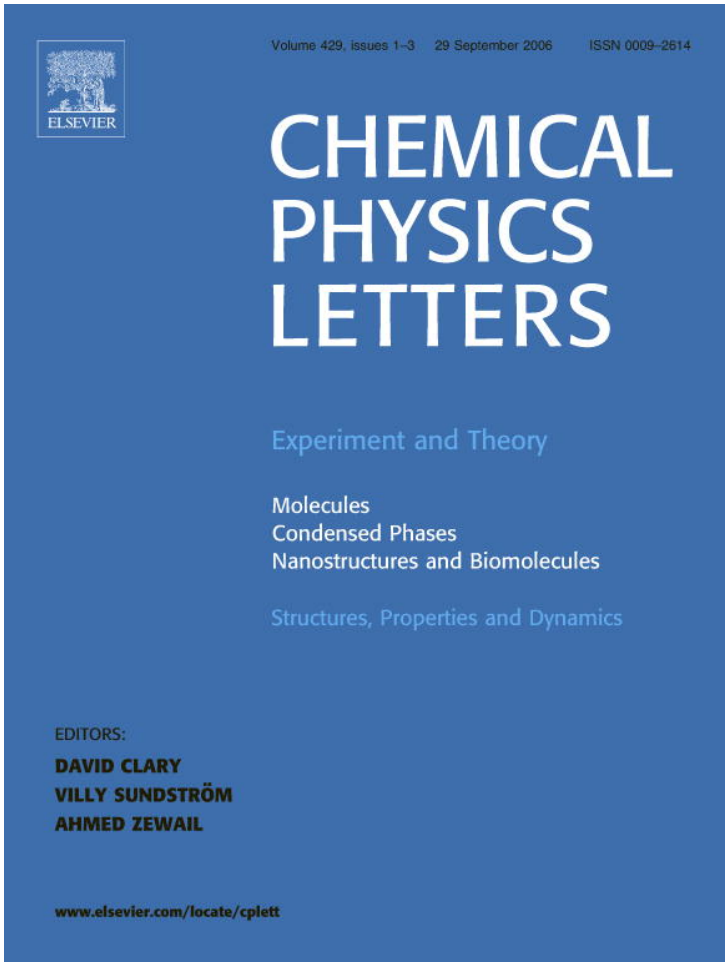

Available online at www.sciencedirect.com
ScienceDirect
Chemical Physics Letters 429 (2006) 250–254
CHEMICAL PHYSICS LETTERS
www.elsevier.com/locate/cplett

Formation of overcharged cationic lipid/DNA complexes

Giulio Caracciolo [a], Daniela Pozzi [a], Ruggero Caminiti [a,*], Heinz Amenitsch [b]

[a] Dipartimento di Chimica, Università degli Studi di Roma 'La Sapienza', P.le Aldo Moro 5, 00185 Rome, Italy
[b] Institute of Biophysics and X-ray Structure Research, Austrian Academy of Sciences, Graz, Austria

Received 31 May 2006; in final form 4 August 2006
Available online 10 August 2006

Abstract

The widely accepted 'counterions release' mechanism predicts the existence of charge-neutral cationic lipid/DNA complexes (lipoplexes). Formation of overcharged complexes, both positively and negatively charged, is supposed to be driven by thermodynamics. Here we report a synchrotron small angle X-ray diffraction and dynamic light scattering study aimed to shed light on the mechanism of formation of lipoplexes. By using a two-step process, consisting in adding excess material to preformed isoelectric lipoplexes, we showed that excess DNA does enter preformed complexes while excess lipid does not. Our findings imply that DNA may play a special role in the formation of overcharged lipoplexes.

1. Introduction

Cationic liposome (CL)-mediated transfection is a useful non-viral tool for in vitro and in vivo gene transfer [1–3]. When DNA is mixed with preformed CLs, multilamellar CL–DNA complexes (lipoplexes) form in a self-assembled manner. Over the last few years, many studies have investigated the physical–chemical parameters that affect the formation and the emerging structure and morphology of lipoplexes [4–6].

Nevertheless, various questions remain unanswered. In particular, the molecular events occurring when DNA interacts with CLs are still poorly understood [7–9]. It is a central point because the mode of formation of the complexes strongly influences the final physical–chemical properties of the lipoplexes and, therefore, modulates their biological activity. Furthermore, understanding the molecular mechanisms underlying lipoplex formation is crucial for their future development and design as efficient gene delivery systems.

DNA and CLs are oppositely charged macromolecules that are surrounded in solution by a diffuse layer of spatially confined 'Manning' counterions [10,11]. Upon approach, the fixed charges tend to neutralize each other with the result that a fraction of (sometimes all) counterions are released into the bulk solution. The condensation of CLs and DNA to form highly-ordered lipoplexes is therefore considered an 'entropically driven' process. However, the so-called 'counterions release' mechanism can only explain the existence of charge-neutral (isoelectric) lipoplexes for which there is perfect compensation between the positive charge of cationic lipids and the negative charge of DNA. On the other hand, several experimental techniques [4–6] have unambiguously shown the existence of overcharged lipoplexes containing excess material (DNA or lipid) into their bulk. Thus, a question immediately arises: how to explain the formation of both positively and negatively charged lipoplexes? The most accredited mode of formation is well described by an 'osmotic pressure model' [4,12]. At the isoelectric point, there is a counterions vacuum inside a charge-neutral lipoplex, because all the lipid and DNA counterions are released into the bulk solution during complex formation. Thus, excess DNA in solution (or excess lipid) is driven into the complex by an osmotic pressure of its confined counterions. Additional DNA molecules (or lipids) can lower their free energy by entering the complex and releasing bound Na^+ (or Cl^-)

* Corresponding author. Fax: +39 06 490631.
E-mail address: r.caminiti@caspur.it (R. Caminiti).

0009-2614/$ - see front matter © 2006 Elsevier B.V. All rights reserved.
doi:10.1016/j.cplett.2006.08.030

counterions into the three-dimensional internal volume of the complex. In this scenario, the intake of excess material is lastly stopped by electrostatic repulsions between DNA molecules (in the case of excess DNA) or between cationic membranes (in the case of excess lipid). The lipoplex is therefore one-phase close to the isoelectric point whereas it is expected to separate into complex plus unbound DNA in the case of excess DNA and into complex plus CLs in the case of excess lipid (Fig. 1).

Pozharki and MacDonald [13] showed that lipoplex formation is endothermic and that the enthalpy change per mole of lipid in lipoplex is positive (~600 cal/lipid mole). More recently, the same authors have also provided the first experimental thermodynamic description of lipoplex formation by isothermal titration calorimetry experiments [14]. The authors have found that binding entropy is about twice larger than the enthalpy change thus providing experimental evidence that lipoplex formation is driven by thermodynamics because the free energy change is negative ($\Delta G = \Delta H - T\Delta S < 0$). In this Letter, we report a combined synchrotron small angle X-ray diffraction (SAXD) and dynamic light scattering (DLS) study aimed to shed more light on the mechanism formation of overcharged lipoplexes.

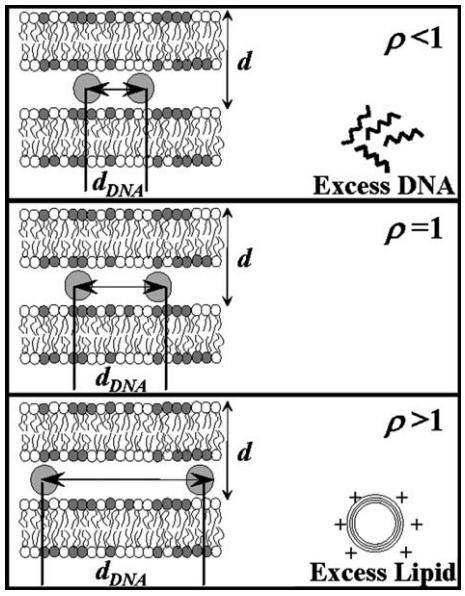

Fig. 1. Schematics of the L_α^C phase: DNA rods are intercalated between cationic lipid membranes in the liquid-crystalline phase composed of cationic (grey) and neutral helper (white) lipids. The lamellar periodicity along the normal to lipid bilayers is indicated as d. The distance between adjacent DNA strands is indicated as d_{DNA}. The currently accepted mechanism of formation of CL–DNA complexes [4,12] relies on the assumption that structure and phase behavior of lipoplexes as a function of the charge ratio, ρ, are driven by thermodynamics. The lipoplex is one-phase (with no excess material) close to the isoelectric point ($\rho = 1$, middle panel). Overcharged lipoplexes adsorb excess material into their bulk thus adjusting the 1D DNA lattice (DNA spacing, d_{DNA}). Electrostatic repulsions set an upper limit on the amount of excess material that a complex can accommodate. As a result, the complex is expected to separate into complex plus excess lipid for $\rho > 1$ (bottom panel) and complex plus excess DNA for $\rho < 1$ (top panel).

2. Experimental section

2.1. Lipoplexes preparation

Calf thymus Na-DNA was purchased from Sigma (St. Louis, MO). 1,2-Dioleoyl-3-trimethylammonium-propane (DOTAP), dioleoylphosphatidylethanolamine (DOPE) and dioleoylphosphocholine (DOPC) were purchased from Avanti Polar Lipids (Alabaster, AL) and used without further purifications. DOTAP–DOPC and DOTAP–DOPE multi-lamellar vesicles (MLVs) were routinely prepared [15]. The molar ratio of neutral lipid in the bilayer was $\Phi = 0.5$ and 0.46 for DOTAP–DOPC and DOTAP–DOPE respectively. Binary mixtures of lipids in chloroform were dried, vacuum desiccated for at least 12 h and finally resuspended in deionized water to reach the desired final concentration (25 mg/ml for SAXD experiments). An aliquot of this was diluted to 0.5 mg/ml (DLS experiments). DOTAP–DOPC and DOTAP–DOPE liposomes were incubated at 30 °C for at least 36 h to achieve full hydration [16]. The nominal composition of the lipoplexes is given by the charge ratio, ρ, between the positive charge carried by DOTAP headgroups and the negative charge carried by DNA. The complex is stoichiometrically charge-neutral when the number of DOTAP molecules and DNA bases are equal ($\rho = 1$) whereas varying ρ may result in the formation of positively ($\rho > 1$) or negatively charged ($\rho < 1$) lipoplexes. The formation of isoelectric DOTAP–DOPC/DNA and DOTAP–DOPE/DNA lipoplexes was promoted by adding appropriate amounts of sonicated Na-DNA solution to liposomal dispersions. On the other hand, overcharged lipoplexes ($\rho = 0.5$ and 2) were formed following two different preparation schedules.

2.2. 'Directly formed' overcharged lipoplexes

In the first case, overcharged lipoplexes were prepared adding proper amounts of NA-DNA solution to liposomal dispersions at the charge ratios $\rho = 0.5$ and 2 and incubated for one week. In what it follows, we shall indicate so prepared complexes as 'directly formed' lipoplexes.

2.3. 'Indirectly formed' overcharged lipoplexes

In the second case, charge-neutral lipoplexes were let to equilibrate for one week. Then, excess material (DNA or lipid) was added to charge-neutral lipoplexes paying attention to reproduce the same nominal ρ values ($\rho = 0.5$ and 2) and the complexes were let to equilibrate for one week. Complexes prepared via such a two-step process will be referred to as 'indirectly formed' lipoplexes.

2.4. Synchrotron SAXD experiments

All SAXD measurements were performed at the Austrian SAXS station of the synchrotron light source ELETTRA (Trieste, Italy) [17]. SAXD patterns were recorded

with a gas detector based on the delay line principle covering a q-range ($q = 4\pi\sin(\theta)/\lambda$) of between 0.05 and 1.5 Å^{-1}. The angular calibration of the detector was performed with silver–behenate [$CH_3(CH_2)_{20}$–COOAg] whose d corresponds to 58.38 Å. The unoriented samples were sealed in a 1.5 mm diameter glass X-ray capillaries. The bulk solution was subtracted as background from the collected data. All SAXD measurements were performed at 26 °C and temperature was controlled close to the capillary by a KPR-Peltier module (Anton Paar, Graz, Austria) with a precision of 0.1 °C.

2.5. Dynamic light scattering experiments

The size of liposomes and lipoplexes were measured at the temperature of 25 °C by means of dynamic light scattering (DLS) technique, using a Malvern Zetasizer4 apparatus. For all the samples investigated, data show a unimodal distribution and represent the average of at least four different measurements carried out for each sample.

3. Results

Fig. 2 (left panel) shows the SAXD patterns of 'directly formed' DOTAP–DOPE/DNA lipoplexes. Lamellar peaks, labelled as (001) and (002), are the first two Bragg reflections of a multilayer L_α^C phase (Fig. 1) with lamellar periodicity $d = 2\pi/q_{001} = 66.1$ Å. The structure consists of smectic-like arrays of stacked bilayers with intercalated monolayers of DNA forming a one-dimensional (1D) in-plane rod lattice with spacing $d_{DNA} = 2\pi/q_{DNA}$ [18–22]. Some of us have recently proposed a theoretical model [22] describing DNA–DNA electrostatic interactions within lamellar lipoplexes. Given a lipid composition, Φ, charge neutrality is the most stable condition and the DNA spacing is given by

$$d_{DNA}^{th} = \frac{A}{2 \times l_B \times (1 - \Phi)} \quad (1)$$

where A is the average lipid headgroup area and $l_B = 1.7$ Å is the distance between two phosphate entities along the DNA axis. Using Eq. (1) relies on the assumption that the area of cationic lipid headgroups, A_C, is similar to that of neutral lipid headgroups, A_N. According to recent findings [23], we used $A_C = A_N = A = 72$ Å^2 and we obtained a predicted DNA–DNA spacing, $d_{DNA}^{th} = 39.2$ Å that is in excellent agreement with the experimental value ($d_{DNA}^{exp} = 39.4$ Å).

Fig. 2 shows that 'directly formed' overcharged lipoplexes adsorbed excess material into their bulk thus adjusting the 1D DNA lattice. As the mobile DNA peak clearly shows (Fig. 2, left panel), DNA–DNA distance of positively charged lipoplexes ($\rho = 2$) was found to be larger ($d_{DNA} = 43.3$ Å) than that of charge-neutral lipoplexes ($d_{DNA} = 39.4$ Å) while DNA–DNA distance of negatively charged lipoplexes ($\rho = 0.5$) was found to be definitely shorter ($d_{DNA} = 30.6$ Å).

The SAXD patterns of 'indirectly formed' overcharged lipoplexes are shown in Fig. 2 (right panel). From the DNA peaks we could calculate the DNA spacings of 'indirectly formed' overcharged lipoplexes ($d_{DNA} = 39.6$ Å at $\rho = 2$ and $d_{DNA} = 30.6$ Å at $\rho = 0.5$). It is evident that the structure of negatively charged lipoplexes is irrespective of preparation, in that the SAXD patterns ($\rho = 0.5$, left and right panels) are identical.

Conversely, the structure of positive lipoplexes was strongly influenced by the protocol of preparation. It is noteworthy to observe that adding excess lipid to charge-neutral complexes did not modify their inner structure thus suggesting that excess lipid did not enter the preformed complex. SAXD experiments (not reported) replicated after longer incubation times (one month) gave the same results and ensured that the equilibrium structure of the complexes was unique.

The mean diameter (D_m) of 'directly formed' DOTAP–DOPC lipoplexes, as revealed by DLS experiments, is shown in Table 1. Two features should be underlined. Firstly, charge-neutral lipoplexes ($\rho = 1$) showed larger size ($D_m \sim 1200$ nm) with respect to pure multi-lamellar lipo-

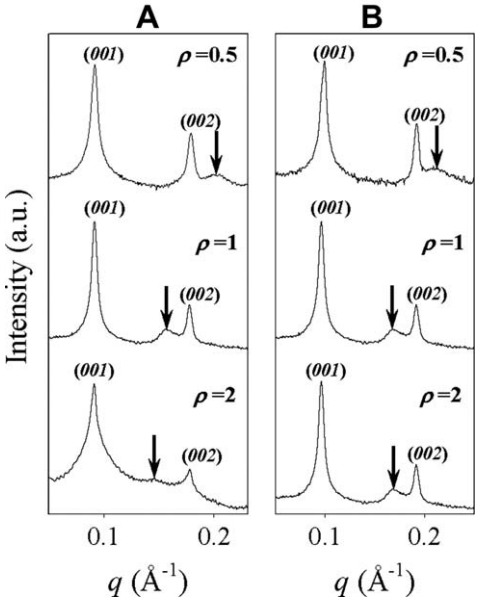

Fig. 2. SAXD patterns of 'directly formed' (A) and 'indirectly formed'(B) DOTAP–DOPE/DNA lipoplexes as a function of ρ. As shown by the DNA mobile peak (marked by arrow), DNA does enter preformed lipoplexes while excess lipid does not.

Table 1
Mean diameter, D_m, of 'directly formed' (A) and 'indirectly formed' (B) DOTAP–DOPC/DNA lipoplexes as a function of ρ

ρ	D_m (nm)	
	A	B
0.5	750	750
1	1200	1200
2	700	1200; 450

somes ($D_m \sim 500$ nm) with no DNA added. Thus, DNA allows the liposomes to come into contact and aggregate by reducing the inter-membrane repulsive barrier essentially due to electrostatic repulsions [7–9]. Close to the isoelectric point, single globules tend to stick when they collide because van der Waals short-range attractions can easily overcome weak electrostatic repulsions. As a consequence, large aggregates of globules form.

Secondly, Table 1 shows that the overcharged lipoplexes, both positively and negatively charged, tend to repel each other, thus leading to much smaller aggregates ($D_m \sim 750$ nm). It is noteworthy to observe that, varying ρ, we may obtain aggregates with the same size but with opposite overall electric charge [24].

Adding excess DNA to isoelectric lipoplexes produced major changes on the mean size of isoelectric lipoplexes. Indeed, the particle mean size decreased from $D_m \sim 1200$ nm to $D_m \sim 750$ nm. It must be evidenced that the average size of negatively charged lipoplexes both 'directly formed' and 'indirectly formed' was found to be essentially the same. Since, for a given ionic strength, the size of the aggregates is controlled by the charge ratio, ρ, our findings imply that excess DNA did enter preformed equilibrium aggregates.

On the contrary, upon adding excess lipid to preformed lipoplexes, no relevant structural changes were observed. Indeed, the mean size of 'indirectly formed' positive lipoplexes was found to be identical to that of charge-neutral lipoplexes ($D_m \sim 1200$ nm) and to coexist with a second population of particles of means size ($D_m \sim 450$ nm) of the same order of magnitude of pure liposomes ($D_m \sim 500$ nm).

DLS findings supplied therefore further evidence that excess DNA does enter preformed lipoplexes while excess lipid does not. SAXD and DLS findings seemingly contradict the universally accepted mechanism of formation of overcharged lipoplexes.

Indeed, if the formation of overcharged lipoplexes was an 'entropically driven' process, excess lipid molecules would enter the preformed complex (as DNA effectively does) due to the entropic gain arising from the release of confined Cl^- counterions into the internal volume of the complex [4,12]. In other words, if formation of overcharged lipoplexes, positive or negative they were, was driven by an entropic gain coming from counterion release one would expect a two-step mechanism to produce the same result than that produced by a direct one-step mechanism. As SAXD and DLS results show, this is not the case.

Mixing DNA and excess lipid (1:2 molar ratio, one-step formation) leads to the spontaneous formation of positively overcharged lipoplexes in the time-scale of milliseconds [25,26], while here we show that adding excess lipid to preformed charge-neutral lipoplexes (fixing the stoichiometry) does not produce the same results within several days. The case of negatively overcharged lipoplexes is unquestionably different since structure obtained by a one-step or a two-step method is exactly the same. It means that, when adding excess DNA to charge neutral lipoplexes, DNA is rapidly incorporated within preformed lipoplex.

When excess lipid (in the form of separate vesicles) is added to preformed lipoplexes several energetics barriers must be overcome, the higher being that due to hydrophobic interactions. Lipid molecules are held in bilayers by strong hydrophobic interactions and a change in organization would be energetically very expensive. How may liposomes break if DNA is embedded within the complex?

On the other hand, our findings imply that DNA may play a special role in the formation of lipoplexes. As recently proposed, DNA molecules may act as a molecular glue in that they may force lipid vesicles, being indistinctly liposomes or preformed lipoplexes, to come into contact and fuse [7,8]. Upon DNA-induced vesicles fusion, large lipid mixing occurs and only mixed lipoplexes exist with highly-specific structural properties [27,28]. It would mean that lipoplexes form by a mechanism that involves necessarily the DNA-induced approach of lipid vesicles. Such a mechanism could explain the formation of 'directly formed' overcharged lipoplexes (both positive and negative) and that of 'indirectly formed' negatively charged lipoplexes. In these cases, there would be in fact naked DNA molecules in solution able to promote formation of lipoplexes. Conversely, since excess lipid cannot play the same role as DNA, it would imply that 'indirectly formed' positively charged lipoplexes cannot form spontaneously because all DNA molecules are embedded within lipid bilayers.

In this scenario, the formation of overcharged lipoplexes would not only be driven by an entropic gain coming from counterion release. According to the 'counterions release' mechanism that can only explain the existence of charge-neutral lipoplexes, we propose that DNA and lipid really interact in a 1:1 ratio even when lipid is 'in excess'. To our opinion, when overcharged lipoplexes are formed by a one-step mechanism, excess lipid molecules are incorporated into the interior of the complex because they are held in bilayers by strong hydrophobic interactions. In terms of free energy, the mixing of hydrocarbon and water would cause a decrease in entropy (another way of saying this is that the entropy of water is decreased at the interface between hydrocarbon and water). As a result, excess lipid molecules do not release their counterions thus modifying the effective surface charge density of lipid bilayers. For that reason, when excess lipids are added to preformed isoelectric lipoplex, they cannot enter the complex due to the presence of such a high, presumably kinetic, barrier.

4. Conclusions

By using a two-step process, consisting in adding excess material to preformed isoelectric lipoplexes, we showed that excess DNA does enter preformed lipoplexes while excess lipid does not (within several days). Our findings imply that DNA may play a special role in the formation of overcharged lipoplexes. DNA, being a single molecule,

does not face the constraints that lipid has. Positive electrostatic charges localized on the surface of both liposomes and preformed lipoplexes create a repulsive barrier which prevails over short-range attractive van der Waals forces and avoids membrane aggregation and fusion. Secondly and most importantly, breakage of liposome and release of molecules by lipid membranes are opposed by the hydrophobic effect. Indeed, lipids are held in bilayers by hydrophobic interactions and a change in supramolecular organization – such as required by lipid molecules to enter preformed lipoplexes – is definitely opposed.

Acknowledgements

We thank Prof. C. Cametti for allowing us to use the Malvern Zetasizer4 apparatus located at the Department of Physics of the University of Rome 'La Sapienza'. We thank Dr. S. Sennato for helping us to treat DLS data. Prof. C. Cametti and Dr. S. Sennato are also gratefully acknowledged for useful discussions.

References

[1] P.L. Felgner et al., Proc. Natl. Acad. Sci. USA 84 (1987) 7413.
[2] P.L. Felgner, Sci. Am. 276 (1997) 102.
[3] D.D. Lasic, Liposomes in Gene Delivery, CRC Press, Boca Raton, FL, 1997.
[4] I. Koltover, T. Salditt, C.R. Safinya, Biophys. J. 77 (1999) 915.
[5] A.J. Lin, N.L. Slack, A. Ahmad, I. Koltover, C.X. George, C.E. Samuel, C.R. Safinya, J. Drug Target 8 (2000) 13.
[6] G. Caracciolo, R. Caminiti, D. Pozzi, M. Friello, F. Boffi, A. Congiu Castellano, Chem. Phys. Lett. 351 (2002) 222.
[7] S. Huebner, B.J. Battersby, R. Grimm, G. Cevc, Biophys. J. 76 (1999) 3158.
[8] M.T. Kennedy, E.V. Pozhaski, V.A. Rakhmanova, R.C. MacDonald, Biophys. J. 78 (2000) 1620.
[9] M.E. Hayes, A.V. Gorelov, K.A. Dawson, Prog. Colloid Polym. Sci. 118 (2001) 243.
[10] G.S. Manning, J. Chem. Phys. 51 (1969) 24.
[11] G.S. Manning, Q. Rev. Biophys. II (1978) 179.
[12] R. Bruinsma, Eur. Phys. J. B 4 (1998) 75.
[13] E.V. Pozhaski, R.C. MacDonald, Biophys. J. 83 (2002) 556.
[14] E.V. Pozhaski, R.C. MacDonald, Biophys. J. 85 (2003) 3969.
[15] G. Caracciolo, D. Pozzi, R. Caminiti, A. Congiu Castellano, Eur. Phys. J. E 351 (2002) 222.
[16] D. Pozzi, H. Amenitsch, R. Caminiti, G. Caracciolo, Chem. Phys. Lett. 422 (2006) 439.
[17] H. Amenitsch, M. Rappolt, M. Kriechbaum, H. Mio, P. Laggner, S. Bernstorff, J. Synchrotron Radiat. 5 (1998) 506.
[18] J.O. Rädler, I. Koltover, T. Salditt, C.R. Safinya, Science 275 (1997) 810.
[19] T. Salditt, I. Koltover, J.O. Rädler, C.R. Safinya, Phys. Rev. Lett. 79 (1997) 2582.
[20] F. Artzner, R. Zantl, G. Rapp, J.O. Rädler, Phys. Rev. Lett. 81 (1998) 5015.
[21] C.R. Safinya, Curr. Opin. Struct. Biol. 11 (2001) 440.
[22] G. Caracciolo, R. Caminiti, Chem. Phys. Lett. 400 (2004) 314.
[23] G. Caracciolo, D. Pozzi, H. Amenitsch, R. Caminiti, Langmuir 22 (2006) 4267.
[24] F. Bordi, C. Cametti, S. Sennato, Chem. Phys Lett. 409 (2005) 134.
[25] P.C.A. Barreleiro, R.P. May, B. Lindman, Faraday Discuss. 122 (2002) 191.
[26] P.C.A. Barreleiro, R.P. May, B. Lindman, J. Phys. Chem. B 107 (2003) 6208.
[27] G. Caracciolo, D. Pozzi, R. Caminiti, H. Amenitsch, Appl. Phys. Lett. 87 (2005) 133901.
[28] G. Caracciolo, D. Pozzi, H. Amenitsch, R. Caminiti, Langmuir 21 (2005) 11582.