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Letters

X-Ray Reflectivity Measurements of Layer-by-Layer Films at the Solid/Liquid Interface

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In this Letter, we present a method for the decoration of layer-by-layer (LbL) structures by heavy metal ions, which allows X-ray reflectivity (XRR) measurements at the solid/water interface. The improved contrast has allowed us to obtain well-structured X-ray reflectivity curves from samples at the liquid/solid interface that can be used for the film structure modeling. The developed technique was also used to follow the formation of complexes between DNA and the LbL multilayer. The XRR data are confirmed by independent null-ellipsometric measurements at the solid/liquid interface on the very same architectures.

Polyelectrolyte self-assembly, known also as layer-by-layer deposition (LbL), is a widespread method for the realization of organic and composite films with thickness controlled down to the nanometer scale.[1−3] It does not require any expensive equipment, and it allows coverage of practically any surface. Soft technological conditions make it very useful also for working with biological samples, such as proteins[4,5] and DNA.[6,7] With this technique, functional structures can be realized completely in an aqueous environment avoiding the exposure of biological molecules to air, which can result in their complete or partial denaturation with consequent loss of biological activity.

X-ray reflectivity (XRR) measurements are very useful to study the structure of thin films (down to monomolecular layers). See refs 8−10 as reviews. This technique has been widely applied to the investigation of LbL and Langmuir−Blodgett films at the surface of solid supports[11,12] and Langmuir monolayers at the air/water interface.[13−15] It seems very important to perform similar measurements at the solid/liquid interface. Clearly, such mea-

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surements would be quite useful to investigate the structure and functioning of nanoscale films with biological molecules that have never been exposed to the air and therefore preserve their biological function. Neutron reflectivity measurements of LbL films at the solid/liquid interface were successfully performed.[16,17] Contrasting in this case was reached by using deuterated water. There is only one publication[16] where XRR was applied for the investigation of the LbL film growth at the solid/liquid interface. Variation of the reflected beam intensity was registered at the fixed incident beam angle. To our knowledge, there are no publications where complete XRR cures, obtained from LbL films at the solid/liquid interface and allowing reliable modeling, were reported. We believe that the main reason for the lack of results is the very low contrast of the sample electron density with respect to the surrounding aqueous medium. In fact, the electron density of polyelectrolyte layers is very close to that of the aqueous solution.

In this Letter, we propose and demonstrate a method to increase the electron density contrast between the deposited LbL layers and the surrounding water, thus allowing the study of the LbL film structure while immersed in the liquid phase. The method is based on the inclusion ("decoration") of heavy metal ions into the layer. XRR curves were measured at the ESRF Synchrotron radiation facility in order to obtain reliable experimental data with an acceptable signal-to-noise ratio. This approach was then applied to study the interaction of DNA molecules with preformed LbL multilayers, investigating the resulting molecular structure for both double-stranded (dsDNA) and single-stranded (ssDNA) DNA. Given the complexity of the system we studied, we felt it was useful to verify our XRR results with an independent technique such as null-ellipsometry.

DNA was chosen as a model biopolymer for checking the applicability of the developed approach for studying biological objects. Previously, it was shown that the interaction of DNA with surfactant monolayers at the air/water interface can vary its structure. It was demonstrated that the presence of the amine headgroup in aliphatic molecules results in the transformation of native DNA into a single-stranded form after interaction with monolayers of such molecules.[18] Such a single-stranded form was maintained also after the transfer of such layers onto solid supports.[19,20] In the case of interaction with monolayers of other molecules, such as, for example, dipalmitoylphosphatidylcholine (DPPC) and hexadecyltrimethylammonium bromide (HTAB), DNA maintained its native double-helix state.[21] Thus, it is interesting to investigate the state of DNA in LbL layers when the sample is in contact with a liquid phase.

Poly(ethyleneimine) (PEI), poly(allylamine hydrochloride) (PAH), and poly(sodium 4-styrenesulfonate) (PSS) were purchased from Sigma. Water was purified with a Milli-Q system and had a resistance of greater than 18.2 MΩ·cm. LbL samples were prepared according to standard techniques.[1−3] Solutions for the layer formation contained 2.0 mg/mL polymers in water. Silicon supports of size 20 × 40 mm^2 were used. Two types of samples were prepared. The first one contained the following sequence of layers:

$$(\text{PEI-PSS})^{H_2O} - (\text{PAH-PSS})^{H_2O} - (\text{PAH-PSS})_3^{Cd^{2+}} - \text{PAH}^{Cd^{2+}}$$

(with the last layer being positive to enhance the successive electrostatic attachment of DNA). The first two polyelectrolyte bilayers were deposited from aqueous solutions of polyelectrolytes without any added salt, while successive three (PAH-PSS) bilayers were formed from solutions containing 0.25 M $CdCl_2$. Control samples were fabricated by depositing all layers from aqueous polymer solutions in the absence of Cd salts. The second type of sample was prepared from aqueous solutions of polymers and contained the following sequence of layers:

$$(\text{PEI-PSS})^{H_2O} - (\text{PAH-PSS})^{H_2O} - (\text{PAH-PSS})_{10}^{Na^{+}}$$

Two XRR measurements of the latter sample were done: the first one was performed immediately after its formation, and the second one was conducted after treating the multylayer in 0.25 M $CdCl_2$ solution for 6 h.

Double-stranded DNA from herring sperm was purchased from Sigma. Single-stranded DNA (oligonucleotide no. 1, 50-mer, molecular weight = 16 011 g/mol; oligonucleotide no. 2, 50-mer, molecular weight = 15 296 g/mol) was from Eurofins MWG GmbH. Deposition of DNA was performed from solutions free from cadmium salt.

XRR measurements were performed at the ID10B station (Troika II) of European Synchrotron Radiation Facilities (ESRF, Grenoble, France) using 22 keV radiation. All measurements were performed in aqueous medium without any salt addition.

The reflectivity data were subsequently analyzed with our own software[13,15,18] which calculates the reflectivity curve for a given model according to the so-called "Parratt recursive approach".[22−24] In the data analysis, we had to introduce a geometrical correction to account for the size of the substrates that could be employed in the solid/liquid cell, which is smaller than the footprint of the impinging X-ray beam at the critical angle. This is due to the high energy of the X-rays needed for the measurements in water, which shifts all the features to small angles: the critical angle for Si/air at 8 keV is 224 mdeg, which reduces to only 59 mdeg for Si/water at 22 keV. We also correct for possible misalignment of the sample, which turns out to be of the order of 2 mdeg.

Ellipsometric measurements[25,26] at the silicon/water interface were performed using high precision null-ellipsometry on a flow cell equipped with amorphous quartz windows normal to the laser beam, positioned by micrometric controls. More details will be given in a forthcoming paper. Here, we wish to note that we used the mean tabulated value $n = 1.51$ for the refractive index of the polymers PEI, PSS, and PAH and $n = 1.33$ (pure water) for the liquid phase.

As expected, X-ray reflectivity of the control samples without decoration (data not shown) revealed a smooth, featureless curve excluding any reliable fitting and interpretation. Instead, in the case of the samples prepared with the use of cadmium salt solutions of polymers, the situation was significantly changed. The experimental X-ray reflectivity curve of these samples together with its best fit are shown in Figure 1. The curve is much

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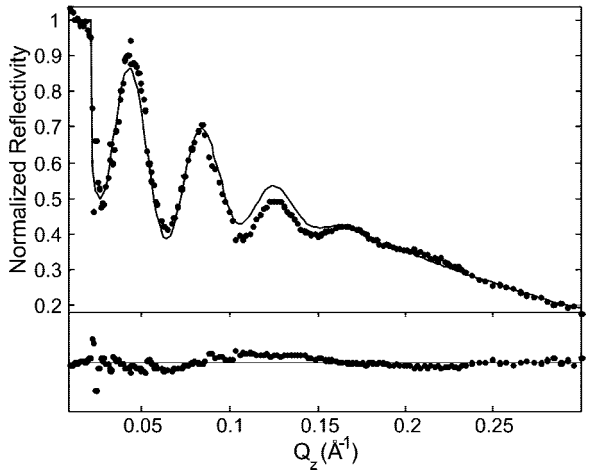

Figure 1. Top panel: Experimental (points) and best fit (line) XRR data for the LbL film $(PEI\text{-}PSS)^{H_2O}-(PAH\text{-}PSS)^{H_2O}-(PAH\text{-}PSS)_3^{Cd^{2+}}-PAH^{Cd^{2+}}$ scaled by the Fresnel reflectivity of the Si/water interface as a function of Q_z. Bottom panel: residues of the fit.

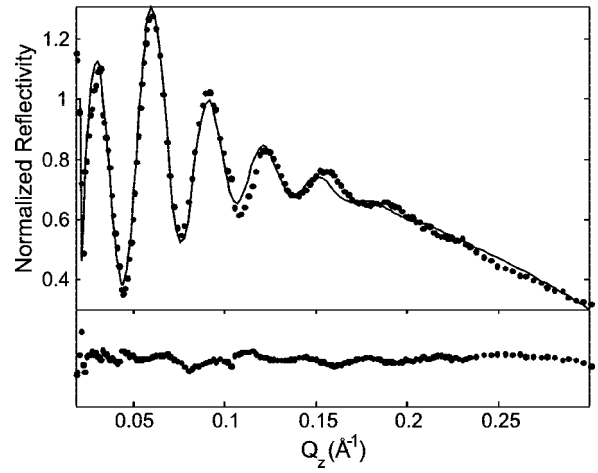

Figure 2. Top panel: Experimental (points) and best fit (line) XRR data for the LbL film $(PEI\text{-}PSS)^{H_2O}-(PAH\text{-}PSS)^{H_2O}-(PAH\text{-}PSS)_3^{Cd^{2+}}-PAH^{Cd^{2+}}-$dsDNA scaled by the reflectivity of the Si/water interface as a function of Q_z. Bottom panel: residues of the fit.

more informative. It allows one to determine the thickness and to build the model. The experimental curve was reproduced with a simple model including water, the LbL film, and silicon. The water used in the experiments contained no salts. Therefore, the values of 0.334 and 0.699 e/Å^3 were taken as electron densities for water and silicon, respectively. Variable parameters were the film thickness, electron density, surface roughness, and geometrical factors. The film thickness obtained (14.9 nm) is in good agreement with the results of our ellipsometric measurements on similar samples. The electron density of the resulting LbL film was found to be 0.423 e/Å^3. This value corresponds to the equilibrium state of the layer. In fact, repeated XRR measurements, performed in pure water, revealed no structural variations of the film structure for 20 h, indicating the strong attachment of cadmium ions to the layer.

The experimental X-ray reflectivity curves of samples in which contrast was obtained by exposure of the LbL multilayer to cadmium chloride solution after the preparation were similar to those of samples deposited from Cd salt-containing polymer solutions (data not shown).

Thus, both methods of contrasting work quite well. However, before using these layers as substrates for biological molecule deposition, it was necessary to check two important points. First, is there any variation of the film thickness during its decoration in salt solutions? Second, is there any release of the attached heavy metal ions back into the aqueous solution? Ellipsometry measurements were performed in order to answer the first question. It was shown that the thickness of the LbL film did not vary within experimental accuracy after exposure of the decorated sample to water for 20 h.

The absence of the back release of ions into aqueous solutions was confirmed by XRR measurements. Experimental curves acquired immediately after the sample preparation and after it was kept in pure water for 6 h had similar features and the same value of the electron density of the layers. This fact seems to be very important for the application of the developed technique to study structures with biological molecules. In fact, some release of the heavy metal ions into the solution during the attachment of proteins or DNA layers could result in the variation of their structure and partial or complete loss of their functional activity.

The sublayers thus characterized were then used as substrates for the subsequent DNA deposition. Experimental XRR data together with the best fit for the double-stranded DNA, incubated

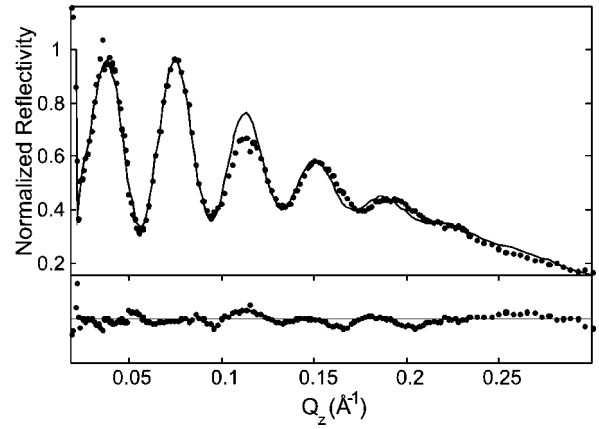

Figure 3. Top panel: Experimental (points) and best fit (line) XRR data for the LbL film $(PEI\text{-}PSS)^{H_2O}-(PAH\text{-}PSS)^{H_2O}-(PAH\text{-}PSS)_3^{Cd^{2+}}-PAH^{Cd^{2+}}-$ssDNA scaled by the Fresnel reflectivity of Si/water as a function of Q_z. Bottom panel: residues of the fit.

for 15 min onto the $(PEI\text{-}PSS)^{H_2O}-(PAH-PSS)^{H_2O}-(PAH\text{-}PSS)_3^{Cd^{2+}}-PAH^{Cd^{2+}}$ sublayer (sample of type 1: the last three (PAH-PSS) bilayers were deposited from cadmium chloride containing solution), are presented in Figure 2. DNA attachment clearly took place, as a comparison of the reflectivity curves of Figures 1 and 2 immediately indicates. Fitting of these data was performed using a four-media model (water, DNA, LbL film, and silicon). The scheme of the realized film is shown in the Table of Contents graphic. The starting values for the fitting parameters for the LbL film were obtained from the previously described fit. Within this model, the thickness of the DNA layer was found to be 5.0 nm. Such a value is larger than the DNA diameter, which seems to suggest that about two molecular layers of DNA were deposited during one cycle, and it is in excellent agreement with our ellipsometric measurements.

We also performed experiments with single-stranded DNA for two reasons: (1) in order to compare the spacing of the resultant films with that obtained for double-stranded DNA and (2) in order to check the possibility of hybridization in LbL layers. In Figure 3, we report our experimental reflectivity curve together with its best fit for the single-stranded DNA (oligonucleotide no. 1) deposited for 15 min onto the $(PEI\text{-}PSS)^{H_2O}-(PAH\text{-}PSS)^{H_2O}-(PAH\text{-}PSS)_3^{Cd^{2+}}-PAH^{Cd^{2+}}$ sublayer (the same as in the previous case).

DNA attachment clearly occurred, and the fitting procedure yielded a thickness of 1.5 nm, that is, substantially smaller than that for dsDNA. We also note that this thickness is larger than expected for a single layer of ssDNA.[18,27]

This sample was then incubated in a solution of the complementary ssDNA (oligonucleotide no. 2) in order to check the possibility of hybridization. The experimental reflectivity curve was practically the same as that reported in Figure 3 for the sample before hybridization. This result indicates that the only driving forces during the LbL layer growth are the electrostatic interactions. Therefore, the conformation and dense packing of oligonucleotides in such films prevent the electrostatic attachment of additional oligonucleotides and do not allow specific binding due to topological reasons.

In summary, the suggested method of the LbL layer decoration has permitted us for the first time to obtain X-ray reflectivity data at the solid/liquid interface, allowing modeling of the film structure. Divalent ions incorporated into the LbL layer are rather strongly bound. This is very important for the experiments when the presence of heavy ions in solution is not desirable. Therefore, only the sublayer must be decorated, while successive layers of biological molecules can be formed from solutions free from heavy metal ions. In fact, the model biological systems have confirmed the validity of the approach. DNA layers were deposited from aqueous solutions and were not exposed to heavy metal ions that could cause some structural variations. Comparison of the data obtained on the samples containing ssDNA and dsDNA allows us to conclude that no DNA denaturation takes place during the LbL layer formation. The film growth is controlled only by electrostatic interactions. The thickness of the resultant layers implies that one cycle results in a layer with an average thickness of about twice the diameter of the DNA used (for both single- and double-stranded forms). We also note that the developed approach will be even more important for studying protein-containing films. In fact, it allows proteins to always remain in their natural environment. Avoiding exposure to air will guarantee the preservation of the structure and functional activity of proteins as well as the absence of any reorganization of the whole layers due to the variation of external conditions. Along with existing neutron reflectivity techniques,[16,17] where contrasting is due to the use of deuterated water, the developed method will help to understand better the organization of such layers. Finally, the method will be very useful also when the final activity of the realized molecular structures will involve specific recognition events (interactions such as antibody−antigen and biotin−streptavidin), when any structural reorganization of the entire layer, which can result from exposure to air, can vary or even block completely the interaction kinetics.

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