Characterization of Pulsed Plasma Polymerization Allylamine as an Adhesion Layer for DNA Adsorption/ Hybridization

Qiang Chen,*,† Renate Förch,‡ and Wolfgang Knoll‡

Institute of Plasma Physics, Chinese Academy of Science, People's Republic of China, and Max-Planck Institute for Polymer Research, Ackermannweg 10, D-55128 Mainz, Germany

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This paper presents an investigation of allylamine polymerization in pulsed radio frequency (RF) plasma as an adhesion layer immobilizing DNA probe for DNA hybridization assay. We looked for a simple and innovative way to improve the stability of pulsed plasma polymerization allylamine (PPPAA) film in phosphate-buffered saline (PBS) solution. To better understand the mechanism of PPPAA stability and the influence on DNA adsorption from films, several techniques were used. Fourier transform infrared (FTIR), atomic force microscopy (AFM), surface contact angle, and surface plasmon resonance (SPR) as well as surface plasmon enhanced fluorescence spectroscopy (SPFS) were all employed to investigate the effect of plasma conditions on the film structure, amine density, and the DNA hybridization reaction. It concludes that polymer deposited at low working pressure is very stable and can be used as adhesion layers for further study of DNA hybridization assay.

Introduction

To date, thousands of different diseases are known to be caused by genetic lesions such as single-point mutations in the human genome.[1] A sequence-specific analysis of the patient's genome is thus highly useful in the detection of pathogenic micro-organisms such as bacteria and viruses by their genetic materials and thus in the diagnosis of infectious diseases.[2] Due to the increasing amount of genetic information to be analyzed in routine medical procedures, there is a high demand for novel DNA detection schemes and efficient screening methods for point mutations in the patient's genome. Conventional gel-based sequencing methods are relatively expensive in terms of equipment and labor costs.[3,4] Novel surface DNA detection formats promise to fulfill the requirement for an optimal biosensor.[5] The commonly applied technique for DNA detection includes the interaction between an immobilized DNA probe sequence on the sensor surface and the target sequence from solution. If fluorescence is involved, a label can be attached covalently to the target sequence itself or it can be added to the sensor after the hybridization reaction.[6] Most of the standard techniques provide an immobilized probe oligonucleotide sequence; for example, a universal binding matrix of streptavidin is first tethered to the self-assembled biotinylated monolayer as an adhesion layer, and then it catches the biotinylated probe oligonucleotide acid (i.e., DNA probe) for future hybridization reaction with DNA target. Such architecture offers a strong binding and a slow dissociation rate ($K_d = 10^{-15}$ M^{-1}) system due to biotin−streptavidin specific affinity.[7,8] In addition the large streptavidin molecule increases the space between the DNA-bound fluorophore and the gold surface, and the fluorescence signal is conducted with less loss and more sensitivity. The drawbacks, however, are the use of an expensive streptavidin, biotinylated-DNA probe and a binary mixed self-assembled monolayer (SAM) as well as the more complex processing in the sample architecture.[8]

It is also known that DNA,[9] a biopolymer, exhibits a negative backbone in physiological pH due to the negative polar phosphate groups. So it can be immobilized on a polycationic surface by electrostatic force. The Coulombic interaction now is widely used in gene delivery systems. Polycations such as poly-L-lysine, poly(allylamine), poly(ethlyenimine), and dendrimer are used as vectors to condense and transfer DNA chips in vivo and in vitro.[10−12] One common approach is based on the layer-by-layer assembly (LbL) method using electrostatic attraction between oppositely charged poly-

* To whom correspondence should be addressed.
† Chinese Academy of Science.
‡ Max-Planck Institute for Polymer Research.

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electrolytes, permitting sequential deposition of these species from diluted solution onto the surface.[13]

Using the highly sensitive surface plasmon enhanced fluorescence spectroscopy (SPFS) technique, now we extend this scheme and develop a novel DNA hybridization biosensor, in which plasma deposits the adhesion layer. The polymer surface is positively charged after the protonation of amine groups in aqueous solution, which attracts the negatively charged DNA probe as an alternative to the biotin−streptavidin−biotinylated DNA probe system.[14] The kinetic reaction of the electrostatically immobilized DNA probe and the hybridization reaction between the DNA probe and the dyed DNA target can be precisely monitored by SPFS just as in a system based on a biotin−streptavidin adhesion layer. One of the basic advantages of an electrostatic adhesion layer is that the substrate can be simply prepared by a normal SAM and DNA probe, rather than the costly biotinylated SAM, macromolecular streptavidin, and biotinylated-DNA probe. Moreover, it can even be reused by breaking the ionic pairs in an alkaline solution in which the pH value is above the film amino group pK_a value.[15]

The widespread use of plasma deposition composite materials is based on on conformal, pinhole-free, adhesive, high-quality films obtained in a relatively simple, all-dry, one-step deposition process. The density of the functional group, such as the amine group, can be easily controlled by plasma power, pulsed plasma duty cycle, plasma working pressure, deposition time, and so on.[16−18] Especially by a pulsed plasma, the surface density of a functional group is much higher than that deposited by conventional continuous wave (CW) plasma in the same plasma conditions because of the longer life of the radicals, the higher polymerization reaction efficiency, and the lower surface etching rate. However, as is noted in many publications,[17,19] the polymer is unstable in solution. X-ray photoelectron spectroscopy revealed the deposited film was an aggregation of the dimer, trimer, and tetramer, particularly film deposited in a low duty cycle pulsed plasma. The weak binding oligomers definitely dissociated when submersed in an aqueous solution.[20] Unstable adhesion layers certainly distorted later hybridization assay results. So the preparation of a nano-thick stable film in aqueous solution with an appropriate functional group on the surface was challenging. In this paper these problems are explored. The paper offers a novel, sensitive, reusable adhesion layer for future DNA hybridization assay.

Experimental Section

Plasma Setup. The plasma reactor and associated electronics employed in this work have been described previously.[19] Our 13.56 MHz capacitively coupled RF plasma discharge apparatus is home-built. The reactor consists of a 300-mm length, 100-mm diameter Pyrex tube with two outside electrodes, which are separated ca. 12 cm from each other. The substrate is located at the midpoint of the electrodes in the chamber. The plasma duty cycle is modulated by a pulsed generator. The plasma on time t_{on} is fixed at 10 ms while plasma off time t_{off} varies from 10, to 20, to 320 ms. As a comparison, we also deposited polymers in CW plasma. The working pressures 0.06 and 0.12 mbar are referred to as the low and high pressures and as candidates to deposit different amine densities on the surface in this experiment.

Sample Preparation. The substrates for SPFS measurement are prepared as in ref 21 except for the adhesion layer. A film of 47-nm Au is vacuum-evaporated on the high refractive index glass slide LaSFN9, and then a SAM forms on this gold surface from 10 mM octadecanethiol in dicholoromethane solution to improve the pulsed plasma polymerization allylamine (PPPAA)-gold adhesive. These substrates are used in subsequent plasma polymerization adhesion layers. After deposition the substrates are immediately mounted on the SPR setup for real-time polymer stability/DNA adsorptive measurement. The thickness of SAM, PPPAA, and DNA probe are simulated from SPR spectra, derived by Fresnel formulation assuming no variation of the refractive index.

To examine the polymer chemical structure and evaluate its stability, FTIR measurement before and after PPPAA immersion in aqueous solution is carried out on a Nicolet 850 instrument. In this case a 30−50-nm PPPAA film is deposited directly on a glass−Cr−gold surface with reflective analysis. After the first FTIR measurement, the samples are immersed in a phosphate buffered saline (PBS) solution for 24 h, then taken out and spun at 500 rps for 1 min, and then heated overnight at 50 °C in a vacuum for the second FTIR measurement.

In the same process samples are prepared for diagnosis of DNA adsorption on PPPAA surfaces by FTIR and UV−visible spectroscopy (Lambda 9) except the PBS solution is replaced by 1 μM DNA solution and the quartz glass is used as a substrate in UV−visible spectroscopy to minimize the basic noise.

For atomic force microscopy (AFM) analysis, the polymer is deposited on gilded glass to measure the surface flatness and homogeneity. However, the contact angle measurement is performed on a silicon substrate by Krüss, DSA10-MK2. The dynamic contact angle is measured in an atmosphere of air at room temperature with Millipore water as solution in 6.32 μL/min increments, maximum volume 3 μL.

PPPAA Dissociation and DNA Hybridization. Kinetic dissociation of PPPAA film in aqueous solution is monitored in real time by SPR. With fitting by Fresnel formulation (assuming no change of the reflectivity index), the time-resolved spectroscopy reveals the reflectivity changes with time, corresponding to the variation of polymer thickness. The DNA probe and target sequences for hybridization research are listed as follows:

Probe sequence: 5′-Biotin-$(TTT)_5$-TGT-ACA-TCA-CAA-CTA-3′

Target sequence: 3′-ACA-TGT-AGT-GTT-GAT-Cy5-5′

These sequences have a hairpin structure considered to be very efficient at discriminating the mismatch base in biotin−streptavidin adhesion layers.[22] Dye Cy5 is terminated in the DNA target for 677-nm fluorescent emission. To avoid signal blanching, the beam is shuttled at 100- or 150-s intervals. The

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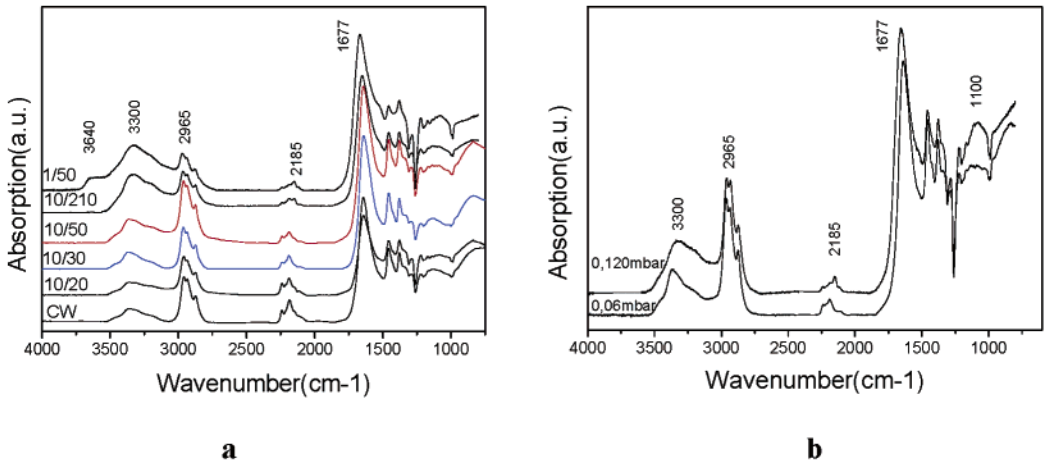

Figure 1. FTIR spectra of PPPAA prepared at (a) low working pressure but different plasma duty cycle $T_{on}/(T_{on} + T_{off})$ (P_{equ} = 20 W); (b) low and high pressures (T_{on} = 10 ms, T_{off} = 40 ms, P_{peak} = 100 W).

90-μL volume flow cell is continuously refreshed by a peristaltic pump during DNA adsorption and hybridization reaction in dynamic conditions at a speed of 3 mL/min. It is worth noting that there is an alternative method for DNA adsorption in this experiment: static adsorption. In this process, DNA solution is only injected into the flow cell with 5 times the cell volume. When DNA adsorption is at equilibrium, a 200 times cell volume PBS solution is injected to rinse the cell and remove excessive molecules.

Note that all chemicals used in this work are from Sigma-Aldrich and DNA from MWG Oligo, Germany, without further purification.

Results

The dependence of amine density on plasma conditions is shown in Figure 1. Note that the duty cycle clearly dominates the surface amine density as in the high-pressure polymerized film.[16] The amine density obviously increases with the elongated plasma off time t_{off} in Figure 1a. From CW to 1 ms/50 ms duty cycle pulsed plasma, the broad band at 3300 cm^{-1}, corresponding to the primary and secondary amine and amide, increases with elongated t_{off}. The peak at 1100 cm^{-1}, attributed to the stretching of the primary and secondary amine, also intensifies with increasing plasma off time, but due to the saturation of cross-linking, the variation from CW to10 ms/30 ms is relatively small. The decay of peak intensity at 2960 cm^{-1} for $-CH_2$ and $-CH_3$ and 2930 cm^{-1} for $-CH_x$ stretching of aliphatic groups indicates that the weak C−H bond is broken and the strong bonds C═C, C═O, C═N, and −C≡N groups in 1677 and 2185 cm^{-1} and cross-linking C−C are formed on the surface.[23,24] It is also noted that in the lowest duty cycle 1 ms/50 ms a visual peak at 3640 cm^{-1}, the independent N−H stretching, appears in the spectroscopy. Table 1 summarizes the above band assignments.

In a comparison to the high pressure ones, Figure 1b shows the PPPAA chemical structures are obviously relevant to the plasma working pressures. The peaks of the primary and secondary amine and the amide group at broad band 3300 cm^{-1} and −NH and $-NH_2$ stretching at 1100 cm^{-1} all relatively decrease at low working pressure. The strong bond −C≡N at peak 2185 cm^{-1} shifts and concentrates in the low-pressure deposited polymer.

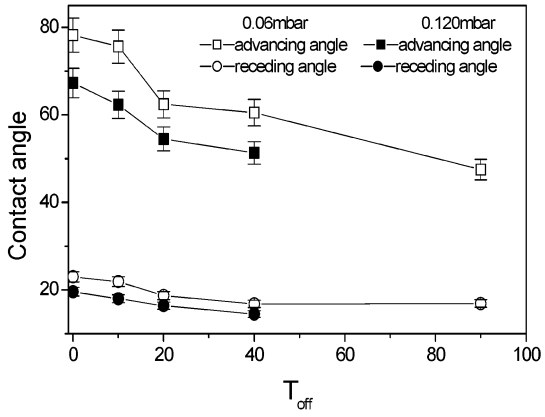

Figure 2. Dependence of contact angle (advancing and receding) on the plasma off time t_{off} and plasma working pressure (P_{equ} = 20 W, t_{on} = 10 ms).

Table 1. Spectral Mode Assignment for PPPAA

absorbance (cm^{-1})	mode assignment
1100	C−N primary amine stretching
1675	C═C alkene
	NH_2
	NH primary and secondary amide
	C═N imine stretching
2185	C≡N nitrile stretching
2875	CH_3 mathyl symmetric stretching
2930	CH_2 mathylene antisymmetric stretching
2960	CH_3 mathyl antisymmetric stretching
3300	NH_2 symmetric and antisymmetric stretching
3640	N−H
	O−H
	hydrogen bond

At low pressure, it is known,[25] aside from ultraviolet (UV) interaction,[26] the electrons, ions, and radicals have long mean free paths and bombard the surface with high momentum, which fracture surface chemical chains and cross-link the surface with dehydrogenation. As a result, the PPPAA deposited at low pressure showed somewhat hydrophobicity. In Figure 2 the advancing and receding

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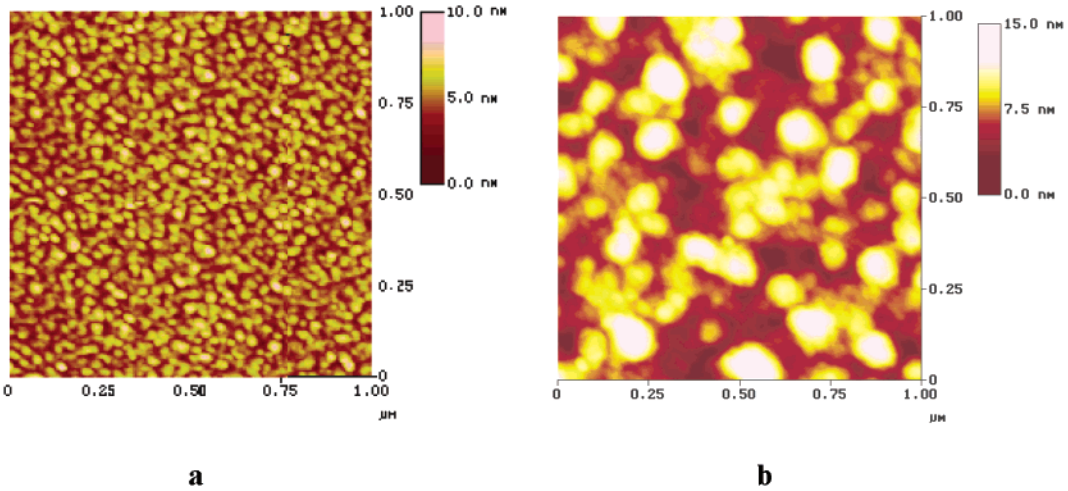

Figure 3. AFM images of PPPAA prepared in different plasma modes: (a) 10 ms/50 ms, 30 s, 0.06 mabr pulsed plasma; (b) CW plasma, 30 s, 0.06 mbar.

angles both increase in such films, about 5° increments for advancing angles.

The result of high-energy radicals' bombardment and etching was identified in AFM images. The mean roughness of each film is determined quantitatively as measured by surface vertical height variation relative to the geometric center plane of the AFM image. Figure 3 reveals that the polymer film completely covered the substrate surface after 30-s deposition time in low-pressure pulsed plasma or CW plasma. But the influence of the plasma mode on the morphology is significant; that is, the surface roughness decreased in the pulsed plasma. Due to the absence of bombardment during the majority of the film formation process, the etching on the surface was small in pulsed plasma. The visual peaks and valleys on the surface of CW plasma polymerization film suggest the radicals heavily bombarded and etched the surface during the deposition process.

Besides the surface morphology affected by plasma modes, the polymer dissociation rate (i.e., thickness variation) in aqueous solution is also relevant to the plasma mode and plasma deposition pressure. In Figure 4 the polymer deposited in CW plasma is remarkably stable. Low pressure causes a surface cross-linking structure; the polymer dissociates significantly more slowly than at high working pressure.

A real-time monitoring of PPPAA stability is shown in Figure 5. It indicates PPPAA deposited at high pressure is continuously dissociated during the entire period of immersion: the reflectivity is decreasing from the beginning. The decay curve can be fitted by a second exponential attenuate as shown in Figure 5a. In contrast, PPPAA deposited at low pressure shows a different result. The polymer is quite stable with only a negligible decrease of reflectivity in SPR spectroscopy. The sensitive scanning spectra in Figure 5b, before and after PPPAA was immersed in PBS solution over 12 h, show that when the polymer is deposited at high pressure, the dip angle shifts are larger to the left side (ca. 0.2°) than they are at low pressure (ca. 0.04°).

It seem one can conclude the stable PPPAA can be prepared at low pressure by the SPR kinetic spectra that the film thickness, calculated from the reflectivity by

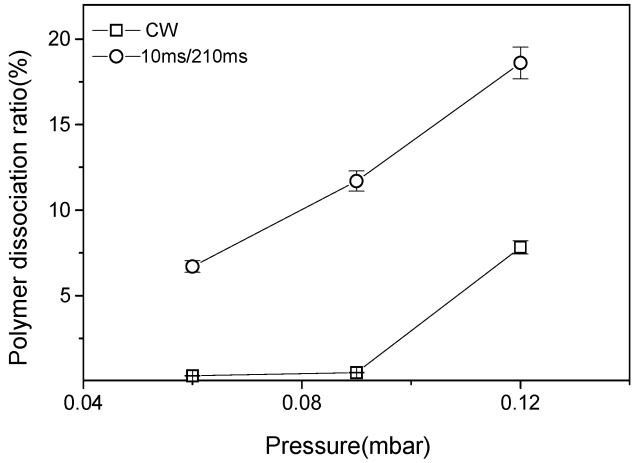

Figure 4. Dependence of the film dissociation rate in PBS solution on the plasma working pressure and plasma model (200-W peak power).

Fresnel formulation, has undergone negligible change after immersion in PBS solution, but the effects on the film's chemical nature are not yet clear. By FTIR spectra Figure 6a seems to indicate that the chemical structure also varies negligibly if the polymer is prepared at low pressure, 10 ms/50 ms duty cycle, and 20-W average power. The relatively slightly weakened intensity at 1677 cm^{-1} for amide I and 2185 cm^{-1} for nitrile stretching vibration may be attributed to the monomer or oligomer leaving the surface after the sample was soaped. The polymeric association of the hydrogen bond between the amine groups and hydroxyl groups from PBS solution causes remarkable increasing, widening, and shifting at broad band 3300 cm^{-1} (about 30 cm^{-1} to the right) as well as a peak increasing at 1100 cm^{-1}.[27]

In a lower duty cycle 1 ms/50 ms, as a comparison Figure 6b indicates that the PPPAA, evenly deposited at low pressure, not only lost more components from its surface but also changed the chemical structure after immersion: all main peak intensities decrease; in particular, the peak at 2185 cm^{-1} nearly disappears. The widening and concentrating peaks at 1300−1500

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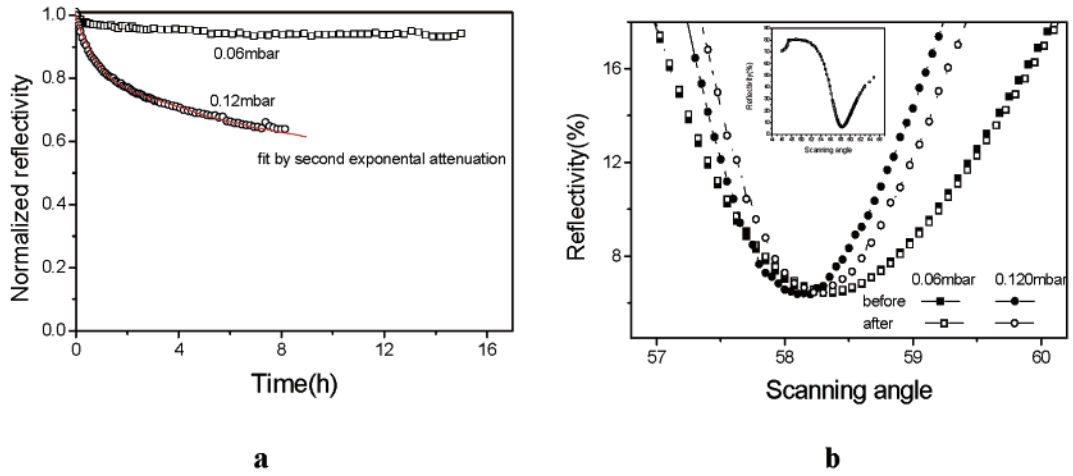

Figure 5. (a) SPR kinetic spectroscopy of PPPAA deposited at low and high pressures; (b) SPR scanning spectroscopy of PPPAA deposited at low and high pressures before and after immersion in PBS for 12 h (P_{equ} = 20 W, 10 ms/50 ms duty cycle).

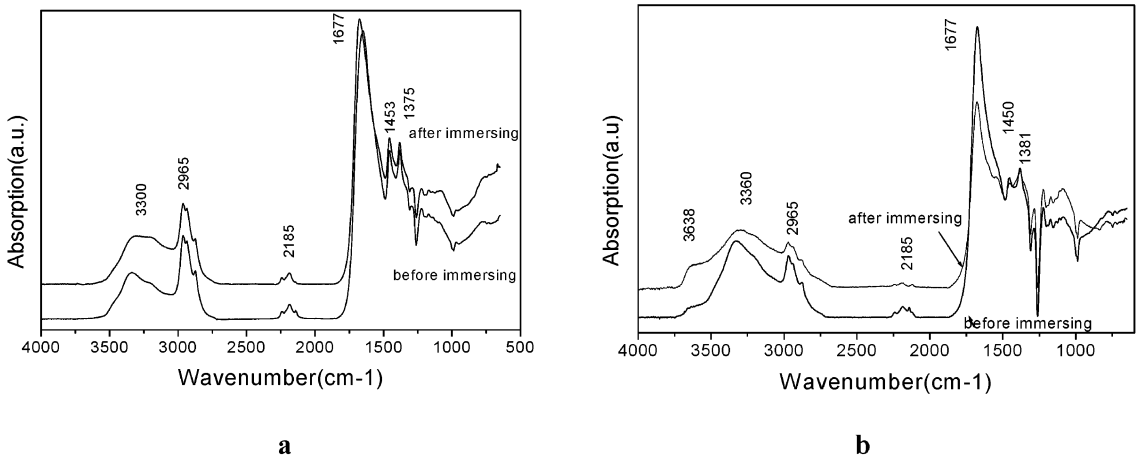

Figure 6. FTIR spectra of PPPAA deposited at 20-W average power, low pressure before and after immersion in PBS solution for 24 h: (a) 10 ms/50 ms; (b) 1 ms/50 ms.

cm^{-1} may indicate the polymer swelling from CH and CH_2 bending. A density peak at 3640 cm^{-1} from N−H and O−H stretching in the spectroscopy confirms the assumption of the polymer swelling in the aqueous solution.

By SPR the DNA probe adsorption has also been time-resolved measured. Figure 7a indicates that the interaction of the positive−negative ionic pairs between PPPAA and DNA probe is very rapid either in low- or high-pressure deposited films (PPPAA in similar thickness). After ca. 20 min the DNA adsorption reaches equilibrium in a PBS solution.

The DNA adsorption is supported by FTIR spectra. Figure 7b shows that the intensities at broad band 3360 cm^{-1}, in particular, at 1141, 1073, 986, and 864 cm^{-1}, significantly increase after DNA attached on the surface. The broad band peak at 3360 cm^{-1}, corresponding to the primary and secondary amino bending, 1140 and 1073 cm^{-1} assignment to $R_3P{=}O-$ and $-PO_4$ stretching, 986 and 861 cm^{-1} assignment to the combination of C═O, C═C, and P-amine stretching, all contribute to the DNA backbone structure.

The UV−visible spectroscopy in Figure 7c also demonstrates the DNA immobilization on the PPPAA surface. The peak intensity at 262 nm assigned to the amino acid group from the DNA structure is obviously higher in PPPAA deposited at high pressure than at low pressure, which means more amine density will immobilize thicker DNA on the surface.

Besides the influence of amine density on DNA adsorption, Figure 8 indicates that the PPPAA thickness also dominates DNA adsorption, that is, the thicker the PPPAA film, the thicker the DNA attached on the surface. The exact reason is unknown but these phenomena were also found in DNA delivery systems: the thicker the polycationic P−L−L, the more DNA was adsorbed on the surface.[28]

After immobilization of DNA probe on the PPPAA surface, the DNA hybridization reaction based on the PPPAA adhesion layer is evaluated. For this purpose the substrates are prepared with different amine densities achieved through changing the plasma working pressure, for example, 0.06 and 0.12 mbar, and different polymer thicknesses through deposition times, 30 and 60 s (10.5- and 23-nm optical thickness).

Figure 9a shows that the plasma pressure, that is, amine density, has an influence on the DNA hybridization reaction. The fluorescence intensity is visually high in a 0.12 mbar working pressure deposited polymer.

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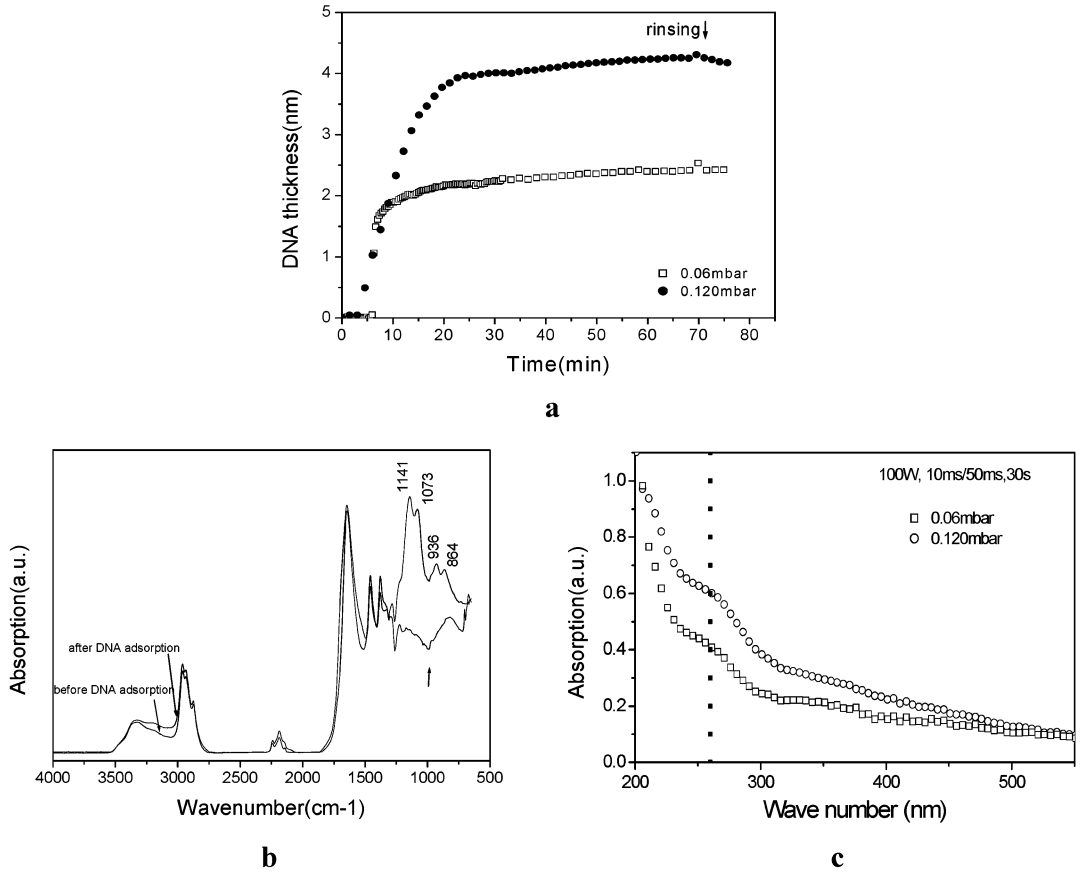

Figure 7. (a) SPR kinetic spectra of DNA adsorption on PPPAA surfaces deposited at low and high pressures; (b) FTIR spectra of DNA adsorption on PPPAA surfaces; (c) UV−visible spectra of DNA adsorption on PPPAA surfaces at low and high pressures.

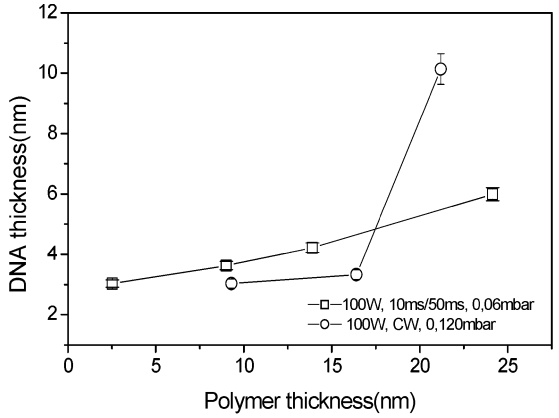

Figure 8. Relationship of the DNA thickness with the PPPAA thickness and plasma mode (in static adsorption).

Data in Table 2 from a simple Langmuir model simulation show, however, the dissociation rate K_{off} (or K_d) and affinity constant K in the two kinds of films are of the same order, without a large variation.

The influence of polymer thickness on the DNA hybridization reaction is shown in Figure 9b. The plasma deposition times were 30 and 60 s, corresponding to the polymer optical thicknesses 10.5 and 23 nm, and adsorbed DNA thicknesses 2.5 and 4.3 nm, respectively, in dynamic adsorption conditions. It indicates clearly that the fluorescence intensity is significantly improved in thick polymers. But the association rate K_{on} and dissociation rate K_{off} as well as the affinity constant K are also analogous in these films as Table 2 shows.

Discussion

A positive charge on the PPPAA surface is essential for DNA adsorption and then for hybridization reaction. But the instability of the plasma deposition polymer in solution, for example, Millipore water, PBS, or PB solution, makes it unusable for a sensitive SPR sensor. There are many methods to increase PPPAA stability,[19] but recent results seem to conclude that low-pressure deposition is very simple and suitable. Polymers deposited at low working pressure show a very stable behavior in aqueous solution possibly because the electron, ion, and radical mean free paths are extended and their momentum are increased, which significantly break the chemical chain and form stable cross-linking structures in the surface. FTIR spectra prove an increasing concentration of the strong bonds −C≡N and C═C and decreasing of the weak bonds $-CH_3$ and $-CH_2$ in the film structure. The contact angle measurement indicates the advancing and receding angles increase in a low-pressure deposited film, about a 5° increment in the advancing angle. This is attributed to the reduction of the hydrophilic groups on the surface and the formation of a hydrophobic cross-linking surface.[26] By SPR scanning spectra this cross-linking film shows a negligible dip angle shift in PBS solution, that is, negligible variation of the polymer thickness.

The optimal density of positive charge on PPPAA to immobilize the DNA probe and demonstrate the DNA hybridization reaction is not clear, but by SPFS and compared with PPPAA deposited at high pressure it is found that PPPAA deposited at low working pressure (0.06 mbar) can provide a remarkable fluorescence

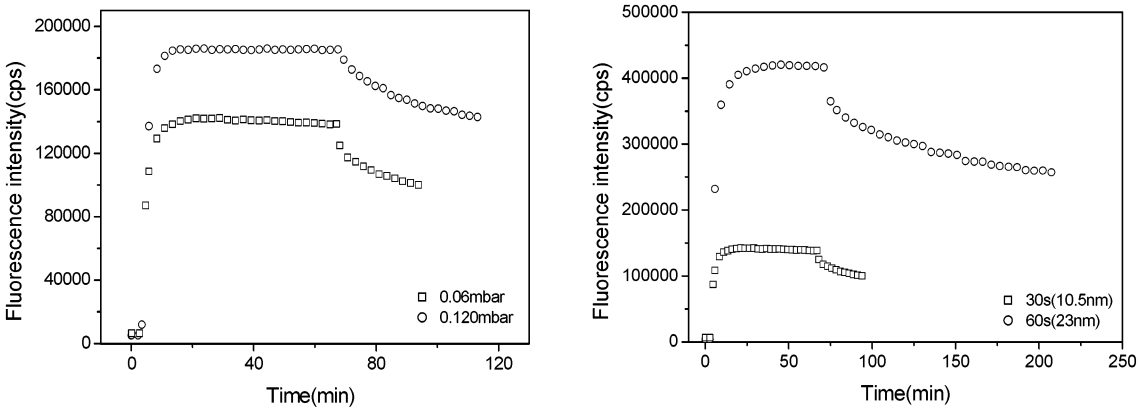

Figure 9. Dependence of kinetic fluorescence spectroscopy of DNA hybridization reaction on (a) working pressure and (b) PPPAA thickness at 100-W peak power, 10 ms/50ms duty cycle.

Table 2. Derivation of Association Rate, Dissociation Rate, and Affinity Constant from Figure 9 by Simple Langmuir Model[14]

hybridization parameters	deposition time (s)		deposition pressure (mbar)	
	30	60	0.06	0.12
K_{on} (M^{-1} s^{-1})	7.3×10^4	4×10^4	7.3×10^4	5×10^4
K_{off} (s^{-1})	6.7×10^{-5}	4.3×10^{-5}	6.7×10^{-5}	4×10^{-5}
K (M^{-1})	1.1×10^9	9.4×10^8	1.1×10^9	1.25×10^9

signal to demonstrate the hybridization reactions. The films deposited at low and high pressures, that is, the different amine densities on the surface, do not show any remarkable influence on the efficiency of the DNA hybridization reaction. The dissociation rate and affinity constant are of the same order for both kinds of samples. An exception is the fluorescence intensity, which correlates with the space between the metal and the fluorophore.[21] These results hint that the efficiency of DNA hybridization reaction based on the electrostatic adsorptive mechanism may not be sensitive to the amino density on the surface; that is, more positive charges on the surface may be not necessary.

Due to the quenching of the fluorescence signal when the fluorophore is near the metal, the intensity is sensitive to the adhesion layer thickness. The fluorescence intensity is significantly improved in thick polymers compared with that in thin ones. But the influence of polymer thickness on the hybridization reaction efficiency based on the data is not significant; more than double thickness of PPPAA film deposited at 60-s pulsed plasma shows the association rate, dissociation rate, and affinity constant are on the same order as the thin one deposited at 30 s, which means that the adsorbed DNA thickness on the low-pressure, 30-s deposited film is enough to demonstrate the DNA hybridization reaction. The negligible influence of polymer thickness on the hybridization reaction efficiency is an interesting subject for future study. Maybe it is the correlation with the electrostatic adsorptive mechanism; in ionic pairs interaction the DNA strand is lying on the surface.

Conclusion

For further DNA hybridization assay investigation, the stability of adhesion layer PPPAA film and the immobilization of DNA probe on the surface are studied. It is found that the PPPAA prepared at the same plasma conditions but at low pressure is extremely stable in PBS solution. FTIR, AFM, and contact angle measurement suggest the formation of a cross-linking structure at low pressure is responsible for the film stability. The DNA probe is very rapidly immobilized on the surface monitored by FTIR, UV−visible, and SPR spectroscopy. The SPFR spectra of a DNA hybridization reaction based on a complementary sequence indicate that the PPPAA prepared at low pressure provides enough functional groups to adsorb the DNA probe and significantly demonstrates the hybridization reaction. It shows that a stable PPPAA film prepared at 0.06-mbar pressure, 20-W average power, and 30-s deposition time can be used as an adhesion layer for further DNA hybridization assay research.

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