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Direct Observation of Conformation of a Polymeric Coating with Implications in Microarray Applications

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The conformation of a three-dimensional polymeric coating (copoly(DMA-NAS-MAPS)) and immobilization and hybridization of DNA strands on the polymer coated surface are investigated. A conformational change, specifically the swelling of the surface adsorbed polymer upon hydration, is quantified in conjunction with the application of this polymer coating for DNA microarray applications. Fluorescently labeled short DNA strands (23mers) covalently linked to the functional groups on the adsorbed polymer are used as probes to measure the swelling of the polymer. A fluorescence microscopy technique, Spectral Self-Interference Fluorescence Microscopy (SSFM), is utilized to directly measure the change in axial position of fluorophores due to swelling with subnanometer accuracy. Additionally, immobilization characteristics of single stranded DNA (ssDNA) and double stranded DNA (dsDNA) probes, as well as hybridization of ssDNA with target strands have been studied. The results show that ssDNA further away from the surface is hybridized more efficiently, which strengthens the earlier analysis of this polymeric coating as a simple but highly efficient and robust DNA microarray surface chemistry.

When molecules come in contact with an interface, their structures may change because of surface energy minimization mechanisms. The influence of the interface, as well as the other local environmental conditions on the conformation of the molecules, is crucial, since molecular interactions are specific not only to the chemical compositions but also to the geometrical structure of the molecules, especially in biomolecular binding.¹ Developing surface chemistries that deliver preserved bioactivity of molecules is therefore of great importance to technologies that rely on surface science for molecular recognition. One such example is microarray research, which benefits significantly from progress in surface chemistries. The ability to do detection at tens of thousands of locations simultaneously with a single measurement stimulated efforts especially in genetics, proteomics, and diag-

nostics research by enabling efficient, fast, and high-throughput results with low analysis cost.^{2–4} While a variety of fluorescent⁵ and label-free^{6–8} detection techniques for microarray applications are readily available, a tremendous effort is expended on developing new techniques and improving the existing ones. Regardless of the detection technique, key factors in preparation of a microarray test can be identified as the characteristics of the solid support surface, the density, activity, and orientation of the immobilized probe molecules and the density and activity of the target molecules to be captured. The surface chemistry applied for the solid support should comply with the requirements of availability of functional groups for probe attachment, minimal non-specific adsorption, and stability to environmental changes, and should ideally be low cost, robust, and easily prepared.⁹ Perhaps even more importantly, the surface chemistry should facilitate probe activity after immobilization for efficient target capture. Among the existing surface chemistries, three-dimensional (3-D) coatings are the most promising in meeting these criteria. One such 3-D polymeric coating, copoly(DMA-NAS-MAPS), has been introduced by Chiari et al. and tested for use in DNA and protein microarray studies.¹⁰ The ter-polymer is obtained by radical copolymerization of dimethylacrylamide with acryloyloxysuccinimide and 3-(trimethoxysilyl)propyl methacrylate, and self-adsorbs to the SiO₂ surface forming a stable hydrophilic coating. Each monomer has a specific function: dimethylacrylamide (DMA) provides self-adsorption to the SiO₂ surface through hydrogen bonding and van der Waals forces, 3-(trimethoxysilyl)propyl

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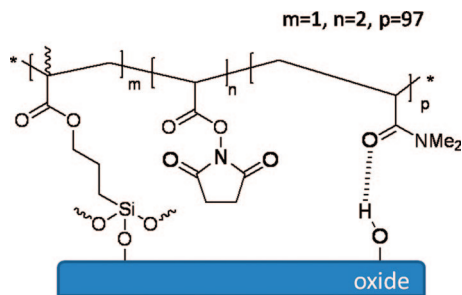


Figure 1. Schematics showing the chemical structure of the terpolymer copoly(DMA-NAS-MAPS) and attachment to the SiO₂ surface. The mole percentages of MAPS, NAS, and DMA monomers within the synthesized ter-polymer are denoted as *m*, *n*, and *p* (respectively). The drawing illustrates the self-adsorption of the polymer to the SiO₂ surface from the DMA group through hydrogen bonding, the covalent attachment to the SiO₂ surface from the MAPS group via silane functionalities, and the NHS esters provided by the NAS group for covalent attachment to amine groups of probe molecules.

methacrylate (MAPS) increases the strength of the binding through covalent attachment to the SiO₂ surface with silane functionalities, and acryloyloxysuccinimide (NAS) provides functional groups (NHS esters) that covalently bind the amine groups of the probe molecules (Figure 1).

Earlier studies with copoly(DMA-NAS-MAPS) have shown an improved performance in probe density and hybridization efficiency of DNA microarrays when compared to existing organosilanization-based microarray surface chemistries.^{10,11} Those studies have demonstrated that the 3-D structure of the polymeric coating provides an increased number of binding sites per area and prevents steric hindrance, therefore resulting in an order of magnitude higher probe density and ~80% hybridization efficiency (compared to 40–50% with silanization). The understanding and optimization of the conformation of the surface adsorbed polymer is extremely important for the interpretation of this observed superior performance and for further improvements. In this study, we present a direct quantified observation of a conformational change, particularly the swelling of copoly(DMA-NAS-MAPS) on SiO₂ surface, and measure the increase in height of the binding sites as 7–20 nm upon hydration.

The swelling, also present in other polymers, depends on the properties of the polymer of interest such as its molecular weight, as well as the environmental conditions such as temperature, or pH. This phenomenon is frequently used for drug delivery and sensor applications.^{12,13} The hydration of several types of polymers in bulk media (in solution) has been characterized previously;^{12,13} however, the characterization on the surface is not trivial.¹⁴ The measurement technique for such surface conformation studies should ideally be non-contact, sensitive to changes in nanometer scale, and should offer detection both in air and in solution. Here, an interferometric fluorescence spectroscopy technique, Spectral Self-Interference Fluorescence Microscopy (SSFM) in combination with a standard white light reflection spectroscopy technique,

is utilized (Figure 2) for characterization of the conformation, specifically swelling, of copoly(DMA-NAS-MAPS) on SiO₂ surfaces. While SSFM can detect the height of fluorescent molecules with subnanometer precision, the white light reflection spectroscopy technique is used to find the average optical thickness of an added bilayer due to additional mass. These two techniques have been successfully used for DNA conformation studies on surfaces earlier.¹⁵ It is also important to note that the environment (air/buffer) may greatly alter the emission intensity and profile of the fluorophores, in addition to factors such as the local fluorophore density or photobleaching. Therefore, when determining the conformational changes due to the presence of buffer, techniques that make use of variations in the total fluorescence intensity to determine the position of the emitters on the surface can be misleading. However, since SSFM utilizes spectral oscillations rather than the total emission intensity, the results obtained with SSFM are immune to factors that can affect the signal such as the local fluorophore density, photobleaching, or change of environment.

MATERIALS AND METHODS

Chemicals. *N,N*-dimethylacrylamide, 3-(trimethoxysilyl) propyl methacrylate, triethylamine, and sodium dodecyl sulfate (SDS) are from Merck (Darmstadt, Germany). Ammonium sulfate, glycidyl propyl silane (γ -GPS), and bovine serum albumin (BSA) are from Sigma-Aldrich (St. Louis, MO). NaCl and sodium citrate (ingredients for the Saline-Sodium Citrate (SSC) buffer) are from Serva (Heidelberg, Germany). *N*-acryloyloxysuccinimide is synthesized as previously reported.¹⁶ All oligonucleotides are synthesized by MWG-Biotech AG (Ebersberg, Germany). Untreated SiO₂/Si wafers (5 μ m oxide on 500 μ m silicon) are purchased from Silicon Valley Microelectronics (Santa Clara, CA). The silicon wafers are chemically and mechanically polished to a roughness of <0.4 nm followed by thermal growth of a 5 μ m thick oxide layer.

Copoly(DMA-NAS-MAPS) Coating. The 10% w/v polymer is synthesized as reported previously.¹¹ Clean SiO₂ surfaces are treated for 30 min with 0.1 M NaOH, immersed in polymer solution (1% w/v in a water solution of ammonium sulfate at a 20% saturation level) for 30 min, washed extensively with deionized water (DI-H₂O), dried with argon, and kept at 80 °C for 15 min.

Epoxy-silanization. Clean SiO₂ surfaces are treated with 0.1 M NaOH followed by γ -GPS (glycidyl propyl silane) (5% in dry toluene) for 4 h at 37 °C and washed with toluene and methanol, then kept at 100 °C overnight.

Oligonucleotide Immobilization. Labeled (5'-amine-modified and 3'-Cy5-modified) single-stranded DNA (ssDNA), unlabeled (5'-amine-modified) ssDNA, and labeled double-stranded DNA (dsDNA) (5'-amine-modified ssDNA hybridized with 5'-Cy5-modified complementary) of 30 μ M concentration in sodium phosphate buffer (150 mM, 0.01% Triton, pH 8.5) are spotted on polymer/epoxysilane coated surfaces using a non-contact microarray spotter (Scienion sciFLEXARRAYER). The substrates are kept at room temperature in a dark chamber saturated with NaCl (~75%

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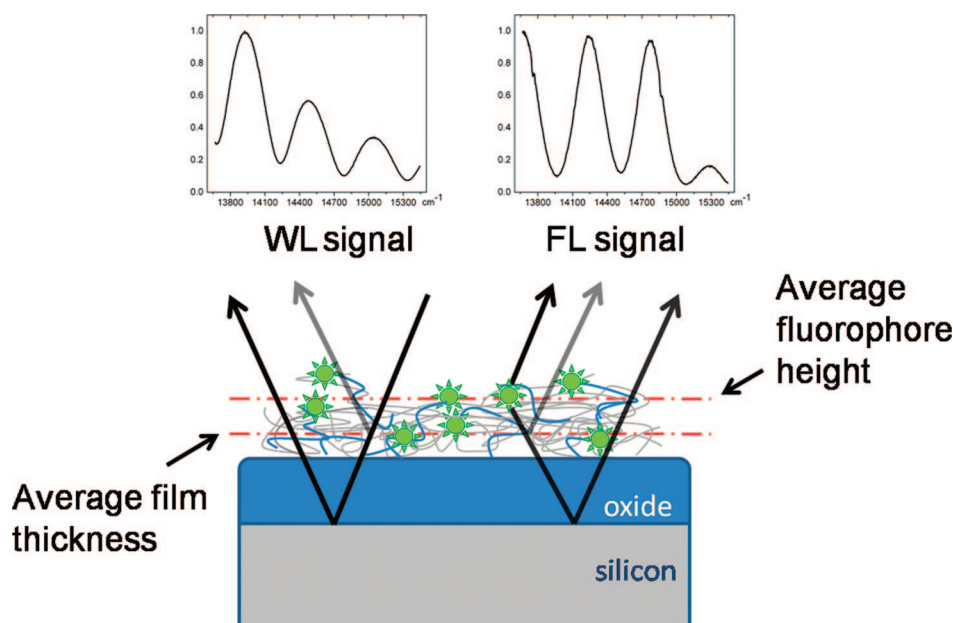


Figure 2. SSFM and WL measurement techniques. In the WL technique, interference of the light reflected from the top surface and the buried SiO₂/Si interface results in periodic oscillations, which are used to determine the bilayer thickness. In the SSFM technique, spectral oscillations due to the self-interference from the direct and reflected emission of the fluorophores are translated into the vertical position of the emitters with subnanometer accuracy. Typical spectral data obtained with the SSFM and WL techniques are shown.

humidity) overnight. The slides are then washed twice for 5 min in 2X SSC/0.01% SDS buffer, at 45 °C, followed by a rinse for 1 min with 0.2X SSC and with 0.1X SSC at room temperature, and finally dried with argon.

Oligonucleotide Hybridization. For hybridization at the polymer-coated and epoxysilanized surfaces, oligonucleotide targets of 1 μ M concentration are dissolved in the hybridization buffer (2X SSC, 0.01% SDS and 0.02% BSA), immediately applied to the spotted ssDNA microarray, and the sample surface is covered with a plastic microscope slide coverslip. The samples are incubated at room temperature in a dark humid chamber for 2 h. After hybridization, the samples are first dipped briefly in 4X SSC at room temperature to remove the coverslips, then washed with 2X SSC/0.01% SDS at 45 °C for 5 min to remove the excess target DNA. This step is repeated, followed by 1 min wash with 0.2X SSC and 0.1X SSC at room temperature, and the samples are dried with argon.

For hybridization in solution, probe and target oligonucleotides of equimolar concentration (30 μ M) were dissolved in the spotting buffer (150 mM sodium phosphate, 0.01% Triton, pH 8.5) and hybridized in a plastic vial in the dark at room temperature for 2 h.

SSFM Setup. The principle of operation of SSFM, the experimental setup for data acquisition, and the processing of the spectra for nanometer accuracy in axial localization have been reported previously.¹⁷ Briefly, the system consists of a continuous wave laser for excitation, a microscope, and a spectrometer. In this study, the laser beam (HeNe at 633 nm) is coupled to the microscope objective (0.12 numerical aperture) through the side port of an upright microscope and focused on to the sample. The fluorescence signal is collected with the same objective, coupled to a spectrometer with 600 grooves/mm grating (spectral resolu-

tion of 3 cm⁻¹ at 700 nm) for spectral acquisition, and the signal is recorded using a thermoelectrically cooled CCD. A halogen lamp with Koehler illumination is used for white light reflectivity measurements. The spectra are then fitted with a custom-built MATLAB application that returns the average height of the bilayer and the average height of the fluorophores from the SiO₂/Si interface. Since the spectral oscillations, rather than the total emission intensity, are used for determining the position of the emitters, the results obtained with SSFM are immune to factors that can influence the total signal.

RESULTS

Various properties such as the density, gyration radius, stability, and contact angle of the copoly(DMA-NAS-MAPS) have been measured and reported previously.^{11,18} According to the AFM scratch tests,¹⁸ the thickness of the dry coated polymer on the SiO₂ surface is \sim 2 nm. The results below summarize the detected swelling of the polymer upon hydration. Fluorescently labeled, amino-modified short DNA strands (23mers) covalently attached to the polymeric coating are used as probes (Figure 3a). An increase in the axial position of the fluorophores indicates an increase in the length of polymer chains due to swelling. SSFM and white light reflectivity measurements are performed before and after hydration of the coated surface.

Measured Swelling with ssDNA Probes. Figure 3c illustrates the swelling upon hydration, where ssDNA are used as probes. As shown (Figure 3b), there is \sim 7.5 nm difference in the detected average fluorophore height obtained at ssDNA spots on the dry and hydrated polymer-coated surface. Considering that ssDNA will not take a fully extended form but rather will orient itself in a random fashion, and the elevation of surface-tethered 21mer ssDNA in solution reported previously using SSFM¹⁷ is

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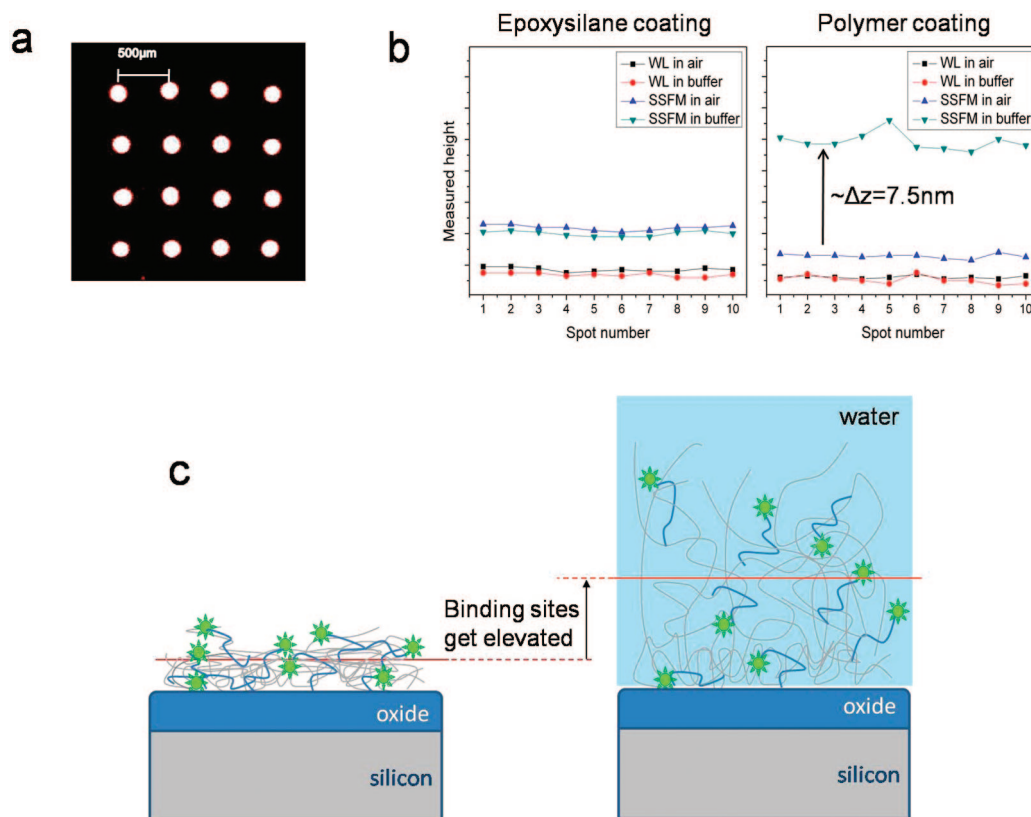


Figure 3. (a) Fluorescence scan of a typical array of ssDNA spots on copoly(DMA-NAS-MAPS) demonstrating spot quality. The spots are $\sim 150 \mu\text{m}$ in size with $500 \mu\text{m}$ pitch. (b) Detected heights with WL and SSFM techniques for 10 different spots on the same sample before and after hydration. The epoxysilanized sample shows no change in WL or SSFM levels before and after hydration. For the polymer coated sample, the WL level is maintained upon hydration, whereas the SSFM level increases by $\sim 7.5 \text{ nm}$. (c) Illustration of the justification for the height change in probe heights immobilized on the polymer. The polymer swells upon hydration, resulting in elevation of the binding sites.

only 1 nm, it is evident that the detected 7.5 nm height difference is not a result of the change in ssDNA conformation due to the presence of the buffer. The height change indicates that, upon hydration, the polymer swells axially, which is also verified by the control measurement performed on the epoxysilanized surface, where no significant increase in average fluorophore height is measured (Figure 3b), indicating, as expected, the absence of any swelling.

Also evident from Figure 3b is that the thickness measured with the white light reflection spectroscopy technique shows no significant change upon hydration of the polymer. This is due to the fact that the white light technique does not measure the absolute height of a layer but rather measures the mass accumulated on the surface. Since there is no additional mass introduced to the surface during the swelling mechanism, the measured average thickness should not change. (see Supporting Information, Figure S1 of ref 7) To demonstrate this, we have fabricated PDMS structures and flowed the polymer within channels formed between the PDMS material and the SiO_2 surface. The surface patterned with the polymer is then analyzed using the spectral reflectance imaging technique in ref 7. As shown in Figure 4, images are taken before and after hydration. The surface-adsorbed mass density plots are obtained by averaging over the selected area in Figure 4 in the direction parallel to the channel and confirm that the detected mass density of the polymer does not change significantly upon swelling.

Measured Swelling with dsDNA Probes. Swelling with dsDNA probes is detected using three different configurations: labeled ssDNA spotted and hybridized on the polymer-coated surface with unlabeled complementary (Case-B), labeled dsDNA spotted (Case-C), and unlabeled ssDNA spotted and hybridized on the polymer-coated surface with labeled complementary (Case-D). Each case is represented by a histogram of the detected swelling at multiple spots (> 25 spots on > 2 samples) in Figure 5, together with the results from the study of ssDNA spots (Case-A) for a comparison. The histograms indicate a mean increase in average fluorophore height upon hydration of $\sim 8 \text{ nm}$ (Case-B), 14 nm (Case-C), and 17 nm (Case-D). Once again, the detected change in fluorophore height is much greater compared to the previously reported¹⁷ height of fluorophores attached to the distal end of surface-tethered dsDNA (21mer) in solution (5.5 nm), indicating swelling of the polymer coating and elevation of the binding sites. Also, note that the values reported previously for the conformation of DNA are on silanized surfaces, in which case the free rotation of the DNA is restricted because of the presence of the surface and steric hindrance. In the case of the polymeric coating, however, the restrictions are reduced because of the elevated probes and the 3-D surface chemistry.

DISCUSSION

The increase in the height of the fluorescent markers attached to the probes is an indication of the swelling but not an absolute

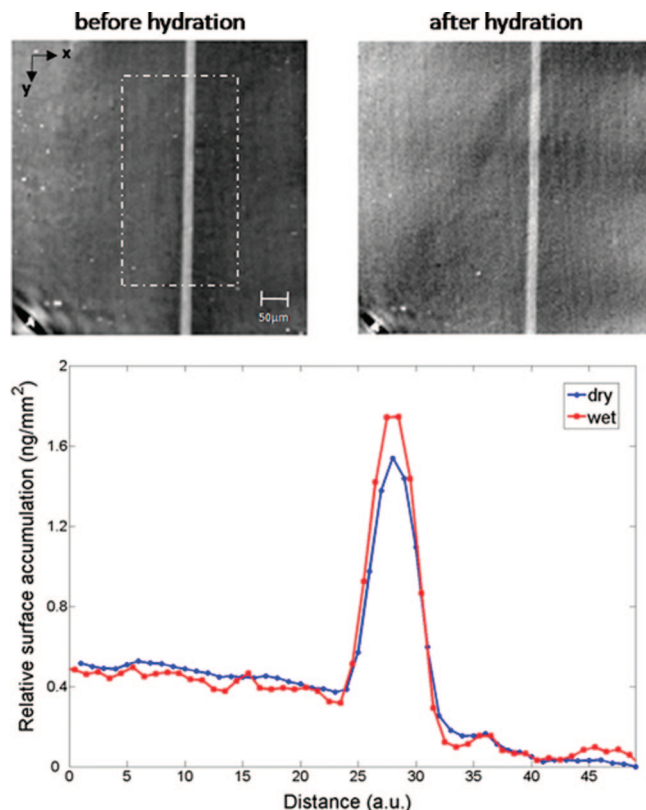


Figure 4. Detection of mass density of the surface-adsorbed polymer. The images before and after hydration are taken with the spectral reflectance imaging technique.⁷ The data represented in the relative surface adsorption plot is obtained by averaging the images inside the dashed rectangle in y-direction, and shows the average mass density in the x-direction. The change in the detected mass density after hydration (0.2 ng/mm^2) is insignificant (1–5%) in comparison to the amount of swelling.

measure, since that depends on the location of the probes within the polymer structure. The differences in the measured change in average fluorophore heights upon hydration for the four different scenarios (labeled ssDNA probes (Case-A), labeled ssDNA probes hybridized (Case-B), labeled dsDNA probes (Case-C), unlabeled ssDNA probes hybridized (Case-D)) are analyzed and discussed below leading to specific conclusions on the conformational change of the polymer, as well as hybridization efficiency of the DNA probes as a function of their displacement from the surface. It is worthwhile to note that no non-specific binding was observed throughout the experiments due to the nonfouling properties of the polymer coating.

Case-A on Polymer versus on Epoxysilane Coating. Using labeled ssDNA as probes on the polymer coated surface, a ~ 7.5 nm increase in average fluorophore height is observed upon hydration. To verify that the measured height change is due to the swelling of the polymer, a control experiment has been conducted on an epoxysilane coated surface with the same probes. As expected, no significant change was observed in fluorophore height upon hydration, indicating that the epoxysilane did not swell. It should be noted that no information on the absolute swelling of the polymer or on the distribution of the probes within the polymer coating can be extracted from this measurement.

Case-A versus Case-B. When the labeled ssDNA probes are hybridized on polymer-coated surface (Case-B), the average height

increase is measured as ~ 8 nm. This shows that the conformational change in the DNA structure (transformation of ssDNA structure from a random coil to a rigid rod because of hybridization) attached to the polymer does not cause a significant increase in the detected height (~ 0.5 nm). This difference between the fluorophore elevation from the surface for ssDNA and dsDNA is lower than previously reported results on silanized surfaces,¹⁷ which might be explained by the reduced steric hindrance and increased freedom in orientation of the dsDNA within the 3-D polymeric coating. Note that in both cases, the signal originates from the initially spotted labeled strands; therefore once again, no additional insight on the probe distribution or activity can be extracted.

Case-B versus Case-D. For the case of unlabeled ssDNA hybridized on polymer-coated surface (Case-D), a ~ 17 nm increase in fluorophore height is measured, which is much greater than for the case of labeled ssDNA hybridized (Case-B). In Case-B, the fluorescence signal originates from all the strands immobilized on the polymer-coated surface, since they are all labeled. However, in Case-D, only the strands that get hybridized with their labeled complementary contribute to the measured signal. Therefore, the difference between the measured elevations in fluorophore positions suggests that the hybridization efficiency is higher for probes which are further away from the surface in comparison to those closer to the surface. These results also indicate that the probes are distributed within the polymer structure in such a way that some probes stay buried within the polymer structure close to the glass surface and thus have low hybridization efficiency, whereas others get elevated and hybridized more readily.

Case-A versus Case-C. In the case of spotted labeled dsDNA (Case-C), the signal will originate from all of the molecules bound to the polymer as it is in the case for spotted labeled ssDNA (Case-A). However, in Case-C, we observe a greater difference in average fluorophore height (~ 14 nm) after hydration when compared to Case-A (~ 7.5 nm). This is an indication that the dsDNA cannot penetrate the polymer in its hydrated state as easily as the ssDNA, and therefore the probes are attached at locations farther from the surface. Calculating the pore size of the adsorbed polymer in the hydrated state would help understand this observed phenomenon but this is not trivial, since the exact microstructure of this polymer is not known. However, if we consider the average molecular weight of the polymer ($\sim 6,000$ Da) and the average mass density of the coating on the SiO_2 surface ($\sim 1.5\text{ ng/mm}^2$), the polymer density can be calculated ($\sim 1.5 \times 10^{11}\text{ /mm}^2$). From here, assuming a homogeneous swelling of 20 nm upon hydration for the sake of simplicity, the pore diameter of the coating in the hydrated state can be approximated as ~ 5 nm. Although this is not a very accurate approximation, it can help us understand the limitation in the penetration of the 23mer dsDNA in rigid-rod form, which has a length (~ 7 nm) that is on the same order as the pore-size.

In these set of experiments, we directly observe the swelling of a polymeric coating upon hydration to understand the effect of swelling on probe immobilization and on hybridization, which influences the performance of this polymer as a microarray support. In addition to providing an understanding to the probe-surface chemistry interactions, these results reveal that, with the

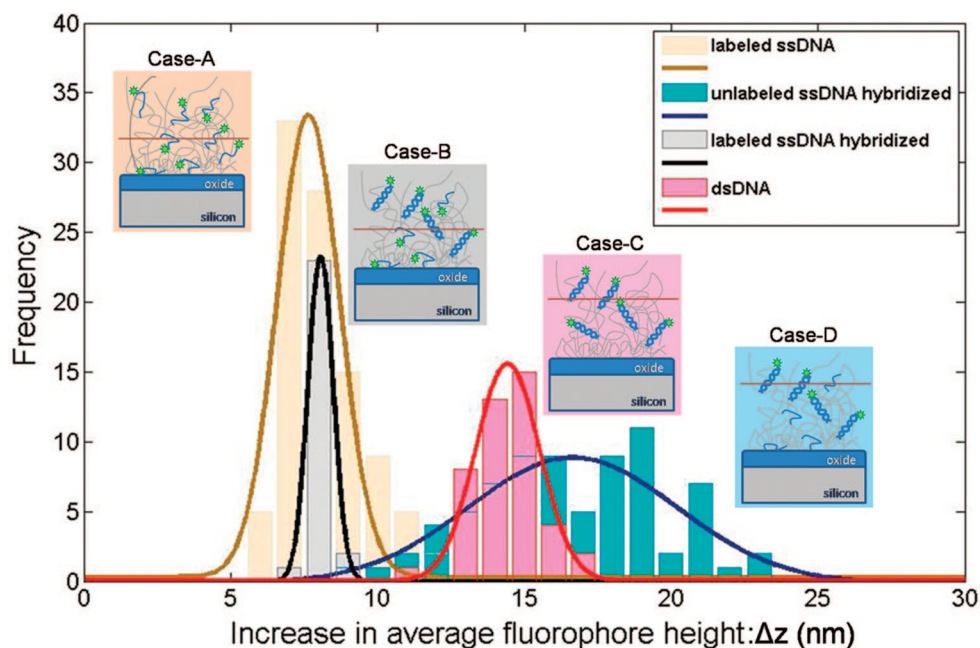


Figure 5. Histograms summarizing the results obtained at spots of labeled ssDNA (Case-A), labeled ssDNA hybridized on polymer-coated surface (Case-B), labeled dsDNA (Case-C), and unlabeled ssDNA hybridized on polymer-coated surface (Case-D). The measured increase in fluorophore heights for several spots on three different samples for Case-A, and on two different samples for Case-B, -C, and -D are included. Each histogram is fitted with a Gaussian curve.

copoly(DMA-NAS-MAPS) coating, hybridization of ssDNA probes with its complementary ssDNA target occurs further away from the surface, which can explain the reported superior hybridization efficiency obtained with these coatings when compared to epoxysilanized surfaces.¹⁰

CONCLUSION

The ability to quantitatively detect conformational changes of molecular structures at interfaces is of great importance for both understanding structural dependence of fundamental interactions and improving conditions such as the solid support itself or the environment in which the interactions take place. Developing surface chemistries that do not hinder the functionality of the immobilized molecules will help increase the efficiency of DNA hybridization on surface, as well as overcome the difficulties in antibody–antigen binding assays. In these lock-key types of interactions, the binding is controlled not only by the chemical compositions of the constituent proteins but also by their geometrical structures. With the use of the polymeric coating, fundamental problems of surface assays such as restricted access to specific regions of the surface immobilized proteins due to random orientation or complete loss of bioactivity due to denaturing of the proteins can be eliminated.

In this work, we have measured the change in the conformation of a surface adsorbed polymer upon hydration and demon-

strated the difference in the axial distribution of immobilized probe molecules (ssDNA and dsDNA) within the polymer structure, the penetration of target molecules within the polymer (ssDNA and dsDNA), and the effect of these on the hybridization. We have shown that the hybridization of the probe with the target occurs further away from the surface. This information suggests that the control and manipulation of the conformation of the polymeric coating will have a significant impact in improving the hybridization efficiency of microarrays. Characterization of existing surface chemistries and development of new ones with the help of techniques such as the one used in this study that can measure conformational changes will enable designing platforms of optimized surface chemistry, probe immobilization, and target detection for individual applications.

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