

Micellar Aggregates from Short-Chain Phospholiponucleosides: A SANS Study

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The structure of micelles formed by a novel group of synthetic phospholipid amphiphiles functionalized with complementary nucleic bases, dioctanoylphosphatidyl-adenosine ($\text{diC}_8\text{P-Ade}$), dioctanoylphosphatidyl-uridine ($\text{diC}_8\text{P-Uri}$), and their 1/1 mole ratio mixture, has been studied at 37 °C by small-angle neutron scattering, SANS, as a function of pH and amphiphile concentration. In the explored pH range (3.5–7.5), pure $\text{diC}_8\text{P-nucleosides}$ form quasispherical micelles with an axial ratio smaller than 2, composed of 30–45 monomers. These micellar aggregates do not grow as lipid concentration increases, while the homologous phosphatidylcholine forms rodlike micelles with an aggregation number of about 500. Micellar charge strongly depends on the pH, indicating a progressive charging of the phosphatidyl nucleoside micelle polar headgroup. The 1:1 mixture of the two phospholiponucleosides shows deviations from the ideal behavior, indicating specific interactions at the micelle aggregate polar headgroups region. The analysis of SANS data shows that the recognition process between the two complementary bases of the phosphatidyl nucleoside, demonstrated by NMR, UV, and CD data, is associated with a consistent decrease of the mean area per polar headgroup, while the aggregation number and the micellar shape remain almost unchanged. The recognition process occurs, as in biological systems, on a local scale without perturbing the overall structure of the system.

Introduction

The balance between hydrophobic and hydrophilic portions of short-chain lecithins supports the existence of L_1 micellar phases, which are preferred to bilayered aggregates formed by longer chain analogues.¹ These nonmembrane-forming phospholipid have proved to be useful to model the mechanism of action of phospholipases,^{2–4} despite the fact that in vivo this class of enzymes that hydrolyzes phospholipid esteric bonds acts on cellular membranes.⁵

A great deal of experimental work has been performed on the structural^{6–8} and aggregational behavior of symmetric and asymmetric series of diacylphosphatidylcholine, i.e., with chain length range $\text{C}_6\text{--C}_{10}$.^{9–12} The properties of the micellar solutions show remarkable dependence on the fatty chain length. $\text{DiC}_6\text{-PC}$ forms spherical micelles that do not grow with concentration,⁶ while diC_7PC micelles have cylindrical shape and show an appreciable growth as concentration is raised.⁷ DiC_8PC forms rodlike particles, whose aggregation number increases dramatically with concentration.¹¹ The simple addition of one methylene group to the lecithin alkyl chain produces a micellar growth that can be successfully modeled using the “ladder theory”.^{13,14}

Recently, a new synthetic route implemented by Shuto and co-workers^{15–17} allowed enzymatic modification of the polar headgroup of various lecithins. The phospholipase D enzyme, in the presence of a strong excess of a nucleophilic acceptor bearing a primary hydroxyl group, catalyzes a transphosphatidyl reaction, exchanging the choline polar head with the primary alcohol. Since nucleosides have a primary hydroxyl

function, it is possible to prepare nucleoside-functionalized lipids as shown in Figure 1. Although these products are not natural, nucleolipids such as CDP-diacylglycerol can be found as an intermediate in several biochemical pathways.¹⁸

Several novel nucleoside-functionalized phospholipids^{19–22} have been synthesized in our laboratory using the Shuto method. These new amphiphilic compounds present aggregational properties as the classical phospholipids. However, major differences can be observed because the capability of the bases to interact with each other through π -stacking and H-bond interactions, and the different hydrophilic–hydrophobic balance, is strongly altered by the insertion of a bulkier polar headgroup that can bear a net negative charge. Our interest in these novel lipids is addressed to the study of their aggregational properties and to the molecular recognition properties possibly displayed by polar headgroups.

It is known that base–base recognition properties of complementary bases determine the transmission of information necessary to the life and the maintenance of the cell.^{23,24} However, base recognition can be only displayed when these bases are anchored to a covalent structure, as in natural nucleic acids²⁵ with the sugar–phosphate covalent backbone or in the synthetic PNA (peptide nucleic acids),^{26,27} where the covalent skeleton is borrowed from proteins. In these natural or synthetic structures, molecular recognition is highly cooperative and the base pairing, due to hydrogen bonds between bases belonging to complementary strands, is stabilized by π -stacking interactions occurring between neighboring bases on the same strand.²⁵ It is worthwhile to mention that, while synthetic PNAs are neutral, in natural nucleic acids each monomeric unit bears a negative charge localized on the phosphate group which is fully

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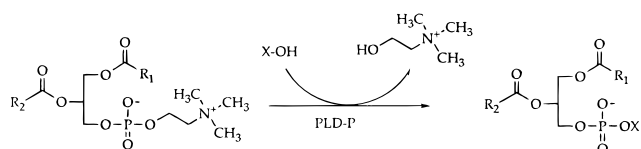


Figure 1. The transphosphatidyl transfer reaction allows an enzymatic exchange of the polar headgroup of phospholipids.

ionized at physiological pH. This charge is screened by cellular cations so that the polyelectrolyte tertiary structure is strictly dependent on the ion concentration and charge.

In aqueous solutions, monomeric nucleosides fail to recognize each other through hydrogen bonding, being the environment too competitive and the cooperative effect provided by the covalent anchoring lacking.²⁵

In colloidal aggregates, the supramolecular organization of amphiphiles is mainly due to the hydrophobic effect, which is considerably weaker than the covalent bond. In previous studies, we have shown selective recognition between complementary bases for dioleoylphosphatidyl-adenosine, dioleoylphosphatidyl-uridine and dioleoylphosphatidyl-cytidine derivatives in monolayer at the air/water interface and in vesicular aggregates.^{20,21}

The ordering of the bases at the vesicles or monolayer interface is such that a selective pattern of hydrogen bonding can be displayed, despite the negative charge of neighboring amphiphiles and the exposure of the polar headgroups to water.

Vesicular aggregates can be considered static metastable structures, since the monomer exchange between the aggregate and the solution occurs on a considerably slow time scale.²⁸ Micelles are equilibrium dynamic structures with a microsecond exchange time scale, and their interfacial curvature is generally higher than that of bilayered structures.

Recently, Kunitake and co-workers²⁹ have shown a dependence of the molecular recognition characteristics on the interfacial structural and dynamical properties of vesicles and micelles formed from guanidinium surfactants. Therefore, base-base interactions in micelles might be different than those observed for monolayers and vesicle.

In a forthcoming paper, we present experimental evidence of base-base recognition at the micellar surface as obtained by classical spectroscopic methods for nucleic acids, such as NMR, UV absorption spectroscopy, and circular dichroism.²²

In this paper, we report a small-angle neutron scattering structural investigation as a function of pH, concentration, and ionic strength of micellar solutions formed by dioctanoylphosphatidyl-adenosine (diC₈P-Ade), dioctanoylphosphatidyl-uridine (diC₈P-Uri) (shown in Figure 2), and their 1:1 mixture. This investigation is necessary since the molecular recognition pattern found for these phospholiponucleosides cannot be correctly interpreted without a structural investigation indicating that the spectroscopic features found via NMR, UV, and CD are not simply due to a possible variation of the overall structure of the supramolecular assembly and therefore to a different orientation of the bases at the micelle interface.

Experimental Section

Materials. Dioctanoylphosphatidylcholine was purchased from Avanti Polar Lipids (Alabama), and its purity was checked by TLC. Since no oxidation or lyso products were detectable, the lecithin was used without further purification. Adenosine and Uridine were obtained from Fluka (Switzerland). Phospholipase D from *Streptomyces* sp. AA 586 was purchased from Genzyme Diagnostics, West Mallory, U.K.. Deuterium oxide

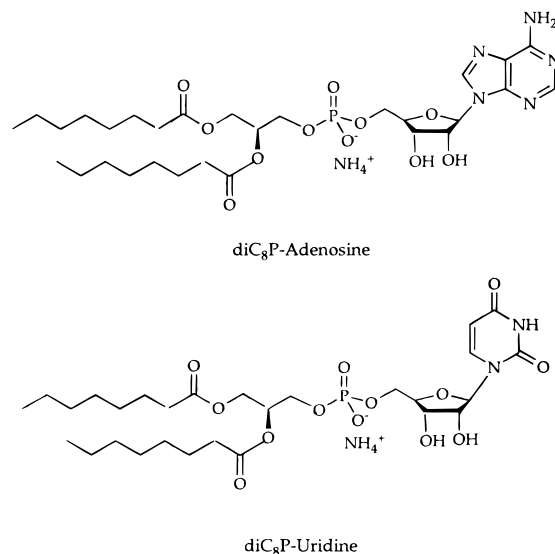


Figure 2. Schematic drawing of diC₈-phosphatidyl-adenosine and diC₈-phosphatidyl-uridine chemical formulas.

>99.5% (product code 31240, analysis number 361691/1 1096), HCl, NaOH, NaCl, CHCl₃, and MeOH were purchased from Fluka. Anhydrous NaH₂PO₄ and Na₂HPO₄ were purchased from Sigma and stored in a desiccator.

Synthesis of DiC₈P-Nucleosides. Short-chain phospholiponucleosides diC₈P-adenosine and diC₈P-uridine were synthesized starting from the corresponding lecithins in a two-phase system, according to the method proposed by Shuto and co-workers for long-chain compounds and were obtained as ammonium salts.

Nuclear Magnetic Resonance. ¹H, ³¹P{¹H}, and ¹³C{¹H} NMR spectra were recorded at 500.132, 202.46, and 125.76 MHz, respectively, on a Bruker Avance DRX-500 spectrometer equipped with a variable temperature control unit accurate to ±0.1 °C. Chemical shifts are relative to tetramethylsilane and to 85% H₃PO₄, respectively, as external references. Downfield values are reported as positive. Double-quantum filtered ¹H COSY experiments were recorded using degassed nonspinning samples with 1024 increments of size 2K covering the full range (ca 5000 Hz) in both dimensions.

DiC₈P-Adenosine. ¹H NMR (DMSO-*d*₆): δ = 0.95 (t, *J* = 8.9, 6H, CH₃); 1.32 (m, 16H, CH₂ aliphatic); 1.58 (m, 4H, CH₂-CH₂-COO); 2.33–2.37 (m, 4H, CH₂-COO); 3.84–3.95 (4H, *sn*-3-CH₂, H5'/5''); 4.10–4.20 (2H, H4', *sn*-1-CH₂); 4.29 (t, 1H, H3'); 4.37 (dd, *J*₁ = 11.0, *J*₂ = 3.3, 1H, *sn*-1-CH₂); 4.69 (t, 1H, H2'); 5.15 (m, 1H, *sn*-2-CH); 5.4–5.7 (bs, 2H, 2' OH e 3' OH); 6.1 (d, *J* = 5.7, 1H, H1'); 7.0–7.3 (bm, 6H, NH₄⁺, NH₂); 8.2 (s, 1H, H2); 8.5 (s, 1H, H8). ³¹P NMR (DMSO-*d*₆): δ = −0.54.

DiC₈P-Uridine. ¹H NMR (DMSO-*d*₆): δ = 0.97 (t, *J* = 6.6, 6H, CH₃); 1.35 (m, 44H, CH₂ aliphatic); 1.62 (m, 4H, CH₂-CH₂-COO); 2.35–2.40 (m, 4H, CH₂-COO); 3.82–3.89 (4H, *sn*-3-CH₂, H5'/5''); 4.02 (t, 1H, H2'); 4.10 (t, 1H, H3'); 4.17–4.21 (2H, H4', *sn*-1-CH₂); 4.39 (dd, *J*₁ = 14.0, *J*₂ = 3.2, 1H, *sn*-1-CH₂); 5.16 (m, 1H, *sn*-2-CH); 5.48 (bs, 2H, 2' OH e 3' OH); 5.67 (d, *J* = 8.1, 1H, H5); 5.90 (d, *J* = 6.4, 1H, H1'); 7.20 (b, 4H, NH₄⁺); 8.04 (d, *J* = 8.1, 1H, H6); 8.5 (b, 1H, NH). ³¹P NMR (DMSO-*d*₆): δ = −0.09.

Small-Angle Neutron Scattering and Light Scattering. SANS measurements were performed on the PAXE spectrometer at Laboratoire Léon Brillouin, Saclay, France. Neutrons with an average wavelength of 5.5 Å and a wavelength spread Δλ/λ < 10% were used. The sample to detector distance was

fixed at 2.0 m, covering a range of magnitude for the \mathbf{Q} vector from 0.01 to 0.32 Å⁻¹. Samples were contained in flat Hellma quartz cells of 1 mm path length. D₂O was chosen as a solvent in order to enhance the scattering contrast and minimize the incoherent background from hydrogen.

The two-dimensional intensity distributions were corrected for the background and the empty cell contributions and then normalized to an absolute intensity scale by a direct measurement of the intensity of the incident neutron beam. Integration of the normalized 2-D intensity distributions with respect to the azimuthal angle yielded the 1-D scattering intensity distributions $I(Q)$ in the units of a differential cross section per unit volume of the sample (cm⁻¹).

The scattering length densities of the phosphonucleosides were determined from the known chemical composition of the samples, taking into account chemical exchange of acidic protons with the deuterated solvent. The experiments were performed at 37.0 ± 0.1 °C.

Cmc were measured by using a light scattering Brookhaven Instrument apparatus (BI 9000AT correlator and BI 200 SM goniometer). The signal was detected by an EMI 9863B/350 photomultiplier, set at 90° with respect to the incident beam on the sample. The light source was a Coherent Innova 90 Ar⁺ laser, at $\lambda = 514$ nm, linearly polarized in the vertical direction, with a power attenuated in order to avoid sample heating. The laser long-term power stability was ±0.5%.

Modeling of the Micelles

Quantitative analysis of absolute scale SANS data was obtained by modeling the micellar solution as composed of charged two-shell particles interacting with each other according to a screened Coulombic potential within the NAR-MMSA (non additive radius mean sphere approximation) model.³⁰ In the fitting procedure, we use an overall amplitude factor which accounts for the calibration factor.

The micellar solution is considered to be composed of uniform sized ellipsoidal micelles with an aggregation number, N , and an effective charge Z . Each micelle has been modeled as formed by a hydrophobic core of spheroidal shape with principal axes a, b, b that contains the surfactant hydrocarbon tails, where the solvent cannot penetrate, and a hydrated hydrophilic shell formed by the polar heads, a fraction of counterions, and some solvent molecules.

Within these assumptions, the scattering intensity as a function of Q can be written as³⁰

$$I(Q) = (C - \text{cmc})N \left(\sum_i b_i - V_m \rho_s \right)^2 \tilde{P}(Q) \tilde{S}(Q) + I_{\text{bgd}} \quad (1)$$

where $(C - \text{cmc})$ is the number of micelles per unit volume, b_i are the scattering lengths of each atom of the surfactant, V_m is the monomer volume, ρ_s is the scattering density of solvent, $\tilde{P}(Q)$ is the orientationally averaged intraparticle structure factor for ellipsoidal particles, $\tilde{S}(Q)$ is the orientationally averaged center-center interparticle structure factor, and the additive term takes into account incoherent scattering and electronic background signal. $\tilde{P}(Q)$ is the orientationally averaged normalized form factor $\tilde{S}(Q)$ is the orientationally averaged center-center structure factor between micelles; they are given respectively by

$$\tilde{P}(Q) = \int_0^1 |F(Q, \mu)|^2 d\mu \quad (2)$$

and

$$\tilde{S}(Q) = 1 + \frac{\langle |F(Q, \mu)|^2 \rangle}{\langle |F(Q, \mu)|^2 \rangle} [S_{\text{MM}}(Q) - 1] \quad (3)$$

where $|F(Q, \mu)|^2$ is the intraparticle structure factor for ellipsoidal two-shell micelles and μ is the direction cosine between the direction of the symmetry axis of the spheroid and the \mathbf{Q} vector,

$$F(Q, \mu) = f \frac{3j_1(u)}{u} + (1 - f) \frac{3j_1(n)}{n} \quad (4)$$

with

$$u = Q[\mu^2 a^2 + (1 - \mu^2)b^2]^{1/2}$$

$$n = Q[\mu^2(a + d)^2 + (1 - \mu^2)(b + d)^2]^{1/2}$$

$$f = V_f(\rho_1 - \rho_2) / \left[\sum_i (b_i - V_m \rho_s) \right]$$

where b is the short axis of the ellipsoid and the long principal axis, a , is determined by equating the volume of the inner core NV_m to the volume of the ellipsoid $(4\pi/3)b^2a$. $S_{\text{MM}}(Q)$ has been calculated by solving the Ornstein-Zernicke equation for the pair correlation function within the NAR-MMSA closure that yields analytical solutions.³⁰

Results and Discussion

Small-angle neutron scattering spectra have been recorded for micellar solutions of diC₈P-Ade, diC₈P-Uri, and their 1:1 mole ratio mixture at two different concentrations and three different pH values, ranging from 3.5 to 7.5.

It has been shown that the aggregational behavior of phosphatidylcholine derivatives,⁹⁻¹¹ that are zwitterionic for pH ≥ 1-2, is quite insensitive to pH. We do not indicate a definite pK_a value for the phosphate group of the lecithin molecule in micellar aggregates, since it has long been established that interfacial ordering can provide a substantially different environment for acid-base equilibria with respect to a bulk solution.

The behavior of phospholiponucleoside is even more complex. The bases themselves can be protonated or deprotonated according to the pH of the micellar solution, while the pK_a for the phosphate group lies in the range indicated above for phospholipids. Since the nucleoside polar head can assume a net charge, phospholiponucleosides are expected to be sensitive to pH. According to the pH of the solution, these phospholiponucleosides can bear a positive, zwitterionic, and eventually negative polar head group. As an example, the case of adenosine-monophosphate is illustrated in Figure 3.

It is worthwhile to mention that even in this case pK_a values might exhibit a deviation from bulk acidic constants since the moiety is confined in an interfacial microenvironment. The equilibrium of the amide-like proton for adenine might be shifted toward higher pK_a s, since there is evidence²⁵ for stabilization of the zwitterion through intramolecular hydrogen bonding. This effect could be more severe in micellar aggregates, since in this case intermolecular charge interactions and hydrogen bonding between neighboring surfactant molecules may be invoked as well.

An important indication of the protonation of the nucleotidic polar head attached to a lipidic moiety has been inferred for dioleoylphosphatidyl derivatives at the air/water interface. We have shown²⁰ that the area per polar head of the DOP-adenosine molecule passes from 107 Å²/molecule at pH = 2.0 to 104 Å²/

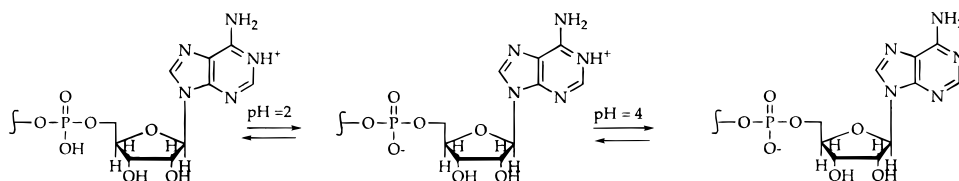


Figure 3. Acidic equilibria involving the polar head of diC₈P-adenosine. Both the phosphate group and adenosine are affected by the pH increase that produces a deprotonation of phosphate at very low pH and of the adenosine at higher pHs.

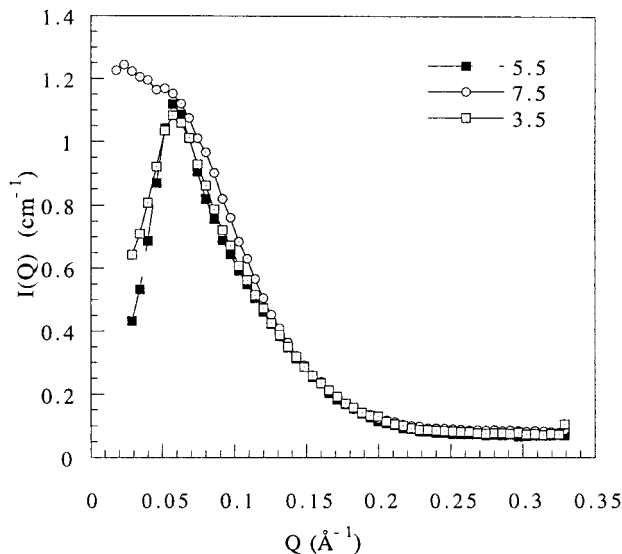


Figure 4. Small-angle neutron scattering spectra obtained from 3.5×10^{-2} M micellar solutions of diC₈P-adenosine at pH = 3.5, 5.5, and 7.5.

molecule at pH = 5.5 to $139 \text{ Å}^2/\text{molecule}$ at pH = 7.5. The almost negligible decrease observed passing from pH = 3.5 to pH = 5.5 has been attributed to charge interactions between the phosphate group and the aromatic ring of the base of phospholiponucleosides polar heads (see Figure 2), while the increase observed at pH = 7.5, which is very close to physiological pH, is ascribable to a deprotonation of the amide on the base aromatic ring together with a full deprotonation of the phosphate, leading to a net negative charge on the polar heads and thus to their mutual repulsion.

An interesting experimental observation for DOP derivatives concern the molecular recognition between Watson-Crick complementary bases at the air/water interface and on the vesicular surface. It is easily predictable that hydrogen bonding formation depends on the charge density present on the bases and thus on the protonation state. The base-base selectivity is displayed only at pH = 7.5, thus pointing out a definite charge density favorable to H bonds and possibly an interfacial orientation occurring at this pH.

In Figures 4 and 5, SANS spectra for 3.5×10^{-2} M diC₈P-adenosine and 3.5×10^{-2} M diC₈P-uridine micelles at pH = 3.5, 5.5, and 7.5 are reported. This concentration exceeds cmc for both surfactants (1.0×10^{-3} for diC₈P-uridine, 4.0×10^{-4} for diC₈P-adenosine, as determined by light scattering).²²

All the experimental small-angle neutron spectra show a more or less pronounced correlation peak, indicating the presence of interparticle interactions mainly due to electrostatic charge repulsions.

The peak position, plotted as a function of concentration on a log-log scale, shows a scaling of $q^* \propto c^{1/3}$, indicating that the shift of the correlation peak with dilution is due to a simple spreading out of the average interparticle distance.

Within the two-shell model, we obtained from the analysis

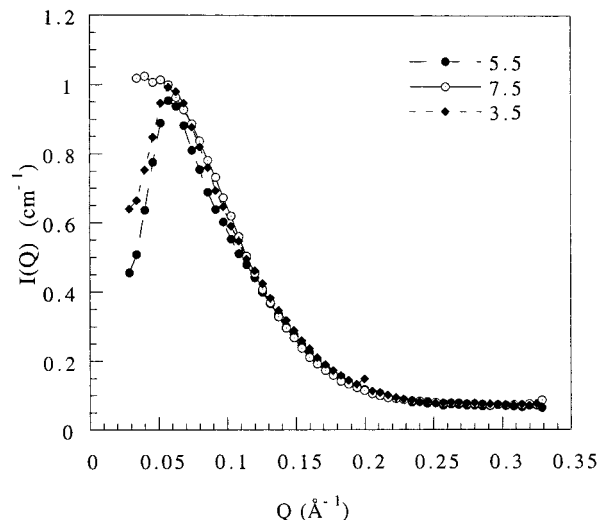


Figure 5. Small-angle neutron scattering spectra obtained from 3.5×10^{-2} M micellar solutions of diC₈P-uridine at pH = 3.5, 5.5 and 7.5.

TABLE 1: Structural Parameters Deduced from Fitting of the Spectra for DiC₈P-Adenosine 3.5×10^{-2} M

pH	N^a	micellar charge ^b and (α^c)	a/b^d	b (Å)	$N_{\text{water/surf}}^e$	shell thickness ^f (Å)
3.5	40	13.1 (0.33)	1.9	12.6	4.9	5.5
5.5	38	13.3 (0.35)	1.7	12.8	7.7	6.0
7.5	45	20.5 (0.45)	1.9	13.1	12.4	7.2

^a Aggregation number. ^b Total micellar charge, Z . ^c Fractional charge, given by Z/N . ^d Axial ratio for the ellipsoidal micelle with axes a , b , b . ^e Estimated number of water molecules per surfactant molecule in the hydrophilic layer. ^f Thickness of the hydrophilic shell.

of the experimental SANS spectra the aggregation number, the charge of the micellar aggregate, and the thickness of the hydrophilic shell occupied by the hydrated polar heads. As already mentioned, we assume for the colloidal aggregate to have either prolate or oblate ellipsoidal shape, with axes a , b , b . Thus, once the short axis, b , has been determined by a best fitting procedure, it is possible to deduce the length of axis a and the axial ratio. The average hydration number can also be inferred from the volume of the hydrophilic shell layer. In the fitting procedure, three variational parameters have been used: the aggregation number, the net charge of the particle, and the thickness of the hydrophilic shell. The length of the short axis has been initially fixed to the Tanford value, corresponding to a fully extended length, given by $L = (1.50 + 1.265n)$, where n is the number of carbons inside the core, and then allowed to vary within a certain range centered around this value to account for possible monomer protrusions from the micelle core.

Pure Phospholiponucleosides. In Tables 1 and 2, the structural parameters deduced from the analysis of SANS spectra of diC₈P-adenosine micellar solutions and the results relative to diC₈P-uridine are shown. In Figure 6, an example of best fit is shown and $S(Q)$ and $P(Q)$, as extracted from the fitting curve, are also reported.

TABLE 2: Structural Parameters Deduced from Fitting of the Spectra for diC₈P–Uridine 3.5×10^{-2} M

pH	N^a	micellar charge ^b and (α^c)	a/b^d	b (Å)	$N_{\text{water/surf}}^e$	shell thickness ^f (Å)
3.5	37	11.1 (0.30)	1.8	12.7	10.4	6.2
5.5	35	10.5 (0.30)	1.6	12.8	10.5	6.2
7.5	43	22.2 (0.55)	2.0	12.7	12.7	7.2

^a Aggregation number. ^b Total micellar charge, Z . ^c Fractional charge, given by Z/N . ^d Axial ratio for the ellipsoidal micelle with axes a , b , b . ^e Estimated number of water molecules per surfactant molecule in the hydrophilic layer. ^f Thickness of the hydrophilic shell.

Since the two nucleoside-functionalized phospholipid surfactants exhibit a very similar behavior with respect to aggregation, we will discuss first the results obtained for both systems and we will then focus on their comparison.

In the explored pH range diC₈P–nucleosides form quasi-spherical prolate micelles, with an axial ratio smaller than 2. No appreciable pH dependence trend can be observed for the shape of both micellar systems, apart from a slight and almost negligible decrease of the axial ratio for pH = 5.5, that is for micelles in pure D₂O without any added salts.

It should be mentioned that a simple scattering experiment cannot discriminate between a weakly polydisperse spherical population and a monodisperse ellipsoidal population with axial ratio less than 2–3.^{31,32} According to this point of view, the deviation of the axial ratio from unity can be also considered as an index of deviation of the system from the spherical monodisperse situation.

The aggregation numbers deduced from the analysis of the experimental spectra range from 35 to 45, and for a given surfactant, the variance with pH and composition of the solution is around 20%. These results highlight the consistent different behavior of this new class of phospholipids as compared to classical phospholipids. The homologous phosphocholine forms rodlike micelles that dramatically grow as concentration and/or temperature are raised and have an aggregation number of about 500 just above the cmc.¹¹ The micelle aggregation numbers of the phospholiponucleosides are consistent with the presence of a bulkier headgroup than that of phosphocholine so that the confinement of the hydrophobic tails is accomplished with a higher curvature of the surfactant film caused by head–head repulsion.

The weak increase in the aggregation number observed as pH is raised can be due to a different charge density of the polar head or to a different screening operated by counterions or dissolved salts.

Both factors act in modulating polar head repulsion, affecting the equilibrium curvature of the aggregate. Their decoupling is not always possible, since the three media in which micellar solutions have been studied differ in ionic strength. A small decrease in the aggregation number is observed for both systems passing from pH = 3.5 to pH = 5.5, while there is a more consistent increase in the number of monomers forming a micelle at pH = 7.5. What was observed would point toward a decrease in head–head repulsion for neutral pH. However, the ionic strength increases in the order $5.5 < 3.5 < 7.5$, and the highest pH medium is thus characterized by the strongest screening efficiency.

Some general conclusions can be drawn correlating the aggregation number to the surface charge. The charge deduced from the analysis of the experimental spectra represents the effective charge of the colloidal aggregate that accounts for counterion condensation that partially neutralizes the micelle. This charge and the related surface charge density parameter

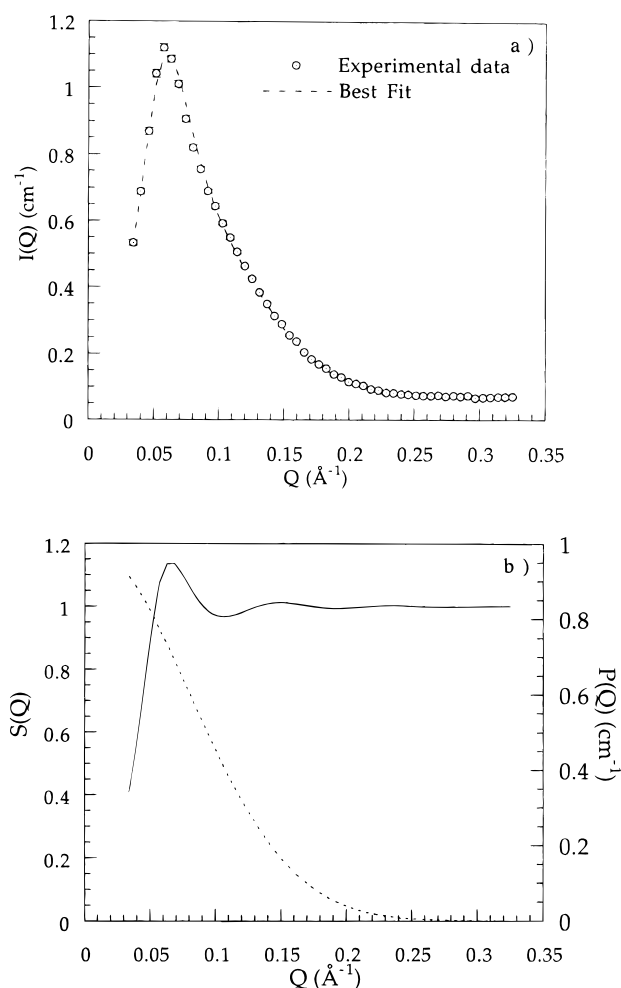


Figure 6. Example of a best fitting of the SANS spectrum obtained for a 3.5×10^{-2} M micellar solutions of diC₈P–adenosine in D₂O, pH = 5.5 (a). In (b) are reported the $P(Q)$ and $S(Q)$ functions.

provide information on the protonation state of the polar head and on the counterions condensation around the colloidal particle. This last parameter, reported in brackets in Tables 1 and 2 for both systems, is defined as the total charge divided by the aggregation number, $\alpha = Z/N$.

The analysis of the tables shows that the charge density increases with pH, regardless of the ionic strength and thus of the screening of the repulsive interactions that increase with ionic strength. This observation suggests that the polar head is subject to a progressive charging until pH = 7.5. This behavior cannot be predicted taking into account the acidic constants of the isolated monomers. It is therefore clear that a cooperative mechanism is acting and that polar head–polar head interactions play an important role. A further support to this hypothesis comes from the comparison of the spectra obtained for diC₈P–Ade and diC₈P–Uri in a pH = 5.5 medium (obtained by NaCl addition) with the same ionic strength as the buffer at pH = 7.5; see Figure 7 and Table 3. The increase of the aggregation number with respect to pure water and the parallel decrease in surface charge account for the screening caused by counterions. The comparison with data at pH = 7.5 shows that surface charge density is almost doubled at this pH, thus confirming that the pH increase leads to the deprotonation of the polar head in the micellar aggregate.

The main difference between adenosine and uridine surfactants concerns a small but reproducible decrease in the aggregation number associated with an increase of the hydration number

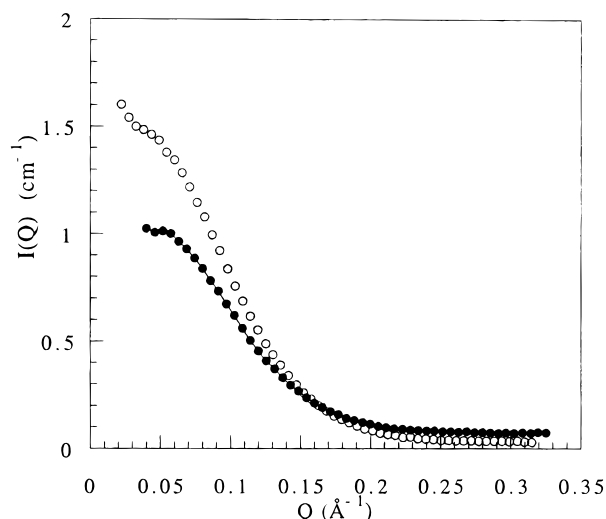


Figure 7. Small-angle neutron scattering spectrum obtained from 3.5×10^{-2} M micellar solutions of diC₈P-uridine in a pH = 7.5 buffered medium (filled circles) compared to the spectrum obtained for the same concentration and ionic strength in a pH = 5.5 unbuffered solution (open circles).

TABLE 3: Structural Parameters Deduced from Fitting of the Spectra for DiC₈P-Adenosine 3.5×10^{-2} M and DiC₈P-Uridine in NaCl ($I = 0.25$)

base	N^a	micellar			$N_{\text{water/surf}}^e$	shell thickness ^f (Å)
		charge ^b and (α^c)	a/b^d	b (Å)		
ade	57	12.5 (0.22)	2.0	13.9	11.8	7.6
uri	44	12.2 (0.27)	2.1	12.7	12.7	7.2

^a Aggregation number. ^b Total micellar charge, Z . ^c Fractional charge, given by Z/N . ^d Axial ratio for the ellipsoidal micelle with axes a , b , b . ^e Estimated number of water molecules per surfactant molecule in the hydrophilic layer. ^f Thickness of the hydrophilic shell.

TABLE 4: Structural Parameters Deduced from Fitting of the Spectra for DiC₈P-Adenosine 1.0×10^{-2} M

pH	N^a	micellar			$N_{\text{water/surf}}^e$	shell thickness ^f (Å)
		charge ^b and (α^c)	a/b^d	b (Å)		
3.5	33	~0 (~0)	2.0	11.7	8.7	5.9
5.5	30	8.9 (0.30)	1.8	11.6	8.7	5.7
7.5	45	20.7 (0.46)	1.9	13.1	4.8	4.8

^a Aggregation number. ^b Total micellar charge, Z . ^c Fractional charge, given by Z/N . ^d Axial ratio for the ellipsoidal micelle with axes a , b , b . ^e Estimated number of water molecules per surfactant molecule in the hydrophilic layer. ^f Thickness of the hydrophilic shell.

per surfactant molecule in passing from the purine to the pyrimidine compound. This result correlates well with the lower polarity and higher affinity of adenosine to give rise to self-stacked structures.²⁴ Stacking interactions can be thought of as hydrophobic interactions, even if for the sake of clarity it should be stressed that stacking is not accompanied by an entropy increase for the system.²⁵ Moreover, base-base interaction could be present even for equally charged headgroups thanks to the high flexibility of the polar head and to the fact that the interaction sites can be sufficiently far from where the charge is localized. These interactions would reduce the average area per molecule and exclude some water molecules from the hydrophilic shell.

The effect of surfactant concentration on shape, size, and charge of the micelles has been investigated in the same pH range. In Tables 4 and 5, the results obtained for 1.0×10^{-2} diC₈P-Ade and diC₈P-Uri, respectively, are reported.

The most remarkable differences with the more concentrated series are related to the samples at pH = 3.5 and 5.5, where a

TABLE 5: Structural Parameters Deduced from Fitting of the Spectra for DiC₈P-Uridine 1.0×10^{-2} M

pH	N^a	micellar			$N_{\text{water/surf}}^e$	shell thickness ^f (Å)
		charge ^b and (α^c)	a/b^d	b (Å)		
3.5	33	~0 (~0)	1.8	11.9	10.5	6.7
5.5	28	7.5 (0.27)	1.8	11.4	14.1	6.3
7.5	44	18.3 (0.46)	1.9	13.0	9.8	6.7

^a Aggregation number. ^b Total micellar charge, Z . ^c Fractional charge, given by Z/N . ^d Axial ratio for the ellipsoidal micelle with axes a , b , b . ^e Estimated number of water molecules per surfactant molecule in the hydrophilic layer. ^f Thickness of the hydrophilic shell.

TABLE 6: Structural Parameters for Pure and Mixed Phospholiponucleosides (3.5×10^{-2} M) Obtained from SANS Spectra (for the Explanation of Symbols See Previous Tables)

	N	micellar charge and (α)	a/b	b (Å)	$N_{\text{water/surf}}$	shell thickness (Å)
(a) pH 3.5 (HCl 3×10^{-4} M)						
ade	40	13.1 (0.33)	1.9	12.6	4.9	5.4
uri	37	11.1 (0.30)	1.8	12.7	10.4	6.2
mix	41	12.1 (0.29)	1.9	12.8	6.2	5.8
(b) pH 5.5 (pure D ₂ O)						
ade	38	13.3 (0.35)	1.7	12.8	7.7	6.0
uri	35	10.5 (0.30)	1.6	12.8	10.5	6.2
mix	35	11.8 (0.33)	1.7	12.9	11.7	6.7
(c) pH 5.5 (NaCl 0.25 M, $I = 0.25$)						
ade	57	12.5 (0.22)	2.0	13.9	11.8	7.6
uri	44	12.2 (0.27)	2.1	12.7	12.7	7.2
mix	54	14.7 (0.26)	2.1	13.6	7.5	6.6
(d) pH 7.5 (phosphate buffer, $I = 0.25$)						
ade	45	20.5 (0.45)	1.9	13.1	12.4	7.2
uri	43	22.2 (0.55)	2.0	12.7	12.7	7.2
mix	46	26.9 (0.58)	2.0	13.0	8.0	6.2

decrease in the aggregation number is present. For the most acidic medium, a considerable reduction of the surface charge density is also observed. For physiological pH, no appreciable dependence from the concentration can be detected. As above anticipated, phospholiponucleosides do not exhibit the tremendous micellar growth observed for diC₈P cylindrical micelles, where even a slight increase in concentration results in an aggregation number rise from about 500 (close to cmc) to 50000,¹¹ in the region close to the phase separation boundary.

Interestingly, small-angle neutron analysis, in addition to the structural parameters characterizing the micellar aggregates, provides information on the effective charge of the supramolecular aggregate and, in this specific case, allows the charge titration "in situ" of the nucleoside polar headgroup to be followed, which is difficult to obtain with other methods.

Mixtures. SANS spectra obtained from 1:1 mole ratio mixed surfactant systems have been analyzed according to two different approaches, corresponding to the following models: (a) mixed micellar two-shell system possessing weight-averaged molecular properties and scattering length densities; (b) mixed micellar two-shell system where the lowest cmc lipid (diC₈P-Ade) is supposed to form micelles (substrate) and the other surfactant is considered an amphiphilic receptor. In the later case, the substrate/receptor ratio has been allowed to vary in the fitting procedure, but invariably the best fit corresponded to the 1:1 adduct with the same structural properties of the mixed micelles obtained from the model a.

In Table 6, the properties of the mixed micellar systems are compared to those of the "pure" systems.

NMR, UV, and CD spectra of dioctanoylphosphonucleosides bearing complementary bases (adenosine and uridine) at the phospholipid polar heads show molecular recognition in mixed

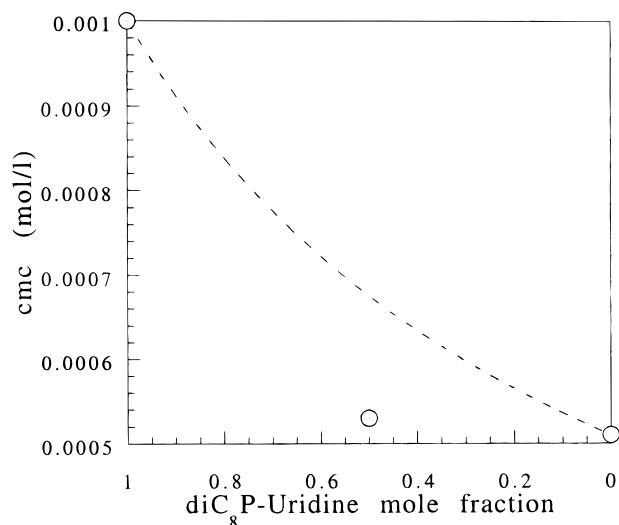


Figure 8. Critical micellar concentration of diC₈-phosphatidyl-adenosine and diC₈-phosphatidyl-uridine and their 1/1 mole ratio mixture. The 1/1 mixture presents a negative deviation from the ideal behavior.

micelles despite the exposure of the nucleosides moieties to the water environment.²² Recognition occurs through Watson–Crick hydrogen bonding between the amino group of adenosine and the carbonyl group of uridine and stacking interactions between the aromatic rings. These results are consistent with previous studies of dioleoylphosphatidyl derivatives in monolayers and in vesicles,^{20,21} where it was also shown that recognition occurs only between complementary bases, at physiological pH only.

In the present study, evidence for recognition comes from the nonideal mixing of the complementary nucleolipids for mixed micelles, i.e., from the negative deviation of the cmc from ideal behavior displayed by the 1:1 system at pH = 7.5 phosphate buffered medium (see Figure 8). Negative deviation from ideal mixing of the cmc values, i.e., negative free energy of mixing, ΔG_{mix} , for the micellization process of the 1:1 mixture, supports attractive interactions between the nucleoside polar headgroups. In fact, departure from ideality in mixed micellar system has been established to be the result of attractive interactions between polar heads,³³ and in some cases a possible hydrogen bonding interactions has also been modeled.³⁴

As far as neutron scattering data are concerned, a possible model for molecular recognition involves a reduction of the average area for a surfactant molecule on the micellar aggregate.

For a prolate ellipsoid, once the principal axes are known, the extension of the surface area is given by

$$\text{area} = \frac{2\pi b a'^2}{(a'^2 - b'^2)^{1/2}} \arcsin\left\{\frac{(a'^2 - b'^2)^{1/2}}{a'}\right\} + 2\pi b'^2 \quad (5)$$

where a' and b' are the long principal and the short axes including the polar shell thickness.

According to this formula, we have evaluated the average area for a surfactant molecule of mixed micellar systems, reported in Table 7, and compared this value to the one predictable for an ideal mixing behavior, reported in square brackets.

The values obtained for the average area are considerably higher than those obtained for monolayer at the air/water interface, as was already noticed for phosphatidylcholines by Chen et al.¹ The higher curvature of the interface allows penetration of solvent molecules in the hydrophilic shell, and

TABLE 7: Mean Molecular Areas for Pure and Mixed Phospholiponucleosides (3.5×10^{-2} M) Obtained from SANS Spectra^a

	mean area ($\text{\AA}^2/\text{molecule}$)			
	pH 3.5	pH 5.5	pH 5.5/NaCl	pH 7.5
DC ₈ P–Ade	146	158	150	163
DC ₈ P–Uri	167	166	159	164
1:1 mixture	152 [157]	170 [162]	141 [154]	149 [164]

^a For the 1:1 mixed micelles the mean areas expected from ideal behavior are also reported in brackets.

base–base interactions are expected to be weaker than those observed for monolayers and bilayered structures.

However, at pH = 5.5 in the presence of salts when an efficient Coulombic screening is acting, and at pH = 7.5, one notices that the average surface area per surfactant molecule is strongly nonideal for mixed micellar systems. We can thus conclude that the structural parameters deduced from SANS analysis well correlate with the presence of base–base interactions at the surface of the mixed phospholiponucleoside. These interactions are responsible for the different behavior of this new class of lipids as compared to the well known phosphatidylcholines.

Conclusions

Novel nucleoside functionalized phospholipids, dioctanoylphosphatidyl-adenosine (diC₈P–Ade) and dioctanoylphosphatidyl-uridine (diC₈P–Uri), where the phospholipid group is connected to the adenosine or uridine nucleobase, have been synthesized by exchanging the choline polar head of the dioctanoyl-lecithin with the nucleobases (adenine and uridine). The structural properties of complementary diC₈P–nucleoside micelles and of the 1/1 mole ratio mixed micelles have been studied by small-angle neutron scattering at 37 °C and as a function of pH.

SANS spectra were analyzed by modeling the micellar solution as being composed of charged two-shell particles interacting through a screened Coulombic potential within the nonadditive radius mean sphere approximation (NAR-MMSA) model.³⁰

Pure phospholiponucleosides form quasispherical prolate micelles, with an axial ratio smaller than 2 and an aggregation number of 35–45. The aggregation number and shape of micellar systems formed from both phospholiponucleosides do not depend on the concentration or the pH of the solution. Micellar charge almost doubles as pH is raised from 3.5 to 7.5, in agreement with a deprotonation of the polar head. Interestingly, neutron analysis allows the charge titration of the nucleoside micelles to be followed “in situ”.

The aggregational properties of phosphatidyl-nucleosides consistently differ from the behavior of classical dioctanoylphosphatidylcholine, which forms rodlike micelles with an aggregation number of about 500 just above the cmc,¹¹ and is almost insensitive to pH, demonstrating how important the capability of protonation/deprotonation of a polar headgroup is in establishing the aggregational properties of these novel nucleosides.

The 1/1 mixture between the complementary adenosine and uridine derivatives shows the recognition process occurring, despite the exposure of the nucleosides moieties to water environment,²² through Watson–Crick hydrogen bonding and stacking interactions between the amino group of adenosine and carbonyl group of uridine, and the aromatic rings, respectively. Recognition, as shown by SANS, is associated with a negative deviation of the cmc of the mixed micellar system with respect to the pure components and with a consistent decrease of the

mean molecular area per polar headgroup, while the aggregation number and the micellar shape remain unchanged. Interestingly, the recognition process occurs, as in biological systems, on a local scale without perturbing the overall structure of the system.

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References and Notes

- (1) Israelachvili, J. N.; Mitchell, D. J.; Ninham, B. W. *J. Chem. Soc., Faraday Trans.* **1976**, 72, 1525.
- (2) El-Sayed, M. Y.; Roberts, M. F. *Biochim. Biophys. Acta* **1985**, 831, 133.
- (3) El-Sayed, M. Y.; De Bose, C. D.; Coury, L. A.; Roberts, M. F. *Biochim. Biophys. Acta* **1985**, 837, 325.
- (4) Lewis, K. A.; Bian, J.; Sweeney, A.; Roberts, M. F. *Biochemistry* **1990**, 29, 9962.
- (5) Dennis, A. E. *The enzymes*, Vol. XVI, *Lipids Enzymology*; Academic Press Inc: New York, 1989; Chapter 9.
- (6) Lin, T.-L.; Chen, S. H.; Gabriel, N. E.; Roberts, M. F. *J. Am. Chem. Soc.* **1986**, 108, 3499.
- (7) Lin, T.-L.; Chen, S. H.; Gabriel, N. E.; Roberts, M. F. *J. Phys. Chem.* **1987**, 91, 406.
- (8) Lin, T.-L.; Chen, S. H.; Roberts, M. F. *J. Am. Chem. Soc.* **1987**, 109, 2321.
- (9) Tausk, R. J. M.; Karmiggelt, J.; Oudshoorn, C.; Overbeek, J. T. G. *Biophys. Chem.* **1974**, 1, 175.
- (10) Tausk, R. J. M.; Van Esch, J.; Karmiggelt, J.; Voordouw, G.; Overbeek, J. T. G. *Biophys. Chem.* **1974**, 1, 184.
- (11) Tausk, R. J. M.; Oudshoorn, C.; Overbeek, J. T. G. *Biophys. Chem.* **1974**, 2, 53.
- (12) Tausk, R. J. M.; Overbeek, J. T. G. *Biophys. Chem.* **1974**, 2, 175.
- (13) Missel, P. J.; Mazer, N. A.; Benedek, G. B.; Young, C. Y.; Carey, M. C. *J. Phys. Chem.* **1980**, 84, 1044.
- (14) Lin, T.-L.; Hu, Y.; Liu, W.-J. *Langmuir* **1997**, 13, 1422.
- (15) Shuto, S.; Ueda, S.; Imamura, S.; Fukukawa, K.; Matsuda, A.; Ueda, T. *Tetrahedron Lett.* **1987**, 28, 199.
- (16) Shuto, S.; Ito, H.; Ueda, S.; Imamura, S.; Fukukawa, K.; Tsujino M.; Matsuda, A.; Ueda, T. *Chem. Pharm. Bull.* **1988**, 36, 209.
- (17) Shuto, S.; Imamura, S.; Fukukawa, K.; Ueda, T. *Chem. Pharm. Bull.* **1988**, 36, 5020.
- (18) Stryer, L. *Biochemistry*, 3rd ed.; W. H. Freeman: San Francisco, 1988; Chapter 23.
- (19) Bonaccio, S.; Wessicken, M.; Berti, D.; Walde, P.; Luisi, P. L. *Langmuir* **1996**, 12, 4976.
- (20) Berti, D.; Franchi, L.; Baglioni, P.; Luisi, P. L. *Langmuir* **1997**, 13, 3441.
- (21) Berti, D.; Baglioni, P.; Bonaccio, S.; Luisi, P. L. *J. Phys. Chem. B* **1998**, 102, 303.
- (22) Berti, D.; Bucci, I.; Baglioni, P. Manuscript in preparation.
- (23) Cantor, C. R.; Shimmel, P. R. *Biophysical Chemistry*; W. H. Freeman and Company: San Francisco, 1980; Chapters 3, 6, and 7.
- (24) Freifelder, D. *Physical Biochemistry*; W. H. Freeman and Company: San Francisco, 1982; Chapters 5 and 6.
- (25) Saenger, W. *Principles of Nucleic Acid Structure*; Springer-Verlag: New York, 1984.
- (26) Egholm, M.; Buchardt, O.; Nielsen, P. E.; Berg, R. H. *J. Am. Chem. Soc.* **1992**, 114, 1895.
- (27) Wittung, P.; Nielsen, P. E.; Buchardt, O.; Egholm, M.; Norden, B. *Nature* **1994**, 368, 561.
- (28) Jones, J. D.; Thompson, T. E. *Biochemistry* **1990**, 29, 1593.
- (29) Onda, M.; Yoshihara, K.; Koyano, H.; Ariga, K.; Kunitake, T. *J. Am. Chem. Soc.* **1996**, 118, 8524.
- (30) Y. C.; Baglioni, P.; Teixeira, J.; Chen, S. H. *J. Phys. Chem.* **1994**, 98, 10208.
- (31) Cabane, B. In *Surfactant Science Series*; Zana, R., Ed.; Marcel Dekker: New York, 1987; Vol. 22, Chapter 2.
- (32) Kotlarchyk, M.; Chen, S. H. *J. Chem. Phys.* **1983**, 79, 2461.
- (33) Nagarayan, R. In *Mixed Surfactant Systems*; Holland, P. M., Rubingh, D. N., Eds.; ACS Symposium Series 501; American Chemical Society: Washington, DC, 1992; p 54-95.
- (34) Maeda, H.; Shiuchi, M.; Kakehashi, R. *J. Phys. Chem. B* **1997**, 101, 7378.