Hybridization of DNA by Sequential Immobilization of Oligonucleotides at the Air−Water Interface

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The hybridization of DNA by sequential electrostatic and hydrogen-bonding immobilization of single-stranded complementary oligonucleotides at the air−water interface with cationic Langmuir monolayers is demonstrated. The complexation of the single-stranded DNA molecules with octadecylamine (ODA) Langmuir monolayers was followed in time by monitoring the pressure−area isotherms. A large (and slow) expansion of the ODA monolayer was observed during each stage of complexation in the following sequence: primary single-stranded DNA followed by complementary single-stranded DNA followed by the intercalator, ethidium bromide. Langmuir−Blodgett (LB) films of the ODA−DNA complex were formed on different substrates and characterized using quartz-crystal microgravimetry (QCM), Fourier transform infrared spectroscopy (FTIR), polarized-UV−vis and fluorescence spectroscopy, as well as thermal melting studies. These measurements clearly showed that hybridization of the complementary single-stranded DNA molecules had occurred at the air−water interface, leading to the characteristic double-helical structure. Furthermore, it was observed that the DNA molecules in the LB films were oriented parallel to the substrate withdrawal direction.

Monolayers of charged surfactant molecules at the air−water interface (Langmuir monolayers) have long been acknowledged to be excellent media for the organization of large inorganic ions,[1] colloidal nanoparticles,[2] and biomacromolecules such as proteins/enzymes[3] and in the growth of oriented crystals.[4] Langmuir monolayers of lipid molecules have frequently been used as models to understand the organization and function of biological membranes.[5] The interaction of DNA with Langmuir monolayers has received considerable attention[6] with a view to understand templated supramolecular organization[5] as well as the transfer of DNA across biological bilayer membranes in gene therapy.[7] Studies on DNA immobilization at the air−water interface have hitherto concentrated on electrostatic complexation of preformed double-helical DNA molecules with cationic lipid Langmuir monolayers[6a,b,d] and hydrogen bonding between alkylated monolayer-forming nucleobases and complementary water-soluble bases[6c] and oligonucleotides.[6e] To the best of our knowledge, the hybridization of complementary single-stranded oligonucleotides (ssDNA) to yield double-helical DNA structures by complexation at the air−water interface has not been demonstrated so far. We show herein that complementary ssDNA strands sequentially immobilized at the air−water interface by electrostatic interaction with cationic octadecylamine (ODA) Langmuir monolayers do indeed hybridize to yield double-helical DNA molecules (Scheme 1).

Oligonucleotides of the sequences GGAAAAAACTTCGTGC (ssDNA-1), GCACGAAGTTTTTACC (ssDNA-2), and AGAAGAAGAAAAGAA (ssDNA-3) were synthesized by β-cyanoethyl phosphoramidite chemistry on a Pharmacia GA plus DNA synthesizer and purified by FPLC and rechecked by RP HPLC. ssDNA-1 and ssDNA-2 are complementary oligonucleotides while ssDNA-1 and ssDNA-3 are noncomplementary. A known quantity of 1 mg/mL concentrated solution of octadeylamine (ODA, used as-received from Aldrich) in chloroform was spread on the surface of a 10^{-8} M concentrated solution of ssDNA-1 in deionized water (pH = 6.8) in a Nima 611 Langmuir−Blodgett trough equipped with a Wilhelmy plate for surface pressure sensing (Scheme 1, step 1). After evaporation of the solvent (typically 20 min after spreading the monolayer), pressure−area (π−A) isotherms were recorded at room temperature as a function of time at a compression/expansion rate of 50 cm^2/min. Curves 1 and 2 in Figure 1 represent the π−A isotherm compression/expansion cycles of the ODA Langmuir monolayer at time = 1 and 12 h, respectively, after spreading the monolayer on the ssDNA-1 subphase. A large, albeit slow, expansion of the monolayer to an area/molecule value of 35 Å^2 is observed which remained constant thereafter. This is to be compared with the ∼21 Å^2/molecule takeoff area for the ODA

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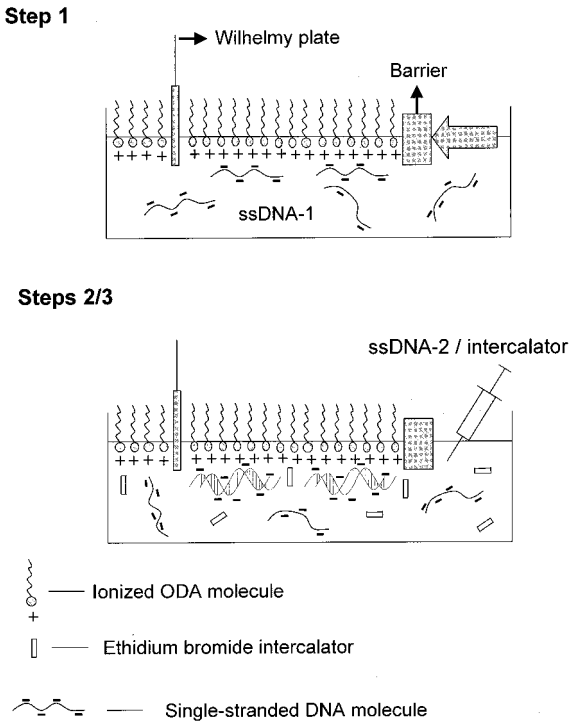
Scheme 1. Diagram Showing the Various Stages of Immobilization/Hybridization of DNA Molecules at the Air−Water Interface with Cationic ODA Langmuir Monolayers[a]



[a] Step 1: spreading of ODA on the ssDNA-1 subphase followed by immobilization of ssDNA-1 at the air−water interface. Steps 2/3: introduction into the trough and complexation of the complementary ssDNA-2−ethidium bromide intercalator molecules with ssDNA-1 molecules immobilized at the air−water interface. The various components in the diagram are explained at the bottom of the scheme.

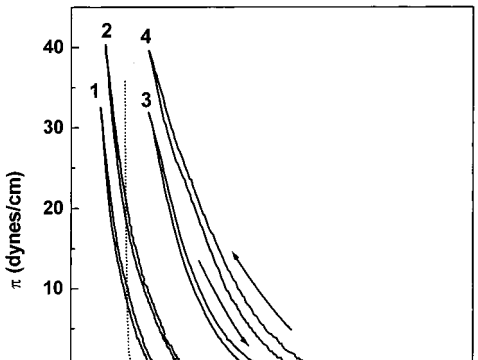


Figure 1. $\pi-A$ isotherms recorded from an ODA monolayer at various times after introduction of DNA/intercalator molecules into the subphase (as per Scheme 1): curves 1 and 2, $\pi-A$ isotherms recorded 1 and 12 h after spreading ODA on the ssDNA-1 subphase respectively; curve 3, $\pi-A$ isotherm recorded 12 h after introduction of ssDNA-2 into the subphase; curve 4, $\pi-A$ isotherm recorded 12 h after introduction of ethidium bromide intercalator into the subphase. The dotted line corresponds to the $\pi-A$ isotherm recorded from an ODA monolayer on a 10^{-8} M solution of ethidium bromide in deionized water as the subphase. See text and Scheme 1 for details.

Langmuir monolayer in 10^{-8} M ethidium bromide solution as the subphase measured 4 h after spreading the monolayer (dotted line, Figure 1).[8] This slow expansion of the monolayer is clearly due to complexation of ssDNA-1 molecules with the ODA monolayer by attractive electrostatic interaction (Scheme 1, step 1) and indicates that the diffusion of the DNA molecules to the air−water interface is extremely slow. We have observed a similar time-dependence in the complexation of surface-modified colloidal silver particles with ODA monolayers at the air−water interface.[9] At pH = 6.8, the ODA molecules (pK_B = 10.8) would be completely ionized, leading to maximum attractive Coulombic interaction with the anionic DNA molecules in solution. We would like to point out that little hysteresis was observed in the compression/expansion cycles of this and other air−water interface complexation experiments discussed below. The above result indicates that the ODA−DNA conjugate monolayers at the air−water interface are stable in time.

After stabilization of the ssDNA-1 density at the air−water interface, the ODA−ssDNA-1 monolayer was transferred by the Langmuir−Blodgett technique[10] onto gold-coated quartz substrates for quartz crystal microgravimetry (QCM) measurements.[11] A plot of the QCM mass uptake recorded as a function of number of immersion cycles in the ODA−ssDNA-1 monolayer is shown in Figure 2A, curve 1. The transfer ratio was close to unity during both the downward and upward strokes of the QCM substrate. Uniform, lamellar growth of the ODA−DNA monolayer is thus clearly seen from QCM and transfer ratio data. The slope of the curve was determined from a linear fit to the QCM data to be 1450 ng/cm^2 per dip. Accounting for the contribution from the ODA bilayer, the concentration of DNA in the interlamellar regions of the ODA bilayer structure can easily be calculated to be 1.17×10^{14} cm^{-2}.[12] Accounting for the charges on the individual DNA molecules (16 per molecule), the charge ratio of DNA/ODA in the bilayers can be calculated to be 1.87×10^{15}:9.52×10^{14}, indicating that there is overcompensation of the positive charge due to ODA by the negatively charged DNA molecules by roughly a factor of 2.[12] Such a charge overcompensation is known to occur during complexation of large inorganic ions such as Keggin anions at the air−water interface[13] as well as in electrostatically formed multilayers of cationic and anionic polyelectrolyte films, in multilayer films of polyelectrolytes and DNA,[14] as well as in multilayers of positively and negatively charged nanoparticles.[15]

(8) In this experiment, both the ODA monolayer and the ethidium bromide molecules are cationic, and therefore, the DNA intercalator does not complex with the ODA monolayer and no expansion of the Langmuir monolayer is observed.

(9) Mayya, K. S.; Sastry, M. *Langmuir* **1998**, *14*, 74.

(10) Ulman, A. *An introduction to ultrathin organic films: from Langmuir−Blodgett to self-assembly*; Academic Press: San Diego, CA, 1991. The monolayer transfer onto different substrates was effected at a surface pressure of 30 dyn/cm for all experiments in this study.

(11) A 6 MHz AT-cut quartz crystal was used in this study together with an Edwards FTM5 frequency counter (resolution and stability of 1 Hz). The frequency change (Δf in Hz) was measured as a function of number of immersion cycles in the ODA−DNA monolayer after careful drying of the crystal and converted to mass uptake using the formula $\Delta m = 12.1\Delta f$ (ng/cm^2).

(12) The molecular weight of ODA is 269.5. The number of ODA molecules in the bilayer is $2/(21 \times 10^{-16})$ and is $\sim 9.52 \times 10^{14}$ cm^{-2}. This then represents the number of charges due to ODA in the bilayer, assuming complete ionization of the ODA monolayer. (The mass of the bilayer of ODA/cm^2 is thus $(9.52 \times 10^{14} \times 269.5)/6.024 \times 10^{23} \sim 426$ ng/cm^2.) The molecular weight of ssDNA-1 is 5280. The contribution to the QCM mass uptake from the DNA molecules is 1450−426 ~ 1024 ng/cm^2. In the manner above, the number of DNA molecules/cm^2 of the film can be shown to be $\sim 1.17 \times 10^{14}$/cm^2.

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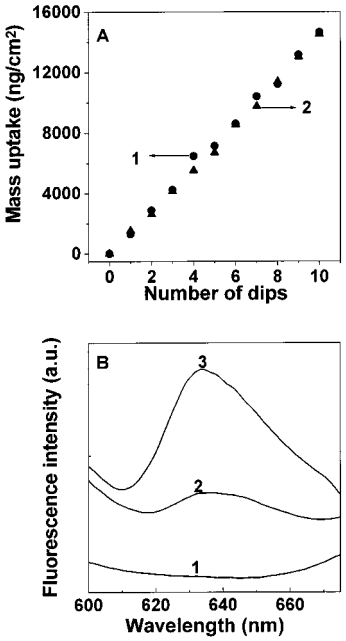

Figure 2. (A) QCM mass uptake recorded as a function of number of immersion cycles in ODA monolayers complexed with ssDNA-1 (curve 1) and ODA complexed with ssDNA-1 and further hybridization with ssDNA-2 along with the intercalator, ethidium bromide (curve 2). See text for details. (B) Fluorescence spectra of (curve 1) a 19 ML LB film of ODA−ssDNA-1 complexed with ssDNA-3 and ethidium bromide intercalator, (curve 2) a 19 ML LB film of ODA−ssDNA-1 hybridized with ssDNA-2 with ethidium bromide as intercalator, and (curve 3) spectrum from the 19 ML LB film shown as curve 2 after further immersion in ethidium bromide solution for 12 h.

After the QCM measurements mentioned above, 4 mL of ssDNA-2 in water was injected into the non-monolayer side of the trough to yield an overall oligonucleotide concentration in the aqueous subphase of 10^{-8} M (Scheme 1, step 2). Care was taken to remove an equal quantity of water prior to ssDNA-2 insertion and maintain the water level in the trough constant. The $\pi-A$ isotherms were recorded as a function of time, and the isotherm recorded 12 h after introduction of ssDNA-2 is shown as curve 3 in Figure 1. A further increase in ODA monolayer area is clearly seen, the takeoff area shifting to a stable 53 Å^2/molecule. Two hypotheses may be advanced to explain this result. The expansion may be either due to direct complexation of the ssDNA-2 molecules with uncoordinated ODA monolayer regions at the air−water interface or due to an elongation due to uncoiling of the ssDNA molecules already immobilized at the air−water interface during step 1 as a consequence of hybridization with the complementary DNA molecules, ssDNA-2. The former is not likely given that charge overcompensation of the ODA monolayer by ssDNA-1 molecules was indicated by QCM measurements. Entropic considerations would lead to replacement of some ssDNA-1 by ssDNA-2 molecules at the air−water interface, but since they are of the same length and equal charge, an expansion of the monolayer would not occur due to such an exchange process. An uncoiling of the immobilized ssDNA-1 molecules by hybridization of ssDNA-2 molecules is thus indicated.

To test whether the expansion observed in the $\pi-A$ isotherms on insertion of the complementary ssDNA-2 is due to hybridization and formation of duplex structures at the air−water interface (curve 3, Figure 1), ethidium bromide was introduced into the trough, as illustrated in Scheme 1, step 3. The concentration of ethidium bromide was adjusted to be 10^{-8} M in the subphase. Ethidium bromide is known to intercalate into the base pairs of DNA double-helical structures, and this process is readily detected by the enhanced fluorescence exhibited by this molecule on intercalation in DNA.[16] The $\pi-A$ isotherm recorded 12 h after introduction of ethidium bromide shows a further expansion in the monolayer with the takeoff area shifting to 70 Å^2/molecule (curve 4, Figure 1). This result indicates intercalation in the DNA double helix formed by sequential adsorption of ssDNA-1 and ssDNA-2 at the air−water interface, thereby leading to a further extension of the double-helical DNA molecules adsorbed at the interface. This observation is consistent with atomic force microscopy studies of interaction of probe molecules with DNA where it was demonstrated that, among the various binding modes of drug molecules with DNA, intercalation leads to elongation of the DNA double-helical structures.[17]

LB films of the ODA−ssDNA-1/ssDNA-2 monolayers after introduction of the intercalator were transferred onto the QCM crystal, and the mass uptake was recorded as a function of number of immersion cycles (Figure 2A, curve 2). A lamellar growth mode is clearly seen for the hybridized DNA−ODA LB films, and interestingly, the mass uptake per dip (slope of curve 2, Figure 2A) is almost identical to that obtained for the ODA−ssDNA-1 LB film. An explanation for this result is postponed until hybridization of the complementary single-stranded DNA molecules at the air−water interface is established.

Nineteen monolayer (ML, 10 dips) LB films of the ODA molecules complexed with ssDNA-1 followed by ssDNA-2 molecules and ethidium bromide were grown on quartz substrates, and the fluorescence spectrum was recorded.[18] Figure 2B shows the spectrum recorded from the above film (curve 2). A clear fluorescence signal at 635 nm can be seen and thus indicates the hybridization of the complementary oligonucleotides ssDNA-1 and ssDNA-2 at the air−water interface to form double-helical structures. The emission wavelength is red-shifted relative to the solution DNA-intercalant value of 580 nm. This shift may be due to differences in the polarity of the fluorescent probe and is consistent with literature observation on ethidium bromide complexes with DNA.[16] Immersion of this film in 10^{-8} M ethidium bromide solution for a further period of 12 h leads to an enhancement in the fluorescence signal (Figure 2B, curve 3). The concentration of ethidium bromide in the DNA subphase is thus clearly not sufficient for complete intercalation in the DNA helical structures formed at the Langmuir monolayer interface, and this result is in agreement with estimates of degree of intercalation from expansion of the ODA Langmuir

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(17) Coury, J. E.; Anderson, J. R.; McFail-Isom, L.; Williams, L. D.; Bottomley, L. A. *J. Am. Chem. Soc.* **1997**, *119*, 3792. The ethidium bromide molecules intercalate into alternate base-pair sites in the double-helical structure, and this process is accompanied by a 3.4 Å increase in the length of the double helix per intercalator molecule insertion (reference above and Lerman, L. S. *J. Mol. Biol.* **1961**, *3*, 18). The increase in the area per ODA molecule on intercalation of the DNA double-helical structures with ethidium bromide (step 3, Scheme 1) from 53 to 70 Å^2 (expansion by ∼32%) may be accounted for if one assumes that roughly five out of the eight sites per DNA molecule immobilized at the air−water interface are intercalated by ethidium bromide molecules. This would lead to an increase in length of the DNA molecules by 17 Å and works out to a ∼31% increase in the length of the DNA molecule (normal length ∼ 55 Å). This result is consistent with further uptake of ethidium bromide indicated in the fluorescence measurements shown in Figure 2B.

(18) The fluorescence measurements were done on a Perkin-Elmer model LS 50-B spectrofluorimeter at 25 °C, with slit widths of 5 nm for excitation at 460 and 10 nm for the emission monochromators. The LB films were grown on quartz substrates cut to fit precisely in the quartz cuvette normally used for liquid samples.

monolayer in step 3 of the protocol (Scheme 1, ref 17). In a control experiment, the sequential immobilization procedure was repeated with a noncomplementary oligonucleotide, ssDNA-3 in Scheme 1, step 2, in place of the complementary ssDNA-2 molecules. The protocol was completed by addition of ethidium bromide into the subphase, as discussed for the ssDNA-2 hybridization case. The fluorescence spectrum measured from a 19 ML LB film formed in this experiment is shown in Figure 2B, curve 1. It is clear that the ethidium bromide molecules do not bind to the DNA molecules immobilized at the air−water interface. This emphatically shows that hybridization to yield the double-helical structure does not occur in the ssDNA-1/ssDNA-3 sequential assembly experiment and, together with the fluorescence results shown in curves 2 and 3, demonstrates the molecular recognition process occurring at the Langmuir monolayer interface.

Through elegant QCM measurements, Okahata and coworkers have recently shown that alkylated monolayer-forming nucleobases recognize linear oligonucleotides introduced into the subphase.[6e] Hydrogen bonding between complementary bases (alkylated-A and linear oligonucleotide-T molecules) leads to the recognition at the air−water interface while such a hydrogen-bonding is not possible in the bulk of the subphase.[6e] As the authors themselves point out, this procedure has the inherent disadvantage that a double-helical structure is not possible due to the restricted mobility of the bases bound to the alkyl chains. In that sense, to term the molecular recognition process as "DNA hybridization" may not be appropriate. In this study, there are no such constraints on the freedom of the individual ssDNA molecules. Even though electrostatically immobilized at the air−water interface, the fluorescence results clearly suggest sufficient mobility of the surface-bound ssDNA molecules to permit formation of double-helical structures upon approach of the complementary DNA molecules. *The hybridization of the complementary DNA single-stranded molecules at the air−water interface demonstrated herein is thus the two-dimensional analogue of hybridization in solution, with the term hybridization being used in the strictest sense to mean formation of double helices*. Another interesting observation is that the hybridization of ssDNA-1 and ssDNA-2 is unlikely to occur in the bulk of the solution in the absence of any salt, as in this study (deionized water). To overcome the repulsion between the negatively charged phosphate backbone, screening of the repulsive interaction with salt (NaCl) is required to promote the hybridization in the bulk solution.[19] The hybridization observed in this study occurs only at the air−water interface, with the cationic ODA Langmuir monolayer screening the repulsion between the surface-bound ssDNA-1 and solution-phase ssDNA-2 molecules.

An important experimental result is that the QCM mass uptake per immersion cycle of the substrate for the ODA-hybridized DNA monolayer (Figure 2A, curve 2) is similar to that obtained for the single-stranded DNA monolayer (Figure 2A, curve 1). Since both ssDNA-1 and ssDNA-2 molecules are identical in terms of charge, it is to be expected that interactions with a charged surface (such as that provided by the ODA Langmuir monolayer) would also be similar. To maintain the DNA/ODA charge ratio at the air−water interface, hybridization of the DNA molecules by hydrogen bonding of ssDNA-2 to surface-bound ssDNA-1 would necessitate detachment of a certain number of surface-bound ssDNA-1 molecules *equal to the number of ssDNA-2 molecules participating in the hybridization events*. Thus, hybridization would not lead to an increase in the effective DNA concentration at the interface (measured in terms of the ssDNA concentration at the air−water interface). However, the monolayer expansion after the hybridization of ssDNA-1 and ssDNA-2 and intercalation of ethidium bromide molecules into the double-helical structures (curve 4, Figure 1, effective ODA area at 30 dyn/cm $\sim$ 33 Å^2/molecule) leads to an effective decrease in the concentration of ODA molecules in the bilayers of the LB films transferred for QCM measurements relative to that for the LB films formed after complexation of ssDNA-1 alone (curve 2, Figure 1, effective ODA area at 30 dyn/cm $\sim$ 20 Å^2/molecule). This should consequently lead to a smaller mass uptake in the case of the hybridized DNA LB films. We believe the contribution to the mass uptake per immersion cycle by the intercalated ethidium bromide molecules in the ODA-hybridized DNA LB films offsets this reduction in density of the ODA molecules and results in the similar mass transfers observed in Figure 1.

Additional evidence of the hybridization of DNA and intercalation of ethidium bromide is provided by Fourier transform infrared spectroscopy (FTIR) analysis of 19 ML LB films of the ODA−ssDNA-1, ODA-hybridized DNA (with ethidium bromide intercalator), and bare ODA films transferred onto Si (111) wafers by the LB method (Supplementary Information, Figure 1).[20] A comparison of the FTIR spectra of LB films of ODA−ssDNA-1 (Supplementary Information, Figure 1, curve 2) and ODA-hybridized DNA (Supplementary Information, Figure 1, curve 3) in the range 1050−1750 cm^{-1} yields an increase in intensity of two prominent features at 1109 and 1719 cm^{-1} in the hybridized DNA LB film. It is to be noted that these features are not present in the bare ODA LB film (Supplementary Information, Figure 1, curve 1) and are therefore clearly signatures from the entrapped DNA molecules in the LB films. The increase in intensity at 1719 cm^{-1}, which is due to resonance in the mainly G-band, has been observed in the case of intercalation of chlorophyllin molecules in hybridized calf-thymus DNA.[21] The increase in intensity of the resonance at 1109 cm^{-1} is due to the deoxyribose band and is also an indicator of the hybridization of the DNA molecules in the LB film.[21] Polarized UV−vis spectroscopy measurements (carried out on a Perkin-Elmer Lambda 15 spectrophotometer) of a 19 ML hybridized DNA−ODA LB film on quartz indicated clearly orientation of the DNA molecules parallel to the substrate withdrawal direction. The dichroic ratio at 260 nm was close to 12, indicating a high degree of order in the DNA molecule orientation.[6a] UV melting experiments of a 19 ML thick ODA-hybridized DNA film yielded a sigmoidal curve characteristic of duplex melting with a transition temperature of 55 °C (Supplementary Information, Figure 2).[22] This is to be compared with the solution melting transition temperature of 41 °C for duplexes of ssDNA-1 and ssDNA-2[23] and indicates stabilization of the double-helix structure in the ODA lipid matrix. We would like to add that LB films of ssDNA-1

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(20) FTIR measurements were made on a Shimadzu PC-8201 PC instrument in the diffuse reflectance mode at a resolution of 4 cm^{-1}.

(21) Neault, J. F.; Tajmir-Riahi, H. A. *J. Phys. Chem. B* **1998**, *102*, 1610.

(22) The UV melting measurements were carried out on a Perkin-Elmer Lambda 15 UV/VIS spectrophotometer fitted with a Julabo water circulator with a programmed heating accessory. The film was heated at a rate of 0.5 °C/min, and the thermal denaturation of the duplex was followed by monitoring changes in the absorbance at 260 nm as a function of temperature.

(23) Pattarkine, M. V.; Ganesh, K. N. *Biochem. Biophys. Res. Commun.* **1999**, *263*, 41.

complexed with the noncomplementary ssDNA-3 molecules did not show any indication of such a denaturation process.

In conclusion, the hybridization of DNA to yield double-helical structures by sequential electrostatic and hydrogen-bonding immobilization of complementary single-stranded DNA molecules at the air−water interface with cationic Langmuir monolayers has been demonstrated. The double-helical DNA molecules in built-up LB films are oriented parallel to the substrate withdrawal direction. This approach is expected to lead to a better understanding of DNA−lipid and DNA−drug interactions, especially in confined spaces. Application in gene-sequencing protocols may also be envisaged.

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Supporting Information Available: Plots of transmittance versus wavenumber and of absorbance versus temperature. This material is available free of charge via the Internet at http://pubs.acs.org.

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