscopy. It will be shown that these spectral changes, despite being less specific than in liposomes, correspond to a novel kind of micellar chemical recognition and spectroscopy.

Materials and Methods

Reagents. Phospholipase D from *Streptomyces* sp. AA586 (PLD) was from Genzyme Diagnosics (West Malling, Kent, UK) and was delivered from batches with different activities (108 and 147 U/mg). Chloroform for the synthesis was stabilized by Amylen (Merck). All the other reagents were from Fluka, Sigma, or Merck at the highest purity grade.

Merck silica gel plates (60 F254) were used for analytical thin-layer chromatography. Chromatographic separations were carried out on 70−230-mesh Merck silica gel.

Instrumentation. UV measurements were conducted using a Varian Cary 1E or an Uvikon 820 spectrophotometer (Kontron, Zurich, Switzerland). The cells were quartz cells from Hellma with a defined path length of 1.0 or 0.5 cm. For the difference spectra special half-cells were used. CD spectroscopy was recorded on a JASCO J-600. The cells used for the measurement had a path length of 1.0 cm.

Ultracentrifugation experiments were performed with an analytical ultracentrifuge (Beckmann Optima XL-A) equipped with scanning absorption optics and full online data capture and analysis facilities (Beckmann, Palo Alto, CA; Gieber 1992). Multichannel cells of 12-mm optical path length were used (3 solution/solvent pairs) and the temperatures quoted were those

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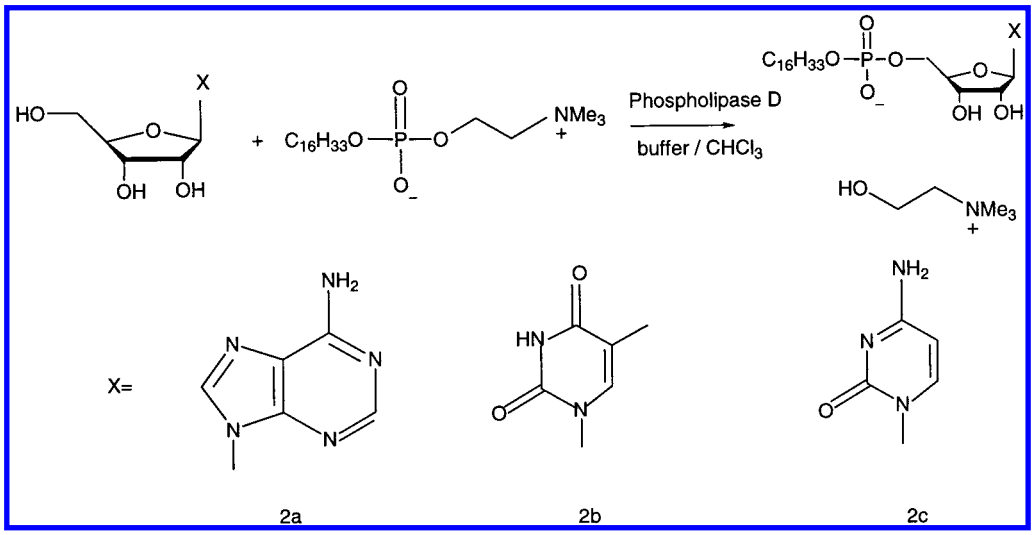
SCHEME 1: Preparation of *n*-Hexadecylphosphonucleosides



reported by the instrument. Densities were measured with a DSA 48 Densitometer from AP Paar, Austria. All data sets were fitted using Origin 2.8 from Microcal.

^{1}H, ^{13}C, and ^{31}P NMR spectra were recorded on 500- and 200-MHz spectrometers (Bruker). Chemical shifts are relative to tetramethylsilane and to 85% H_3PO_4 (external), respectively.

The starting material *n*-hexadecylphosphorylcholine was synthesized following a procedure of Erukulla et al.[9] Purification was made by flash chromatography (p = 0.2 bar, MeOH) on silica gel yielding 73%. The purification was proved by NMR.

Synthesis. *General Procedure.* The nucleosides (8 mmol) and PLD (900 units) were dissolved in 20 mL of an appropriate buffer (**2a** and **2b** = 250 mM $CaCl_2$, 200 mM acetate buffer, pH = 5.8; for **2c**, the pH was adjusted to pH 4.5) and *n*-hexadecylphosphorylcholine (1.9 mmol) in 60 mL $CHCl_3$ was added. The so obtained turbid suspension was stirred at 45 °C. The reaction was monitored by thin-layer chromatography and stopped after 5−6 h by adding 5 mL 0.1 M HCl. The mixture was extracted with $CHCl_3$/MeOH (2:1). The product was in the organic phase.

*n-Hexadecylphosphoryladenosine (**2a**).* The synthesis was done as described in the general procedure. After removal of the solvent under reduced pressure, the product was purified by chromatography on silica gel ($CHCl_3$/MeOH/NH_4OH = 85:15:2), R_f ($CHCl_3$/MeOH/NH_4OH = 85:10:2) = 0.12. ^{1}H NMR: (δ-dimethyl sulfoxide [DMSO]): 8.32 (1H, s); 8.15 (1H, s); 7.42 (2H, b); 5.91 (1H, d, J = 5.45 Hz); 4.59 (1H, d, J = 5.2 Hz); 4.20 (1H, d, J = 4.2 Hz); 4.08 (2H, m); 3.98 (1H, m); 3.80 (2H, d, J = 6.8 Hz); 1.49 (2H, m); 1.22 (26H, m); 0.84 (3H, t, J = 6.7 Hz). ^{31}P NMR: (δ-DMSO): 0.13. ^{13}C NMR: (δ-DMSO): 150.6 (*s*, C-6); 148.9 (*s*, C-2); 144.2 (*d*, C-4); 142.6 (*d*, C-8); 89.3 (*s*, C-1′); 84.1 (*s*, C-4′); 75.4 (*s*, C-2′); 70.5 (*d*, C-3′); 68.0 (*t*, C-5′); 66.0 (*t*, C-α (CH_2); 32.2 (*t*, 2C); 30.7 (*t*); 30.6 (*t*); 29.9−29.5 (several *t*), 25.8 (*t*, 2C); 22.9 (*t*; 2C); 14.2 (*q*; Me).

*n-Hexadecylphosphoryluridine (**2b**).* The synthesis was done as described in the general procedure. After removal of the solvent under reduced pressure the product was purified by chromatography on silica gel (400 mL $CHCl_3$/MeOH = 3:1, then $CHCl_3$/MeOH = 3:80; p = 0.2 bar). The organic solvent was evaporated to half. This solution was treated with NH_4OH and concentrated in a vacuum. R_f ($CHCl_3$/MeOH/NH_4OH = 80:30:2) = 0.24. ^{1}H NMR in DMSO: δ = 0.83 (3H, t, J = 6.7 Hz); 1.21 (26H, m); 1.42 (2H,m); 3.60 (1H, d, J = 6.3 Hz); 3.77 (2H, b); 3.91 (2H, m); 4.02 (2H, m); 5.56 (2H, m); 5.76 (1H, d, J = 5.5 Hz); 7.90 (1 H, d, J = 5.98); 11.27 (1H, b).^{31}P NMR: (δ-DMSO): −0.56. ^{13}C NMR: (δ-DMSO): 163.5 (s, C-4); 151.2 (s, C-2); 141.4 (d, C-6); 102.3 (d, C-5); 88.3 (d, C-1′); 84.2 (d, C-4′); 73.9 (d, C-2′); 70.9 (d, C-3′); 64.6 (t; C″-α); 31.8 (t); 30.9 (t); 29.5 (several t); 29.20 (t); 29.2 (t); 25.7 (t); 22.54 (t); 14.40 (q; Me).

*n-Hexadecylphosphorylcytidine (**2c**).* The synthesis was done as described in the general procedure. After removal of the solvent under reduced pressure the product was purified by chromatography on silica gel ($CHCl_3$/MeOH/NH_4OH = 65:80:8). The lipid was converted into the NH_4-salt by dissolving it in 40 mL $CHCl_3$/MeOH (1:1) and adding 15 mL NH_4OH. After 5 min stirring at room temperature the solvent was slowly evaporated. The remaining lipid was dried overnight (HV). R_f ($CHCl_3$/MeOH/NH_4OH = 68:80:8) = 0.36. ^{1}H NMR in $CDCl_3$/MeOH (1:1): δ = 0.88 (3H, t, J = 6.77 Hz); 1.26 (26H, b); 1.63 (2H, m); 3.87 (1H, d, J = 6.4 Hz); 4.12 (2H, b); 4.16 (2H, m); 4.23 (2H, m); 5.96 (1H, d, J = 5.25 Hz); 8.1 (1H, d, J = 7.52 Hz). ^{31}P NMR ($CHCl_3$/MeOH: 0.7235). ^{13}C NMR: (δ-DMSO): 156.55 (*s*, C-4); 155.3 (*s*, C-2); 141.1 (*d*, C-6); 142.6 (*d*, C-5); 89.3 (*s*, C-1′); 84.1 (*s*, C-4′); 75.4 (*s*, C-2′); 70.5 (*d*, C-3′); 68.0 (*t*, C-5′); 62.9 (C2″-α); 31.75 (t); 30.40 (t); 29.05 (several t); 28.94 (t); 28.65 (t); 25.45 (t); 22.00 (t); 13.83 (q, Me).

Critical Micelle Concentration Determination. Using the colorimetric method with pinacyanol chloride and rhodamine 6G, we carried out the critical micelle concentration (cmc) determinations. Eight microliters of a 1.3 mM methanolic solution of pinacyanol chloride (respectively, rhodamine 6G) were added to 1 mL of an appropriate aqueous solution of the phosphate-containing surfactant. In pinacyanol chloride the absorbance at 605 nm was plotted against surfactant concentration, and the cmc value was taken as that surfactant concentration at which the absorbance at 605 nm increases. In rhodamine 6G the wavelength with the maximal absorbance was plotted against the concentration. Unless otherwise specified, the final concentration of pinacyanol chloride was always 10 μM.

Results

Preparation and Characterization. Synthesis of the *n*-Hexadecylphosphoryl Nucleosides. The three *n*-hexadecylphosphoryl nucleosides mentioned above were synthesized enzymatically in a two-phase $CHCl_3/H_2O$ system from *n*-

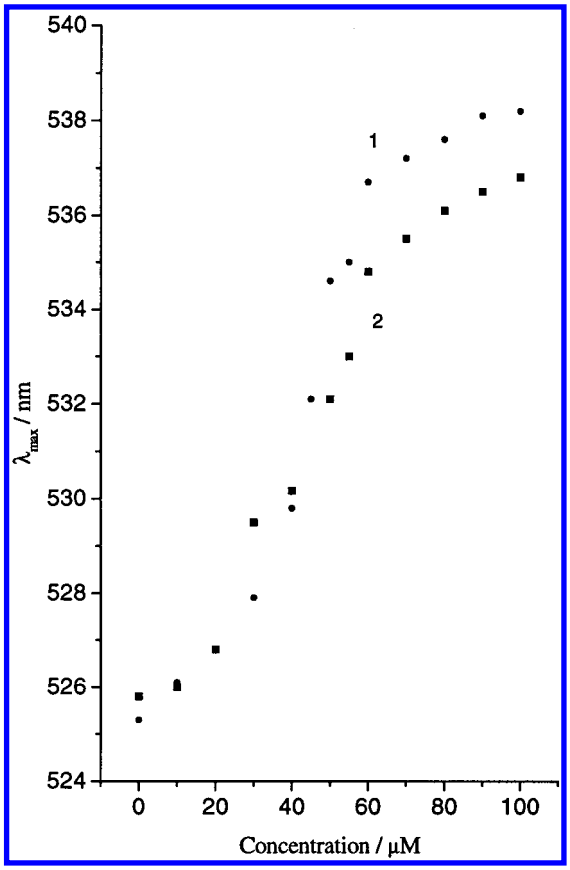


Figure 1. CMC determination of (1) C16-UMP and (2) C16-CMP with rhodamin 6G. The wavelength at which maximal absorbance occurs is represented as a function of the lipid concentration of the solution.

hexadecylphosphorylcholine and the corresponding acceptors (Scheme 1) using PLD from *Streptomyces* sp. AA 586 as a catalyst.[10,11]

	acceptor		aq phase	yield, %
2a	adenosine	(C16-AMP)	A	54
2b	uridine	(C16-UMP)	A	56
2c	cytidine	(C16-CMP)	B	63

A, 250 mM $CaCl_2$, 200 mM acetate buffer, pH = 5.8.
B, all as in A, but pH adjusted to pH 4.5.

Whereas this enzymatic reaction was successful for the three bases adenosine, uridine, and cytidine, we could not obtain any significant yield with guanosine. The reason is still unclear.

The three surfactants so prepared are able to build micelles in the neutral-alkaline pH range. The cmc was determined by UV spectroscopy, based on the color changes of hydrophobic dyes, in particular pinacyanol chloride and rhodamine 6G, both of which were used previously.[12] Typical results are given in Figure 1.

The cmc values, as indicated in Table 1, are all in the range of 20−35 μM. It appears that the chemical structure of the aromatic base does not influence the cmc value significantly.

The aggregation state of C16-AMP was determined using analytical ultracentrifugation at several concentrations and speeds (Table 2). The final concentrations were determined by UV absorption at 260 nm (ϵ = 12 027 L mol^{-1} cm^{-1}), 280 nm (ϵ = 2933.9 L mol^{-1} cm^{-1}), 285 nm (ϵ = 1199.7 L mol^{-1} cm^{-1}). The partial specific volume of C16-AMP (0.823 cm^3 g^{-1} mol^{-1}) was determined using eq 1 at three concentrations.[13]

TABLE 1: CMC Values and Extinction Coefficients of *n*-Hexadecylnucleosides

compound	cmc μM	ϵ/10^3 L mol^{-1} cm^{-1}
C16-AMP	35	12.0
C16-UMP	20	8.1
C16-CMP	15	6.8
HDP-Cholin	13	
AMP		15.4
UMP		10.0
CMP		9.1

TABLE 2: Conditions of the Analytical Ultracentrifugation Experiments

	mass at		
conc. mM	15 000 rpm	20 000 rpm	25 000 rpm
0.120	40377	40190	39151
0.160	40130	40651	40046
0.200	41381	39422	38539
0.241	40346	39029	37888
0.281	40102	39892	40173
0.321	40032	38983	38711
0.361	39153	38043	38568
	$\bar{x}$ = 40181.4	39458.6	39010.9
	±541.7	807.2	775.6

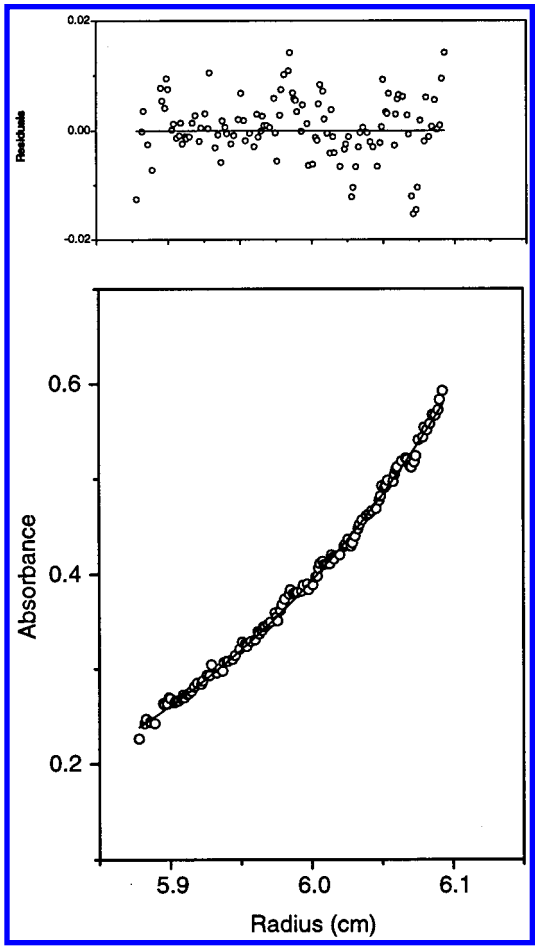


Figure 2. Typical data of the analytical ultracentrifugation studies.

$$\bar{v} = \frac{1}{\rho_0}\left(\frac{\rho - \rho_0}{m}\right) \qquad (1)$$

where $\bar{v}$ = partial specific volume lipid solution, ρ = density of solution, and m = monomer mass.

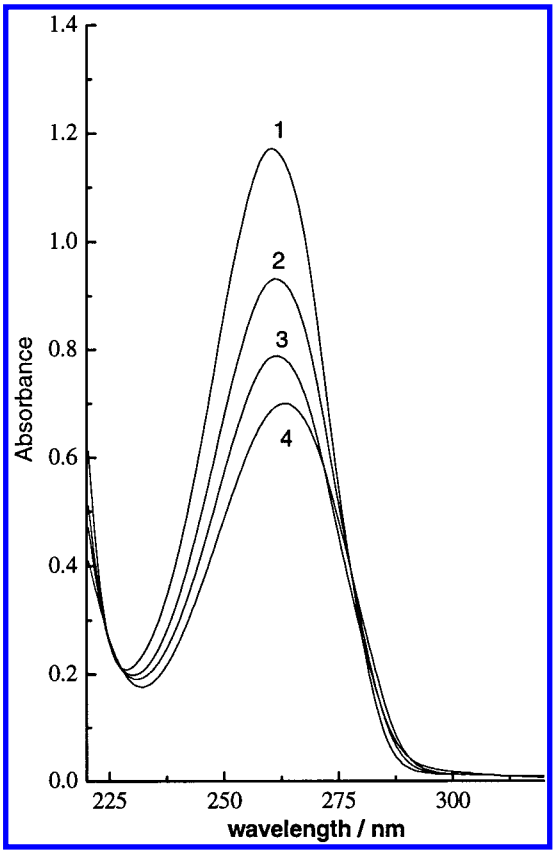


Figure 3. UV spectra of micelles from *n*-hexadexylphosphonucleosides: (1) C16-AMP, (3) 1:1 mixture and (4) C16-UMP measured in 2 mM NaOH. The average UV spectrum (2) of the two components C16-AMP and C16-UMP is shown for comparison. The lipid concentration of all the solutions was 90 μM.

The mass of the Scatchard component was calculated with eq 2.[13]

$$M_s = M_{Lipid} + \frac{1}{2}z\left(\frac{m_1}{m_1+m_2}\right)M_{Cation} + \frac{1}{2}z\left(\frac{m_1}{m_1+m_2}\right)M_{Cation2} + \frac{1}{2}zM_{Anion} \quad (2)$$

Since under these conditions (100 mM NaCl, 50 mM Tris, pH 8.2) the monomer concentration is very small compared with the micelle concentration, we used a monomer model for the fitting procedure.

$$c = c_0 e^{M(1-\upsilon\rho)\omega^2(r^2-r_0^2)/2RT} \quad (3)$$

where c = concentration of the lipid solute at any radial distance, c_0 = concentration of the lipid solute at the reference radial distance r_0, M = molecular weight, ω = rotational velocity in radians/s, R = gas constant, and T = absolute temperature.

A typical fit is shown in Figure 2. The mass was corrected for charge effects by the following procedure[14] (eq 4) to get the aggregation number **69** $\pm$ **1**.

$$N = \frac{\frac{s}{A_2'}}{1 - \frac{z^2 m_2 s}{[2(m_3+m_5)A_2']}} \quad (4)$$

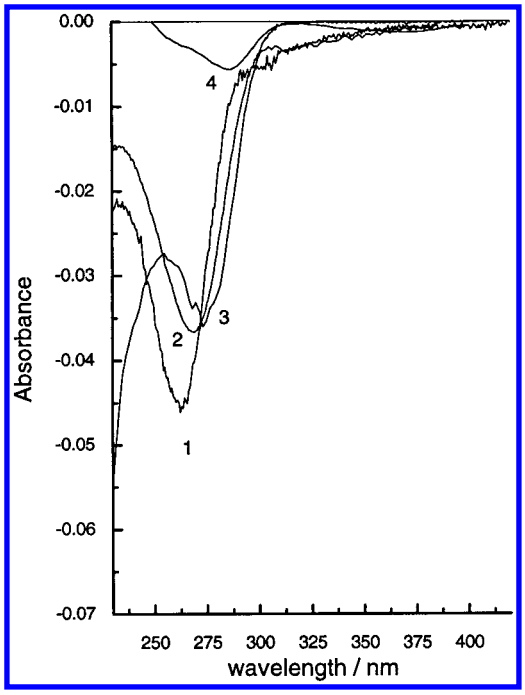


Figure 4. Interaction between complementary and noncomplementary bases: (1) C16-AMP and C16-UMP, (2) C16-UMP and C16-CMP, (3) C16-AMP and C16-CMP, and (4) C16-AMP and C16-choline. The special half-cells allowed us to take the baseline of two different micellar solutions without mixing them. After shaking, the two solutions could mix, and the difference spectra could be measured. All samples had a total lipid concentration of 100 μM.

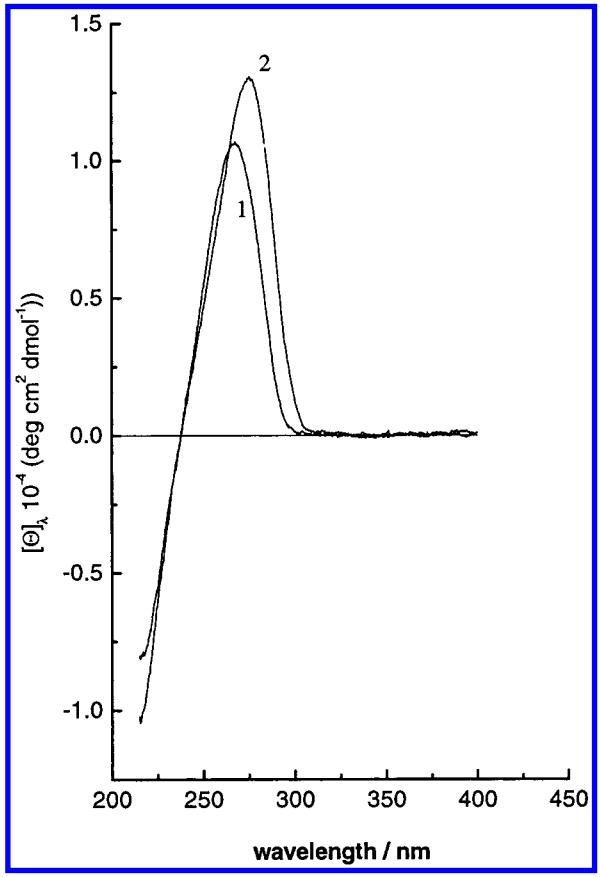


Figure 5. Comparison of the spectra of micelles from C16-CMP (1) with the spectra of the corresponding nucleotides (cytidine monophosphate) (2) in water.

$S = \mathrm{d}\ln(c)/\mathrm{d}r^2$; $A_2' = M_2'(1-\rho)\omega^2/2RT$; M_2' = molecular mass of the monomeric Scatchard component.

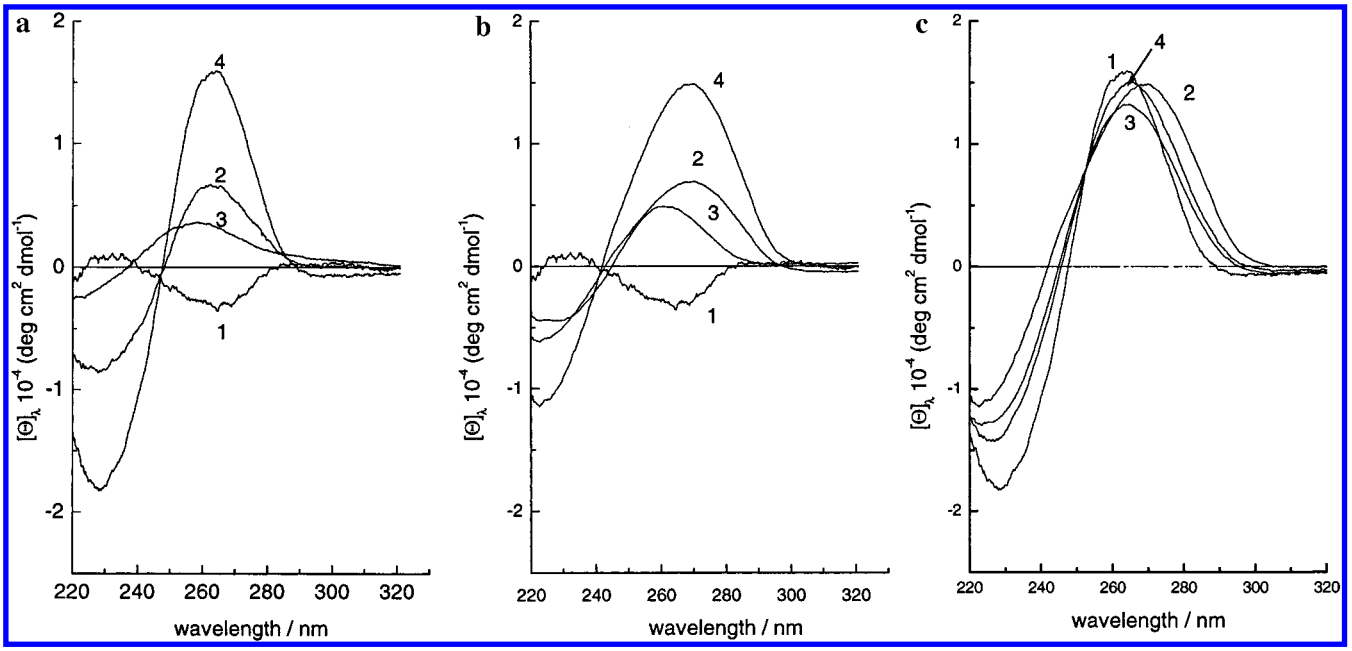


Figure 6. CD spectra of different micellar solutions. The total lipid concentration of all the solutions was 90 μM. (a) (1) C16-AMP, (3) 1:1 mixture, and (4) C16-UMP measured in 2 mM NaOH. The average UV spectrum of the two components C16-AMP and C16-UMP is (2) shown for comparison. (b) (1) C16-AMP, (3) 1:1 mixture, and (4) C16-CMP measured in 2 mM NaOH. The average UV spectrum of the two components C16-AMP and C16-CMP is (2) shown for comparison. (c) (1) C16-UMP, (4) 1:1 mixture, and (2) C16-CMP measured in 2 mM NaOH. The average UV spectrum of the two components C16-CMP and C16-UMP is (3) shown for comparison.

As far as the structure of the micelles is concerned, we can reasonably assume that the hydrophobic alkyl chains build the core of the micelles. The hydrophilic phosphate groups, as well as the ribose units, are exposed to water; therefore, the bases also should be at least partially facing water.

In principle this structure could be checked by spectroscopy, for example, by NMR. However it was not possible to compare the NMR signals of the micellar solution with the NMR signals of the corresponding monomeric monophosphate in water because of concentration limits. The NMR sensibility was not sufficient in the micromolecular concentration range (at which micelles are formed), and in the concentration range required for NMR oil droplets were formed.

UV-absorption spectroscopy was carried out. Note from Table 1 that the UV-absorption extinction coefficients of the bases in the micellar solutions are smaller than those of the corresponding monophosphate. This could be ascribed to self-stacking in the micelle, resulting in a small hypochromic effect. An indication of this could be obtained by adding salt to the solution to disrupt the micelles. Consequently, the absorbance of the surfactant solution rises to the value of the corresponding monophosphate. The absorption properties of the micellar solutions have been measured in a wide range of concentrations, and the optical density increases linearly with concentration. Of some interest is whether the nucleobases in the micelles are able to display the classic complementary binding, i.e., in our case (lacking guanine) a specific adenine−uracile binding. In fact, C16-AMP and C16-UMP micelles can be seen as polymeric aggregates of A and U, respectively, and in principle, then, capable of displaying the complementary binding observed between polyA and polyU.

To study the possible complementary binding, we studied the absorption of the mixed micellar solution obtained by mixing the micelles from C16-UMP with those of C16-AMP. Note from Figure 3 that the absorption is lower than the value calculated as the algebraic average. This may suggest an electronic perturbation due to a specific interaction between the complementary bases A and U. However, a similar effect, although of smaller intensity, is observed also by mixing C16-UMP micelles with C16-CMP (see Figure 4), i.e., with noncomplementary bases. It is not easy to give a precise interpretation of these spectroscopic effects. Apparently, this is a heterologus interaction (e.g., between chemically different bases) which is larger than the self-stacking effect; and it appears also that the effect due to complementary base interaction is the largest effect. However, the later is not as large as one would have expected, and it is actually less specific than that found in liposomes obtained from phosphatidyl nucleosides.[8]

CD spectra were recorded for the micellar solutions and compared with the spectra of the mononucleotides in water. This kind of experiment permits one to address the question of whether the conformation of the chromophor-bearing moieties is changed in the micellar aggregate with respect to the situation of the free monomer. Studies along these lines have been presented before for other systems, most typically for the lecithin systems.[17]

Typical results are shown in Figure 5. The slight changes between the micellar solution and a solution of the related monophosphate could reflect the chemical difference between the lipidic nucleotide and the free monophosphate.

We also examined the mixed micelle solutions both in UV-absorption and CD spectroscopy. As shown in Figure 6a the CD spectrum in micelles obtained from the mechanical mixture of C16-AMP and C16-UMP is somewhat different from the calculated spectrum obtained from the algebraic average of the two homomicellar solutions. The anomaly is a little smaller in the noncomplementary bases C16-AMP and C16-CMP (respectively, C16-UMP and C16-CMP), as shown in Figure 6b (respectively, Figure 6c).

Discussion

An interesting question is related to the structure of the aqueous micelle, in particular to the relative distance and geometry of the bases. They are most likely exposed to water,

as expected on the basis of the structure of the surfactant and as elicited from the analysis of the CD spectra, which are substantially the same for the micelles as for the free mononucleotides in water. The chemical nature of the base does not seem therefore to influence significantly the aggregation behavior of the micelles. This is at variance with the situation found earlier with the liposomes of 1,2-dipalmitoyl-*sn*-glycero-(3)-phosphatidyl nucleosides;[2] as in this case significant differences in stability and size distribution were observed with different bases.

Concerning the spectroscopic and binding properties, we expected that the complementary base binding would be more visible and faster than in the liposomes. This turned out not to be so, actually the aqueous micelles did seem to facilitate the binding. We did notice an effect in both UV-absorption and CD spectra on mixing micelles from C16-AMP with micelles from C16-UMP; however, changes were also observed when noncomplementary bases were used. The point can be made that the signal is more intense in the complementary bases; the situation appears complex. The spectral changes in the micelles are within mixing time and cannot be recorded by normal spectroscopy (a stopped-flow instrumentation would be necessary). That suggests the interactions are indeed very fast, contrary to the case of liposomes.[8]

More in general, these studies show that supramolecular surfactant aggregates bearing nucleobases are able to display a particular kind of recognition chemistry. Micelles (or liposomes) built by different nucleobases bind and fuse with each other, giving two mixed systems containing the two different bases in stoichiometric amount. The kinetics of this process, as well as the specificity, depends strongly on the nature of the chemical aggregate, which is very different in micelles and in liposomes. Micelles and liposomes are high molecular weight aggregates consisting of many monomeric mononucleotides, which (in contrast to DNA or RNA) are not linked with each other by covalent bonds. The other interesting difference from DNA lies in the fact that the recognition chemistry takes place within a spherical and no longer within a linear topology.

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