# DNA Hybridization on Plasma-Polymerized Allylamine

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ABSTRACT: Surface Plasmon Enhanced Fluorescence Spectroscopy (SPFS) has been used to monitor the hybridization reaction of fluorophore-labeled 15mer target oligonucleotides from solution to probe DNA previously immobilized on plasma-polymerized allylamine films (ppA). The surfaces were treated with 254 nm UV light to covalently link the probe DNA to the ppA films. Since strong unspecific adsorption would occur between target DNA and unreacted amino groups on the polymer surface, any remaining reactive moieties in the polymer film were neutralized by rinsing the substrate surface with succinic anhydride after probe adsorption and before target hybridization. The SPFS data were utilized to determine hybridization affinity constants ($K_A$) for complementary targets (MM0) as well as mismatch 1 (MM1) and mismatch 2 (MM2) hybridization reactions. Complementary target (MM0) DNA hybridization was shown to have a significantly higher $K_A$ than that MM1 and MM2. The three-dimensional network of the ppA appears to allow a similar hybridization mechanism in comparison to the more conventional two-dimensional systems.

## Introduction

DNA hybridization has been extensively studied by a number of groups worldwide and already shows broad commercial applications in biosenor technology. The most common systems used for DNA sensing include, for example, the streptavidin model,[1] monoclonal antibodies,[2] or cationic lipid membranes.[3] These systems characteristically rely on a two-dimensional surface, and the orientation of hybridization is generally assumed to occur perpendicular to the substrate surface. The analytical methods utilized for such studies include Atomic Force Microscopy (AFM),[4,5] Quartz Crystal Microbalance measurements (QCM), and Electrocatalysis.[6,7] Much more recently, SPFS has been shown to provide a very high sensitivity for the study of DNA association and dissociation behavior.[1,8] With the aid of simple Langmuir models, the rate constants for the association and dissociation of the oligonucleotides can be calculated and the hybridization conditions can thus be optimized.

Our group has recently described the use of plasma polymerized amine functionalized films (ppA) and their application as supports for the immobilization of single-stranded DNA.[9] The plasma polymer network was shown to exhibit a three-dimensional interface structure, and reactive sites to an approximate depth of 40 nm were shown to participate in the immobilization reaction. The plasma-assisted polymerization of allylamine can be controlled such that the chemical structure and the density of amino groups within the films can be tailored with reasonable precision.[10−12] In general, films deposited at a relatively high energy (high input power, $P_{peak}$, high equivalent power $P_{eq}$) will be highly cross-linked and have little to no resemblance to the precursor molecule. However, low-energy plasma deposition processes ($P_{eq}$ < 5 W) have been shown to lead to polymeric networks with a high retention of the monomer structure and thus a high density of amino functional groups. In the present work, optimal deposition conditions for high $NH_2$ group retention and DNA adsorption were obtained at 5 W cw and a pressure of 0.1 mbar.

When subjected to an aqueous buffer, the plasma polymer films undergo a swelling process which leads to an open and mobile polymeric matrix that bears some resemblance to functionalized polymer brushes or hydrogels.[9] At a physiological pH of 7.4, the amino groups will undergo the following reaction:[13,14]

$$R{-}NH_2 \overset{H^+}{\rightleftharpoons} R{-}NH_3^+$$

leading to a positively charge matrix that can undergo electrostatic interactions with negatively charged oligonucleotides. The highly swollen and positively charged polymer thus provides a reactive interface in three dimensions. This was shown to lead to a 6-fold increase in the amount of DNA probe adsorbed on a 13 nm thick plasma polymer in comparison to a more conventional polylysine surface. The DNA loading density was found to be influenced by the thickness of the plasma polymer layer,[15] and it was shown that for a 30mer oligomer it could be increased up to the equivalent of 15−20 monolayers for plasma polymer films of 50 nm thickness.

The present paper describes a continuation of the previous work on oligonucleotide adsorption and carries on to the investigation of the hybridization reactions occurring in this swollen 3D polymer network. This includes hybridization reactions with a fully complementary target (MM0), as well as with target sequences exhibiting one mismatch (MM1) or 2 mismatches (MM2). Conventional methods of DNA cross-linking and amino group deactivation after probe adsorption have been adapted in the procedures described below.

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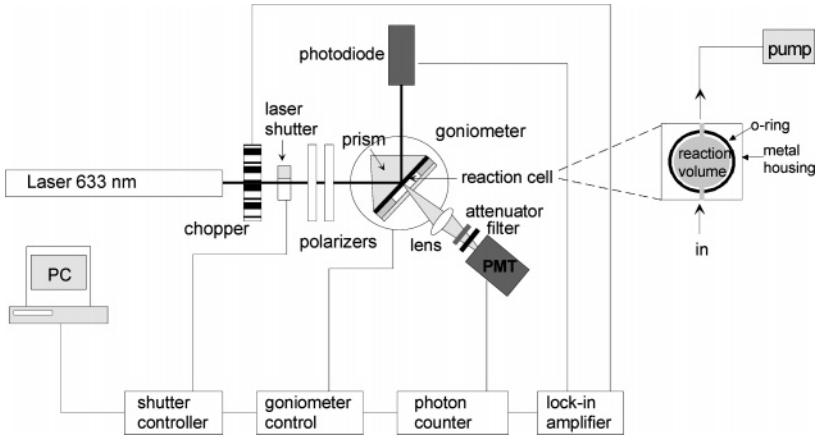


**Figure 1.** Schematic of the experimental SPR/SPFS set up.

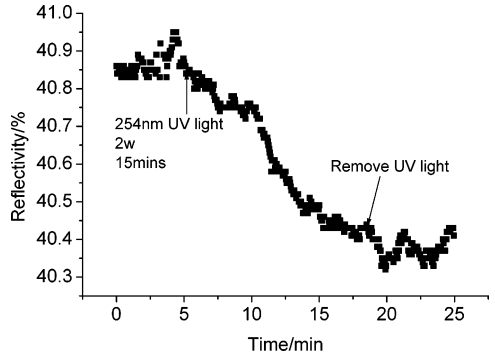


**Figure 2.** SPR reflectivity changes observed during UV irradiation.

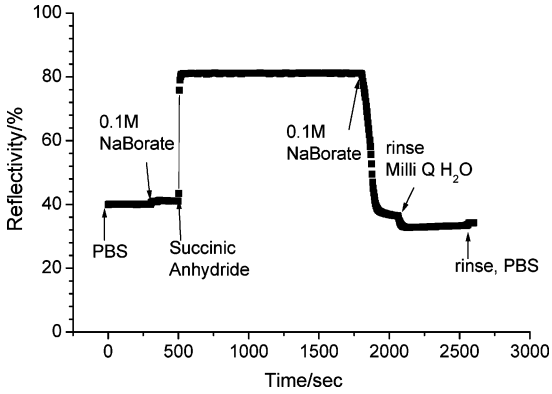


**Figure 3.** SPR measurements of the neutralization reaction of the surface using succinic anhydride.

## Experimental Section

**Plasma Polymerization.** The allylamine monomer was purchased from Sigma-Aldrich, Drisenhofen, Germany. It was polymerized in a home-built (30 cm long, ∅10 cm) Pyrex plasma reactor using an excitation frequency of 13.56 MHz. This has been described in detail in previous papers.[10] Using an input power of 5 W and a deposition time of 7 min led to films of approximately 30 ± 5 nm in thickness. These deposition parameters were used throughout this work and lead to films of relatively high amino group densities.[15] Before probe immobilization, the films were immersed in buffer for 5 h to allow for the swelling of the film.

**Surface Plasmon Enhanced Fluorescence Spectroscopy.** SPFS is a recently developed technique[16] which makes use of the field enhancement mechanisms that operate at the already well-established resonant excitation of surface plasmons at a gold/dielectric interface.[17] The highly enhanced electromagnetic field of the surface plasmon mode is used for the excitation of surface-confined chromophores. It was recently demonstrated that hybridization reactions between surface-immobilized DNA probes and complementary target strands carrying a fluorescent label can be detected using SPFS.[8] The exploitation of this "surface light" for the excitation of chromophores near or at a gold/dielectric interface has since then been described and discussed in a number of articles in the study of hybridization reactions at interfaces.[1,18] SPFS was used in the present work to study the reactions between adsorbed probe DNA and labeled target strands occurring on and within a plasma polymer network.

The home-built spectrometer allows for both SPR and SPFS measurements, Figure 1. The spectrometer was equipped with a photodiode to detect the reflected laser light at the angle $\theta$ (SPR) and with a photomultiplier to measure the photons emitted by the chromophore labels (SPFS). An attenuator was placed between the photomultiplier and the reaction chamber since the signal observed during the experiments was greater than the photomultiplier range and thus created a possible source of error. All fluorescence data presented in this paper show the attenuated signal intensities. The stainless steel wet cell had a volume of 5 cm$^3$ with an inlet and outlet, allowing for a continuous flow of reagents throughout the experiments. The small wet cell was sandwiched between the reactive surface to be investigated and a quartz plate.[19] The sample was irradiated with monochromatic, linearly (p-) polarized light (He−Ne, $\lambda$ = 633 nm) such that under total internal reflection the surface plasmon is excited at the gold/dielectric interface at a certain angle of incidence. If the refractive index or thickness of the medium just outside the gold surface changes, then a proportional change in the angle can be observed and measured. Near the surface plasmon resonance angle, the fluorescence intensity of chromophores within the polymer coating increases strongly with peak intensities about 2 orders of magnitude above the background. This simultaneous measurement of reflectivity and fluorescence intensity offers a unique method for monitoring the hybridization process of chromophore-labeled oligunucleotides with surface-attached DNA strands.

**DNA Probe Immobilization.** The oligonucleotides were purchased from MWG BIOTECH AG, Ebersberg, Germany. The probe oligonucleotide sequences (30mers) were as follows

5′-TTT TTT TTT TTT TTT TGT ACA TCA CAA CTA-3′

5′-TTT TTT TTT TTT TTT TGT ACA TCA CAA CTA-cy5-3′

The probe sequences consisted of a labeled chromophore (cy5) and an unlabeled sequence. The labeled probe was used in a control experiment. The DNA solutions were made up using phosphate buffer at a pH of 7. After an initial swelling process, probe immobilization was monitored in-situ using SPR;[15] any physisorbed probe was removed by thorough rinsing with excess buffer solution.

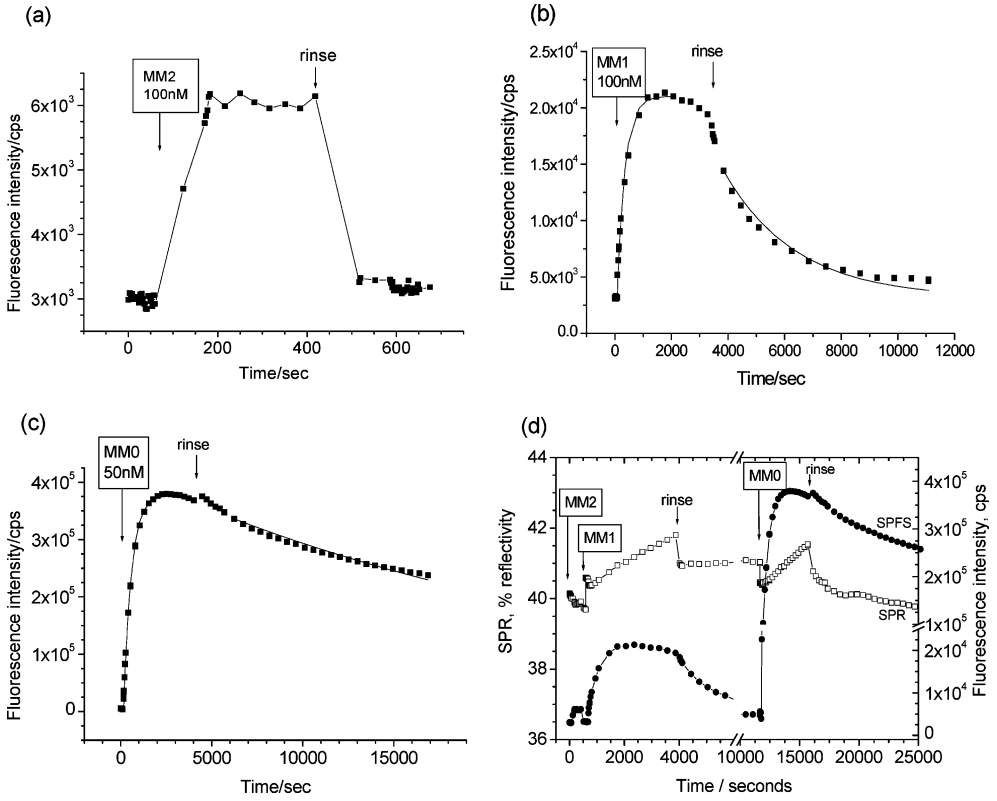


**Figure 4.** SPFS measurements of the hybridization reaction using (a) cy5-labeled MM2, (b) cy5-labeled MM1, and (c) cy5-labeled MM0 target DNA. The signal for the MM0 hybridization was attenuated by a factor of $10^2$. (d) SPFS data for the three hybridization reactions in combination with the associated SPR data. Calculated $K_A$ constants are given in Table 1.

**Cross Linking and Neutralization.** In accordance with standard hybridization protocols,[20] the oligonucleotides were cross-linked with the polymer matrix using a short exposure to UV light. A 254 nm, 2 W UV light was mounted behind the SPR wet cell, and the sample was irradiated though the quartz window and a 5 mL solution (solution depth ca. 4 mm) for 15 min. The total distance between the UV lamp and the ppA/probe surface was 36 mm. The power density of UV light was 10.2 W/cm$^2$.

The neutralization of any remaining amino groups within the ppA network, which may interfere with the subsequent hybridization reaction, was achieved by treatment of the samples with succinic anhydride and sodium borate ($Na_2B_4O_7 \cdot 10H_2O$) after the UV treatment.[20] For each experiment, fresh succinic anhydride was dissolved in 1-methyl-2-pyrrolidinone. A 0.1 M $Na_2B_4O_7$ solution was first circulated for 2 min to displace the buffer present in the reaction cell. The blocking solution, consisting of succinic anhydride in sodium borate, was circulated in the SPR wet cell for at least 20 min, followed by rinsing with a 0.1 M $Na_2B_4O_7$ solution, then Milli-Q water, and finally PBS buffer solution.

**Hybridization.** Hybridization reactions were carried out using the target sequences listed below. The unlabeled target was used in a control experiment with the labeled probe.

Unlabeled (MM0) 15 mer:

5′-TAG TTG TGA TGT ACA-3′

Labeled (MM0) 15 mer:

5′-cy5-TAG TTG TGA TGT ACA-3′

Labeled (MM1) 15 mer:

5′-cy5-TAG TTG TGA **C**GT ACA-3′

Labeled (MM2) 15 mer:

5′-cy5-TAG TTG T**C**A **C**GT ACA-3′

Stock solutions of the oligonucleotides in phosphate buffer at pH 7 with a concentration of $c_t = 1 \times 10^{-7}$ M were prepared and used throughout this work. Complementary target (MM0) and MM1 solutions with concentrations ranging from $c_t = 1 \times 10^{-9}$ M to $c_t = 1 \times 10^{-6}$ M were prepared for the Langmuir adsorption isotherm discussed below.

## Results and Discussion

**DNA/Polymer Cross-Linking.** After probe immobilization,[15] the samples were subjected to UV light to initiate covalent bonding between the probe oligonucleotides and the ppA. The changes in the bonding environment occurring throughout the plasma polymer and at the ppA/DNA interface as a result of the UV treatment were monitored using SPR. The measurements shown in Figure 2 indicate a small but measurable change in the SPR reflectivity. Unfortunately, it is rather difficult to attribute this change to any specific reaction since neither FTIR nor XPS were able to provide any reliable accompanying chemical information. Side effects due to the UV treatment, such as cross-linking within the polymer network or the reaction of active sites with water molecules can, however, be considered irrelevant for the subsequent hybridization reactions.

**Neutralization.** The reaction cell was flushed with 0.1 M sodium borate (pH 8) followed by a mixture of succinic anhydride in 0.4 M sodium borate. In contrast to the on-line protocols, the concentration of both the succinic anhydride and the $Na_2B_4O_7$ solution had to be increased substantially in order to achieve complete neutralization of the amino groups within the plasma polymer network. A significant increase in the SPR reflectivity could be observed, Figure 3, which reflects the changes in the refractive index of the environment within the flow cell as the PBS buffer is exchanged with basic solvent and reactant. After rinsing, first with sodium borate and then with Milli-Q water, the SPR

**Table 1. Kinetic Data Showing the Association Constant, $K_{on}$, the Dissociation Constant, $K_{off}$, and the Affinity Constant $K_A$ ($=K_{on}/K_{off}$) for the Reaction of a Surface-Attached Probe with MM0 and MM1 Target Oligomers**

| | MM0 (Langmuir simulation) | MM1 (Langmuir simulation) | MM0 (titration) | MM1 (titration) | MM0[1] | MM1[1] |
|---|---|---|---|---|---|---|
| $k_{on}/M^{-1}\,s^{-1}$ | $4.6 \times 10^4$ | $3.3 \times 10^3$ | | | $3.7 \times 10^4$ | $8.9 \times 10^3$ |
| $k_{off}/s^{-1}$ | $4 \times 10^{-5}$ | $3.9 \times 10^{-4}$ | | | $7 \times 10^{-6}$ | $3.7 \times 10^{-4}$ |
| $K_A/M^{-1}$ | $1.15 \times 10^9$ | $8.46 \times 10^6$ | $1 \times 10^8$ | $6.7 \times 10^6$ | $5.3 \times 10^9$ | $2.4 \times 10^7$ |

reflectivity was slightly below its original value. This may suggest some loss of material from the surface or changes in the optical properties of the multilayer.

**Hybridization.** Upon hybridization with a chromophore-labeled target, the number of surface-bound oligonucleotides should increase and so should the observed thickness and the fluorescence intensity. SPFS and SPR measurements during the hybridization reactions of the three target oligonucleotides with the surface-attached probe are shown in Figure 4. With a decreasing number of mismatches, from (a) MM2 to (b) MM1 to (c) MM0, the maximum fluorescence intensity increased, indicating the improved hybridization conditions between probe and target. Upon passing the MM2 target over the surface-attached probe, a small rise above the background (<1 order of magnitude) was observed in the fluorescence intensity, Figure 4a. The rapid decrease in the fluorescence signal upon rinsing with buffer suggests only unspecific binding of the target and a monitoring of bulk contributions from the evanescent tail.

The addition of MM1 target showed an increase in the fluorescence intensity (approximately 1 order of magnitude), Figure 4b, indicating some hybrid formation. These dissociated completely after rinsing with excess buffer. However, when comparing the dissociation curves for the MM2 and MM1 situation, the shape of the curves shows that the dissociation of the MM1 target already bears a close resemblance to a typical Langmuir model, suggesting partial hybridization rather than just unspecific binding of the target to the surface. The MM0 hybridization lead to a large increase in fluorescence intensity, as shown in Figure 4c. Rinsing of the surface with excess buffer leads to only a very slow loss in the fluorescence intensity, suggesting that hybridization had occurred and stable hybrids were formed within the plasma polymer network.

When comparing the SPFS data with the accompanying SPR data in Figure 4d, moderate increases in the optical thickness can be observed for each of the MM2, the MM1, and the MM0 situations. For each of these reactions, rinsing led to a decrease in the optical thickness because the SPR technique cannot distinguish between the single strand probe attached to the plasma polymer and the double helix attached to the polymer.

A simple Langmuir model can be used to describe the association and dissociation behavior during the process of hybridization with MM1 and MM0. The fluorescence data in Figure 4 were used to determine the two reaction rate constants, $k_{on}$ and $k_{off}$, as well as the affinity constant $K_A = k_{on}/k_{off}$. The rate constants, Table 1, for the hybridization of the MM0 and MM1 targets show that, with increasing mismatch, the association rate constant ($k_{on}$) decreases and the dissociation rate ($k_{off}$) increases. The $k_{on}$ and $k_{off}$ values obtained are close to previously published values for similar hybridization reactions occurring on 2D surfaces. The affinity constant, $K_A$, for the MM0 situation decreases by about 2 orders of magnitude if one single base is replaced in the complementary target strand.

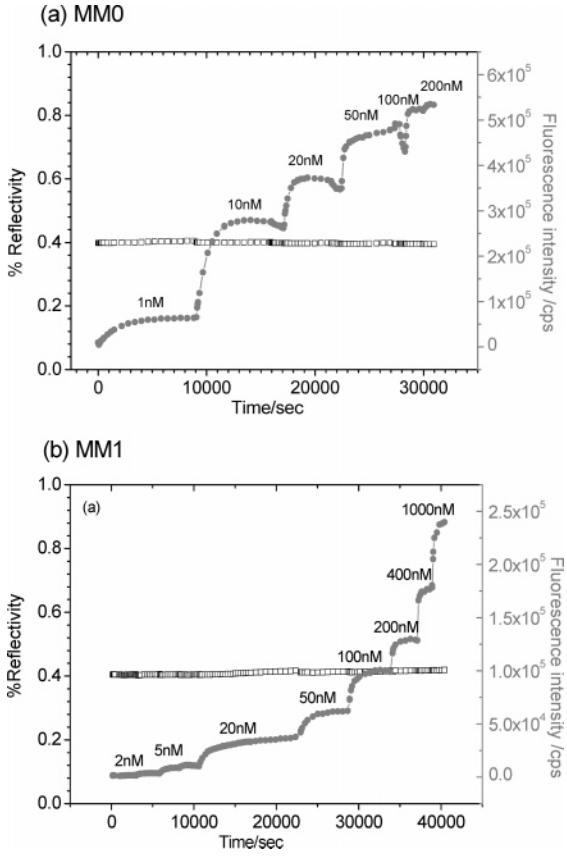


**Figure 5.** SPR and SPFS data for titration experiments in which the surface-bound probe oligomers are subjected to (a) MM0 or (b) MM1 target oligomer solutions of increasing concentration.

An alternative way to investigate the hybridization process is given by the analysis of titration experiments in which the surface-bound probe oligomers are subjected to MM0 or MM1 target oligomer solutions of increasing concentration, Figure 5. By a stepwise increase in the target concentration ($c_t$) new equilibrium coverages are reached which are associated with a particular fluorescent intensity ($I_{fl}$). The fluorescent intensity observed after equilibration of each step allows for a quantitative evaluation of the affinity $K_A$. If the fluorescence intensity is plotted as a function of the target concentration, or as the linear version of the Langmuir isotherm, $c_t/I_{fl}$ versus $c_t$, Figure 6, then the affinity constant $K_A$ can be calculated. Using this procedure, the $K_A$ for the MM1 hybridization reaction was found to be $6.7 \times 10^6$ $M^{-1}$, while for the MM0 reaction it was $1 \times 10^8$ $M^{-1}$, Table 1. The values obtained from both Langmuir simulations and experimental titration for DNA hybridization within a 3D polymer matrix are in fairly close agreement with the association and dissociation behavior determined for a 2D biotin−streptavidin model surfaces.[1,21] The ad-

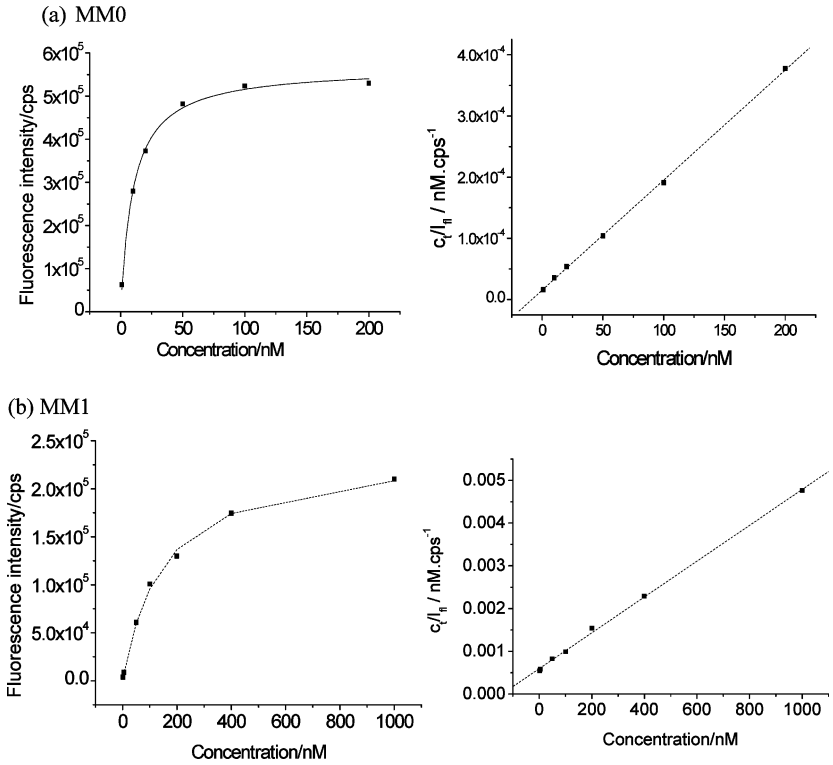


**Figure 6.** Langmuir adsorption isotherm of (a) MM0 and (b) MM1 target hybridization with surface-attached probe plotted as the fluorescence intensity $I_{fl}$ versus the target concentration ($c_t$) and as $c_t/I_{fl}$ versus $c_t$ allowing for the calculation of the affinity constant $K_A = 8.46 \times 10^6$ M$^{-1}$.

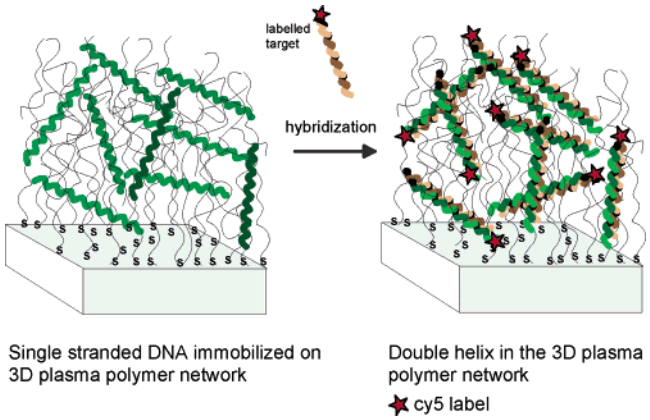


**Figure 7.** Schematic of the hybridization reactions of labeled target strands with surface bound DNA probe.

ditional, increased loading capacity of the 3D matrix suggests the low duty cycle plasma-polymerized matrixes to be promising candidates for future sensor technology.

Figure 7 shows a schematic of DNA hybridization on plasma-polymerized films as discussed in this work. So far, it has been assumed that the increased fluorescence intensities observed upon hybridization always reflect the formation of hybrids that remain within the polymer matrix. The possibility of a substitution reaction of labeled target molecules with the labeled hybrid can, however, not be distinguished in the above data and needs to be ruled out. To investigate this, a control experiment was performed in which a labeled surface attached probe was coupled with an unlabeled target. The SPR and SPFS data for this experiment are summarized in Figure 8.As the concentration of the labeled probe was increased in consecutive immobilization experiments, an increase of the fluorescence signal was observed indicating an increase in the absorbed probe oligomers at the plasma polymer/solution interface, Figure 8. Even though the concentration was doubled with the addition of probe at $c_p = 100$ nM, the fluorescence signal did not increase significantly. This is probably because a maximum loading density of oligomers within the 3D matrix has been reached. For this reason, the concentration of the probe was not increased further. The significantly higher sensitivity of the SPFS data in comparison to the SPR data can immediately be seen in the data and was used here merely for additional on-line monitoring of the reaction. After the surface had stabilized, the neutralization of remaining amine groups was carried out using succinic anhydride in sodium borate, as described earlier. Hybridization was carried out in two steps: (i) using labeled MM2 target to confirm that no unspecifically bound target remains on the surface after rinsing and (ii) using unlabeled MM0 target to investigate replacement reactions. The addition of labeled MM2 target to the labeled surface attached probe (at $t = 33\,000$ s) led to an increase in the SPFS signal. The complete loss of the labeled MM2 target upon rinsing with excess buffer (at $t = 34\,500$ s) confirms that no unspecific binding of the MM2 target on the plasma polymer film occurs. The addition of complementary, unlabeled target (at $t = 35\,000$ s) surprisingly led to an increase in the fluorescence intensity. Since the addition of unlabeled target does not increase the chromophore density but does raise the charge density, the observed change in the signal may imply swelling of the polyelectrolyte-like matrix, which allows previously undetectable chromophores within the plasma polymer network to become "visible". Rinsing of the surface appears to perturb the surface and also leads to an increase in the observed

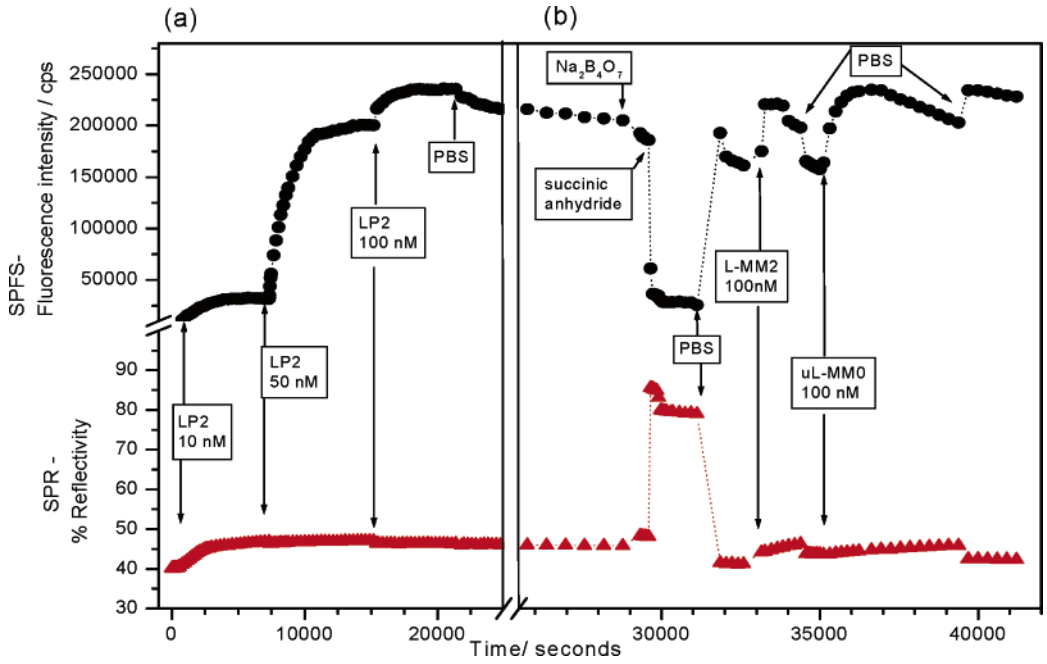


**Figure 8.** SPR and SPFS data for consecutive immobilization, neutralization, and hybridization experiments using labeled probe and unlabeled target molecules as depicted in Figure 7.

fluorescence, again suggesting there to be a very sensitive equilibrium between the density of the adsorbed oligonucleotides and the behavior of the polymer matrix in the solution.

The relatively stable fluorescence intensity observed during the hybridization reactions of the labeled probe with the target oligomers on the plasma polymer surface show that no replacement reaction take place in which either the labeled or unlabeled target replaces the probe or the hybrid after hybridization.

## Conclusions

The 3D matrix of thin plasma-polymerized films allows not only improved immobilization of single-stranded DNA but also enables the successful hybridization of the surface-attached probe with complementary target molecules. Conventional cross-linking and neutralization techniques led to covalently bound probe DNA, which could not be removed by rinsing or by competing oligomers. SPFS has shown that mismatch situations (MM1 and MM2) can easily be distinguished from the hybridization process with complementary targets (MM0). MM1 and MM0 hybridization reactions on the plasma polymer matrix could be described by a simple Langmuir model. Rate constants for the association and dissociation in the 3D polymer matrix were found to be in close agreement with previously published values for 2D biotin−streptavidin model surfaces. The combination of improved DNA loading density and similar association and dissociation rate constants in comparison to other systems suggests such plasma-deposited films to have a potential for future DNA sensing.

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