Research Article

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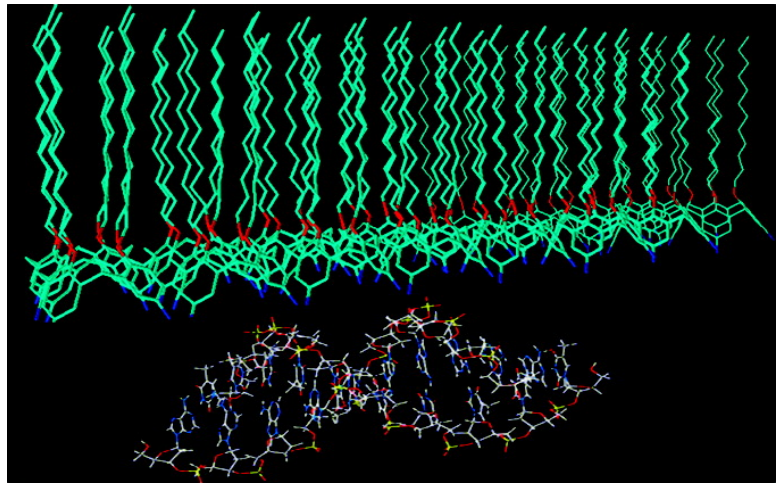

Amino-Substituted Amphiphilic Calixarenes: Self-Assembly and Interactions with DNA

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The amphiphilic 5,11,17,23-tetramino-25,26,27,28-tetradodecyloxycalix[4]arene is shown to self-assemble as stable and well-defined Langmuir monolayers at the air−water interface. The effect of the presence of DNA in the subphase reveals interactions taking place at the interface between the positively charged surface and the negatively charged DNA, causing an expansion of the monolayers and a phase transition from a liquid-condensed to a liquid-expanded phase; a slight decrease in the stability of the monolayers is also observed. The title compound is shown to self-assemble, with the absence of a cosurfactant, as stable colloidal suspensions. Photon correlation spectroscopy, ζ-potential measurements, and atomic force microscopy reveal that these colloidal suspensions present a monodisperse size distribution and are composed of positively charged solid lipid nanoparticles (SLNs), with an average hydrodynamic diameter of 190 nm and a surface potential of +13.2 mV. The interaction of these SLNs with double-stranded DNA is demonstrated.

Introduction

The increasing promise of gene therapy revealed the need to develop efficient carrier systems capable of delivering efficiently genetic-information-bearing molecules through the cell membrane and the cytoplasm to the nucleus of cells. Since more than 20 years, because of some drawbacks of virus-based vectors, nonviral transporting systems for synthetic drugs are under development and some of them have been successfully used in therapy. In view of the large variety of drug carriers already developed and in spite of the fact that the class of molecule to transport is different, it seems obvious that the new generations of drug carriers for gene therapy will be adapted from existing systems.[1] Among them, self-assembled vectors are prepared taking advantage of the outstanding self-organization properties of amphiphiles.[2–4] In addition to the large variety of natural amphiphiles available, a number of synthetic systems have been developed in order to improve several key properties of these carriers including high stability, enhanced loading capabilities, low production costs, improved bioavailability, and so forth. Among these synthetic amphiphiles, micelle-forming polymers are the subject of high interest and are used in therapy for hydrophobic drug formulation.[5–9] More sophisticated amphiphiles such as peptides[10–12] or peptidyl-nucleic acid[13] have been shown to form micelles, but their high production costs may be a limiting factor for their commercial use. In the case of amphiphilic peptides, it has been demonstrated that the self-assembly process may take place when the amphiphiles are injected from water to a given physiological milieu.[4] This approach opened up new promises for the application of amphiphilic peptides specifically designed in order to make only the self-assembled form biologically active. Regarding the more conventional approach which consists of using the self-assembled system as drug carrier, amphiphilic macrocycles based on calixarenes, cyclodextrins, or crown ethers have been shown to self-assemble in water as micelles,[14–16] liposomes,[17–23] or solid lipid nanoparticles (SLNs).[24–26] These molecules have been widely used in supramolecular chemistry[27] because of their remarkable molecular recognition properties which could also be highly beneficial for the transporting systems they form. Calixarenes are macrocyclic molecules produced by the base catalyzed reaction of formaldehyde and *p*-substituted phenols.[28,29] When suitably modified,

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(1) Allen, T. M.; Cullis, P. R. *Science* **2004**, *303*, 1818.
(2) Israelachvili, J. N. *Intermolecular and Surface Forces: With Applications to Colloidal and Biological Systems*; Academic Press: London, 1985.
(3) Nandi, N.; Vollhardt, D. *Acc. Chem. Res.* **2007**, *40*, 351.
(4) Palmer, L. C.; Velichko, Y. S.; Olvera De La Cruz, M.; Stupp, S. I. *Philos. Trans. R. Soc. London, Ser. A* **2007**, *365*, 1417.
(5) Bramer, T.; Dew, N.; Edsman, K. *J. Pharm. Pharmacol.* **2008**, *59*, 1319.
(6) Li, W.; Szoka, F. C., Jr *Gene Transfer* **2007**, 441.
(7) Mahmud, A.; Xiong, X.-B.; Aliabadi, H. M.; Lavasanifar, A. *J. Drug Targeting* **2007**, *15*, 553.
(8) Narang, A. S.; Delmarre, D.; Gao, D. *Int. J. Pharm.* **2007**, *345*, 9.
(9) Torchilin, V. P. *Pharm. Res.* **2007**, *24*, 1.
(10) Raman, S.; Machaidze, G.; Lustig, A.; Aebi, U.; Burkhard, P. *Nanomedicine* **2006**, *2*, 95.
(11) Santoso, S. S.; Vauthey, S.; Zhang, S. *Curr. Opin. Colloid Interface Sci.* **2002**, *7*, 262.
(12) Yamnitz, C. R.; Gokel, G. W. *Chem. Biodiversity* **2007**, *4*, 1395.
(13) Lazar, A. N.; Coleman, A. W.; Terenzi, S.; Strazewski, P. *Chem. Commun.* **2006**, 63.
(14) Auzely-Velty, R.; Pean, C.; Djedaieni-Pilard, F.; Zemb, T.; Perly, B. *Langmuir* **2001**, *17*, 504.
(15) Kellermann, M.; Bauer, W.; Hirsch, A.; Schade, B.; Ludwig, K.; Boettcher, C. *Angew. Chem., Int. Ed.* **2004**, *43*, 2959.
(16) Lee, M.; Lee, S.-J.; Jiang, L.-H. *J. Am. Chem. Soc.* **2004**, *126*, 12724.
(17) De Lisi, R.; Milioto, S.; Muratore, N. *J. Phys. Chem. B* **2002**, *106*, 8944.
(18) Houmadi, S.; Coquiere, D.; Legrand, L.; Faure, M. C.; Goldmann, M.; Reinaud, O.; Remita, S. *Langmuir* **2007**, *23*, 4849.
(19) Markowitz, M. A.; Bielski, R.; Regen, S. L. *Langmuir* **1989**, *5*, 276.
(20) Micali, N.; Villari, V.; Consoli, G. M. L.; Cunsolo, F.; Geraci, C. *Phys. Rev. E: Stat. Phys., Plasmas, Relat. Interdiscip. Top.* **2006**, *73*, 051904/1.
(21) Strobel, M.; Kita-Tokarczyk, K.; Taubert, A.; Vebert, C.; Heiney, P. A.; Chami, M.; Meier, W. *Adv. Funct. Mater.* **2006**, *16*, 252.
(22) Tanaka, Y.; Mayachi, M.; Kobuke, Y. *Angew. Chem., Int. Ed.* **1999**, *38*, 504.
(23) Falvey, P.; Lim, C. W.; Darcy, R.; Revermann, T.; Karst, U.; Giesbers, M.; Marcelis, A. T. M.; Lazar, A.; Coleman, A. W.; Reinhoudt, D. N.; Ravoo, B. J. *Chem.—Eur. J.* **2005**, *11*, 1171.
(24) Dubes, A.; Parrot-Lopez, H.; Abdelwahed, W.; Degobert, G.; Fessi, H.; Shahgaldian, P.; Coleman, A. W. *Eur. J. Pharm. Biopharm.* **2003**, *55*, 279.
(25) Shahgaldian, P.; Cesario, M.; Goreloff, P.; Coleman, A. W. *Chem. Commun.* **2002**, 326.
(26) Shahgaldian, P.; Da Silva, E.; Coleman, A. W.; Rather, B.; Zaworotko, M. J. *Int. J. Pharm.* **2003**, *253*, 23.
(27) Steed, J. W.; Turner, D. R.; Wallace, K. J. *Core Concepts in Supramolecular Chemistry and Nanochemistry*; John Wiley and Sons: Chichester, 2007.
(28) Asfari, Z.; Bohmer, V.; Harrowfield, J.; Vicens, J.; Eds. In *Calixarenes 2001*; Kluwer Academic Publishers: Dordrecht, 2001.

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they possess amphiphilic properties and therefore are capable of self-assembly at interfaces and in water. In addition, they have been shown to possess recognition properties of biological molecules.[30,31] In this paper, we report on the amphiphilic and molecular recognition properties of *p*-amino tetradodecyloxy-calix[4]arene; it is shown that this amphiphile is able to self-assemble at the air−water interface as stable Langmuir films, which possess DNA recognition properties. The possibility to prepare solid lipid nanoparticles from this amphiphile is demonstrated by photon correlation spectroscopy and confirmed by atomic force microscopy; these systems also possess the ability to interact with DNA through electrostatic interactions.

Experimental Section

Synthesis. *p*-Nitro-tetradodecyloxy-calix[4]arene was prepared as previously described.[32] The reduction of nitro groups was carried out using a procedure described by Dudic et al. for water-soluble calixarenes.[33] Briefly, in 50 mL of ethanol was added 2 g of *p*-nitro-tetradodecyloxy-calix[4]arene, hydrazine (2.2 mL), and a catalytic amount of palladium on activated charcoal (10%). The mixture was refluxed, and the disappearance of the starting material was followed by thin layer chromatography (TLC; $CHCl_3$/hexane/acetone, 10:20:3). When the reaction was complete, the reaction mixture was filtered and evaporated under vacuum; the yellowish solid formed and crystallized from ethanol is **1** (95% yield). Analytical data (^1H and ^{13}C NMR) are in perfect agreement with those published.[32]

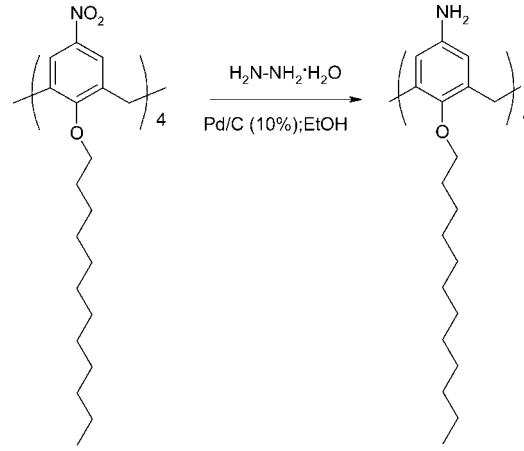
Solid Lipid Nanoparticle (SLN) Preparation. To 3 mL of a solution of **1** in tetrahydrofuran (THF) (5 mg/mL), under vigorous magnetic stirring, was added 50 mL of water at a flow rate of 500 mL/min. The resulting milky suspension was stirred for an additional minute, and the THF was evaporated under reduced pressure at 40 °C.

Particle Size and ζ-Potential Measurements. Particle size measurements were carried out using photon correlation spectroscopy (PCS), diluting SLN samples at a concentration of 30 mg/L in nanopure water at 298 K. The ζ-potential of the SLNs dispersed in water at a concentration of 30 mg/L was determined by electrophoretic mobility measurements (Zetasizer Nano ZS, Malvern Instruments Ltd., U.K.). For both PCS and ζ-potential measurements, the measurements were done in triplicate to ensure the reproducibility of the results.

Langmuir Monolayer Studies. Langmuir film experiments were carried out using a Nima Technology 112D system (Coventry, U.K.). The trough and the barriers were cleaned with analytical grade chloroform and purified water (resistivity ≥18 MΩ·cm). Surface tension was monitored using a Wilhelmy plate system. Compressions were performed continuously at a speed rate of 5 cm^2/min. DNA subphases were freshly prepared by dissolving the appropriate amount of low molecular weight DNA from salmon sperm (BioChemika) in pure water. Compression without amphiphiles spread on the surface were performed in order to check the absence of surface-active molecules in the subphase; in no case was a change in surface tension observed. Spreading solutions were prepared by dissolving **1** in chloroform at a concentration of 1 mg/mL, and they were stored at 4 °C in order to prevent solvent evaporation. A total of 15 μL of this solution was spread on the aqueous subphase using a Hamilton gastight microsyringe, and a time of 30 min was allowed for total evaporation of the solvent and equilibration of the amphiphiles at the interface.

Atomic Force Microscopy. Samples were prepared by spreading 10 μL of **1**-based SLNs at a concentration of 300 mg/L on freshly cleaved mica surfaces, and then they were dried overnight at room temperature. Imaging was carried out in noncontact mode in air using a NTegra Prima system (NT-MDT, Moscow, Russia) equipped with silicon rectangular cantilevers with a force constant of 2.5−10 N/m (NT-MDT).

Scheme 1. Synthetic Route to 1



DNA Interactions. Plasmid DNA pPICZα (Invitrogen) was prepared from *E. coli* cultures using a QIAprep Spin Miniprep kit (Qiagen) and linearized by treating 10 mg of the plasmid DNA with EcoRI (10 U) and NotI (10 U) (Roche Molecular) in a buffer containing Tris-HCl EDTA buffer (Tris-HCl, 50 mM; $MgCl_2$, 10 mM; NaCl, 100 mM; dithioerythritol, 1 mM; pH 7.5) for 1 h at 37 °C. The resulting material was purified on a nucleospin column (Macherey Nagel), and the quantity of DNA was measured spectrophotometrically (OD 230, 260, and 290). The DNA in solution in water was diluted with a **1**-based SLN suspension (300 mg/L) at 3:100 (v/v), incubated for 30 min under shaking at 25 °C, and centrifuged at 1300 rpm for 12 min. A total of 10 μL of the supernatant was loaded on an agarose gel (0.9% agarose (ethidium bromide, final concentration 0.5 μg/mL) in TAE buffer (40 mM Tris, 20 mM acetic acid, 1 mM EDTA, pH 8.0)) submitted to 130 mV tension for 30 min.

Results and Discussion

p-Nitro-tetradodecyloxy-calix[4]arene was prepared using the literature procedure.[32] *p*-amino-tetradodecyloxy-calix[4]arene, **1**, was prepared via the reduction of the tetra-nitro derivative using a two-electron reducing agent, hydrazine, in the presence of palladium on activated charcoal as catalyst, as previously described for water-soluble calixarenes (cf. Scheme 1),[33] with a yield of 95%.

The amphiphilic behavior of **1** at the air−water interface was studied by means of the Langmuir balance technique; the compression isotherm measured on a pure water subphase is presented in Figure 1.

The compression isotherm carried out on a pure water subphase shows that **1** can form stable monomolecular layers at the air−water interface; the collapse pressure value measured at 45.3 mN/m reveals a relatively high dynamic stability of the monolayer. The apparent molecular area of 105 $Å^2$ is in good agreement with that reported for tetra-dodecyloxy-calix[4]arene in the cone conformation and reveals that the molecules when compressed at the interface present an orthogonal orientation regarding the interface; the molecular area is governed by the size of the macrocycle. It also shows that the positive charges of the amine groups (at neutral pH) do not cause significant repulsive interactions within the monolayer. The compressibility modulus (C_S^{-1}) value of 290 mN/m reveals that the film is macroscopically in a liquid-condensed state.

(29) Gutsche, C. D. *Calixarenes Revisited*; The Royal Society of Chemistry: Cambridge, 1998.
(30) Coleman, A. W.; Perret, F.; Moussa, A.; Dupin, M.; Guo, Y.; Perron, H. *Top. Curr. Chem.* **2007**, *277*, 31.
(31) Perret, F.; Lazar, A. N.; Coleman, A. W. *Chem. Commun.* **2006**, 2425.
(32) Jakobi, R. A.; Boehmer, V.; Gruettner, C.; Kraft, D.; Vogt, W. *New J. Chem.* **1996**, *20*, 493.
(33) Dudic, M.; Colombo, A.; Sansone, F.; Casnati, A.; Donofrio, G.; Ungaro, R. *Tetrahedron* **2004**, *60*, 11613.

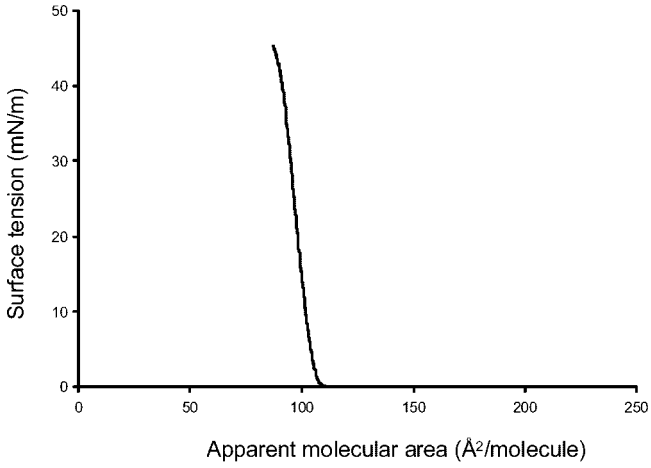


Figure 1. Π/*A* isotherm of **1** on a pure water subphase.

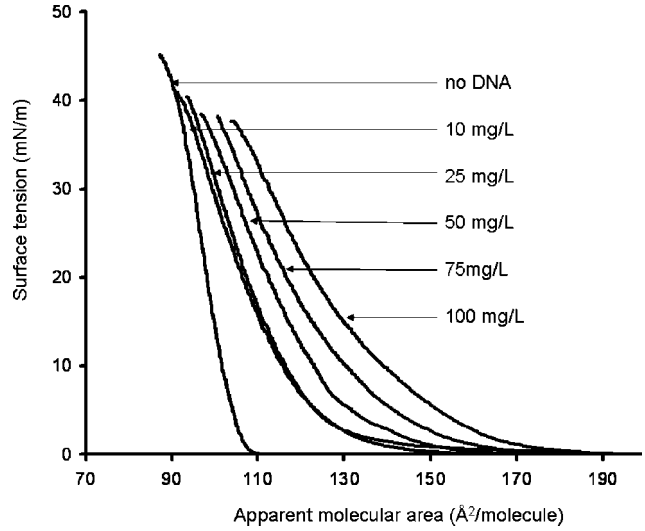


Figure 2. Π/*A* isotherms of **1** on water subphases containing 100, 75, 50, 25, and 10 mg/L DNA (for clarity, the isotherms measured for concentrations of 1 and 0.1 mg/L are omitted here and are provided in the Supporting Information).

In order to investigate the interactions of **1**-based monolayers with DNA, compression isotherms were carried out on subphases containing double-stranded low molecular weight DNA with concentrations ranging from 0.1 to 100 mg/L; the isotherms are presented in Figure 2, and the isotherm characteristic values summarized in Table 1.

From the isotherms, it could be clearly seen that the presence of DNA in the subphase causes an expansion of the monolayer with values of apparent molecular area increasing from 105 Å²/molecule for the monolayer compressed on pure water to 113, 114, 115, 119, 127, 132, and 140 Å²/molecule for concentrations of 0.1, 1, 10, 25, 50, 75, and 100 mg/L, respectively. This expansion is even more obvious when studying A_0 values, which increase even more. This indicates that there is an interaction of the DNA with the monolayer and this interaction causes an expansion of the film. When following the compressibility modulus values, the film, naturally in a liquid-condensed phase (i.e., on pure water), undergoes a phase transition to be, at high DNA concentrations, in a liquid-expanded phase. The study of the values of the collapse pressure confirms this interaction: a clear decrease is observed when increasing the concentration of DNA from 45.3 mN/m for the film prepared on water to 44.2, 41.8, 41.0, 40.9, 38.4, 38.2, and 37.6 for concentrations of DNA of 0.1, 1, 10, 25, 50, 75, and 100 mg/L, respectively. From these results, one can assume that, during the compression of the film, electrostatic interactions between negatively charged DNA molecules and positively charged amphiphiles are taking place. These interactions prevent the closed-packed compression of the amphiphile and cause an apparent expansion of the film which is no longer in a liquid-condensed but in a liquid-expanded state. The absence of a clear phase transition on the isotherms suggests that, in the conditions used in these experiments, the interactions at the interface are strong enough to prevent the phase transition between the liquid-expanded and the liquid-condensed phases. This prevents the amphiphiles from packing closely, as illustrated in Figure 3. We have previously demonstrated that the interactions of monovalent ions with a monolayer of *para*-dodecanoylcalix[4]arene causes its stabilization, with an exponential increase of the collapse pressure

Table 1. Characteristic Values Extracted from Langmuir Compression Isotherms of 1 on Subphases Containing Increasing Concentrations of DNA[a]

[DNA] (mg/L)	Π_c	A_c	A_{lim}	A_1	A_0	C_S^{-1}
0	45.3	87	105	107	108	290
0.1	44.2	86	113	119	125	195
1	41.8	89	114	138	165	170
10	41.0	91	115	144	162	160
25	40.9	93	119	143	160	160
50	38.4	95	127	147	172	130
75	38.2	100	132	160	175	126
100	37.6	103	140	170	192	90

[a] Π_c, collapse pressure; A_c, collapse area; A_{lim}, extrapolation of the linear part of the isotherm on the *x*-axis; A_1, surface area at a pressure of 1 mN/m; A_0, surface area at the surface pressure takeoff; and C_S^{-1}, compressibility modulus. Π_c and C_S^{-1} are expressed in mN/m and area values in Å²/molecule, respectively.

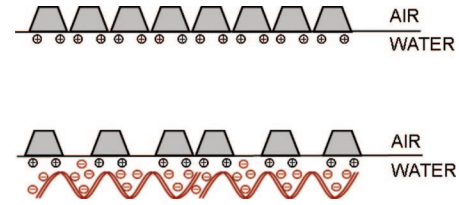


Figure 3. Schematic representation of monolayers of **1** compressed on a subphase with or without DNA.

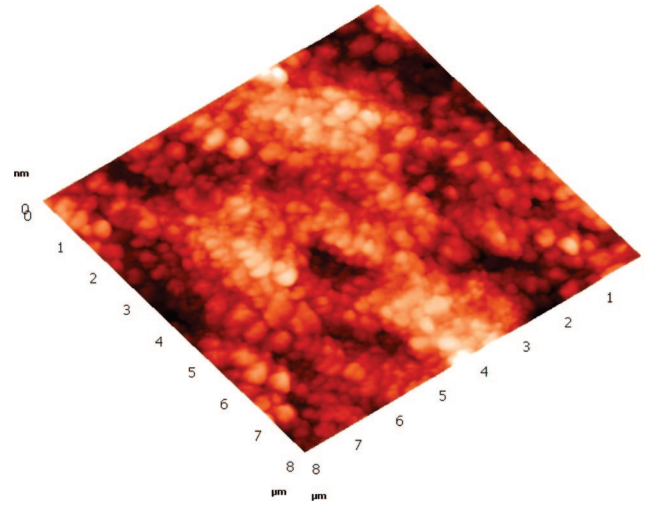


Figure 4. Atomic force micrsocope image of **1**-based SLNs spread on mica and imaged in air in noncontact mode.

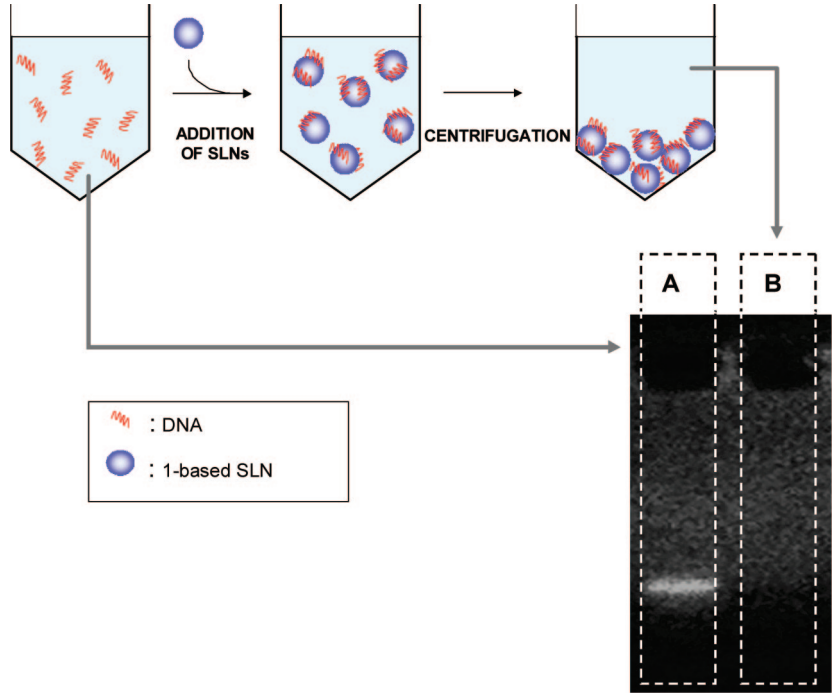


Figure 5. Chromatogram of linearized plasmid DNA pPICZα incubated (A) without or (B) with **1**-based SLNs.

measured.[34] In the present study, the phase transition between the liquid-expanded and the liquid-condensed phases prevents application of a consistent model for the evolution of the characteristic values of the isotherm.

In order to study the self-assembling properties of **1** in water, the solvent displacement method was applied using tetrahydrofuran as the organic solvent and pure water as the dispersant, as described elsewhere.[26] The suspension formed was studied by means of photon correlation spectroscopy (PCS); the results demonstrated that the suspension is formed by particles of 190 nm in diameter and a polydispersity index of 0.15. This result is comparable to that previously reported for *para*-acylcalix[4]arene,[25] and it shows that the possible repulsive interactions between the positively charged polar groups (ammonium) of **1** do not prevent the self-assembly of **1** in water. The ζ-potential of these submicrometer sized objects was studied by means of electrophoretic mobility measurements. It is shown that the particles present a net charge of +13.2 mV, a value which is in good agreement with the hypothesis that the polar groups of calixarene-based nanoparticles exhibit their polar functions at the surface of the particles.[35] To confirm the solid structure (matrix-like arrangement of the particles), a suspension of **1**-based SLNs was sperad on a mica surface, dried, and imaged using atomic force microscopy in noncontact mode; an image obtained in air in noncontact mode at a scan range of $8 \times 8\ \mu m^2$ is presented in Figure 4.

It could be clearly seen that the particles are present on the surface as round-shaped objects and they have a size of 80 ± 10 nm in height and 200 ± 20 nm in width. The absence of substantial flattening, already observed for *para*-dodecanoylcalix[4]arene SLNs, is attributed to their solid structure.[25] Similar results were obtained when imaging was carried out at higher concentrations (not shown). These values are in agreement with those measured by PCS.

It has been demonstrated that different kinds of positively charged nanoparticles are able to interact with DNA to form a stable complex which could be used for DNA transport. For example, it has been demonstrated than fluorescent core−shell silica nanoparticles coated with polyethylenimine are able to complex double-stranded plasmid DNA.[36] Nafee et al. demonstrated that cationically modified poly(D,L-lactide-*co*-glycolide) (PLGA) nanoparticles are able to complex oligonucleotides and they have been proposed as an antisense nucleotide transport system for cancer therapy.[37] In order to investigate the interactions of **1**-based SLNs, they were incubated with plasmid DNA for 30 min and spun-down, and the supernatant was analyzed using gel chromatography; a schematic summary of the experiment and the chromatogram are presented in Figure 5.

From Figure 5, it could be seen that, at the detection limit of the experiment, no plasmid DNA can be detected after incubation of **1**-based SLNs. One can then conclude that all the DNA was centrifuged down with the nanoparticles and no DNA remains in the supernatant. This confirms the interaction and complexation properties of **1**-based SLNs with double-stranded DNA. This may open new prospects for the use of calixarenes-based SLNs for gene delivery applications.

Conclusion

The study of the self-assembly of the amphiphilic 5,11,17,23-tetramino-25,26,27,28-tetradodecyloxycalix[4]arene at the air−water interface shows that this macrocycle is able to form stable Langmuir monolayers and presents at the interface in a cone conformation and in an orthogonal orientation regarding the interface. The investigations of the compression isotherm of this molecule on a subphase containing DNA revealed the presence of molecular interactions, which cause an expansion and a slight

(34) Shahgaldian, P.; Coleman, A. W. *Langmuir* **2001**, *17*, 6851.
(35) Ehrler, S.; Pieles, U.; Wirth-Heller, A.; Shahgaldian, P. *Chem. Commun.* **2007**, 2605.
(36) Fuller, J. E.; Zugates, G. T.; Ferreira, L. S.; Ow, H. S.; Nguyen, N. N.; Wiesner, U. B.; Langer, R. S. *Biomaterials* **2008**, *29*, 1526.
(37) Nafee, N.; Taetz, S.; Schneider, M.; Schaefer, U. F.; Lehr, C. M. *Nanomedicine* **2007**, *3*, 173.

destabilization of the monolayer. This amphiphile is also capable of self-assembly as solid lipid nanoparticles which possess molecular recognition properties of DNA.

Acknowledgment. The financial help of the FHNW-Forschungsfond is gratefully acknowledged. The authors wish to dedicate this article to the memory of Prof. Dr. Ernst Hungerbühler, former director of the Institute of Chemistry and Bioanalytics of the FHNW, who passed away in December 2007 after a long sickness.

Supporting Information Available: Langmuir isotherms of **1** measured on subphases containing 1 and 0.1 mg/mL DNA. This material is available free of charge via the Internet at http://pubs.acs.org.

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