Journal of Macromolecular Science, Part A
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597274

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Online publication date: 30 November 2001

To cite this Article Minehan, Daniel S. , Marx, Kenneth A. and Tripathy, Sukant K.(2001) 'DNA BINDING TO ELECTROPOLYMERIZED POLYPYRROLE: THE DEPENDENCE ON FILM CHARACTERISTICS', Journal of Macromolecular Science, Part A, 38: 12, 1245 — 1258
To link to this Article: DOI: 10.1081/MA-100108381
URL: http://dx.doi.org/10.1081/MA-100108381

J. MACROMOL. SCI.—PURE APPL. CHEM., A38(12), 1245–1258 (2001)

DNA BINDING TO ELECTROPOLYMERIZED POLYPYRROLE: THE DEPENDENCE ON FILM CHARACTERISTICS

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This manuscript is dedicated to the memory of our colleague,
Professor Sukant K. Tripathy.

ABSTRACT

Evidence is presented that suggests the binding of DNA to electropolymerized polypyrrole (PPy) is directly related to the presence of positive charge carriers in PPy. The adsorption rate of short radiolabelled ØX174 DNA fragments (average size 300 bp) onto thin films of electroxidized PPy doped with p-toluene sulfonate was found to vary as a function of: film storage conditions, film age, electrochemical reduction, but not synthesis current density. DNA bound more readily to films stored under dry conditions. DNA binding varied only slightly with the current density used during PPy electropolymerization. The DNA binding levels were significantly reduced with increasing PPy film age. This observation correlated qualitatively, but not quantitatively, with the known loss of PPy conductivity and charge defects with aging. DNA adsorption levels fell off far more rapidly with film age than does conductivity. Oxidized PPy films bound DNA at a higher rate than films which had been reduced subsequent to electroxidative synthesis, thereby removing a large fraction of the surface positive charge defects. These results support the idea

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that DNA binding is brought about by the presence of positive charge defect carriers in the PPy film.

Key Words: Polypyrrole; DNA binding; Electropolymerization; Thin films; Charge defects; Conductivity

INTRODUCTION

We have previously demonstrated that the binding kinetics of DNA to PPy is diffusion limited [1-4), a property shared by other biopolymers binding on two-dimensional surfaces [5]. In those studies we demonstrated that DNA binds to PPy with [DNA] and $t^{1/2}$ dependent kinetics and a low total activation energy suggesting that there was little change necessary in the DNA conformation or PPy surface structure in the process of binding to the PPy surface. In addition, it was observed that DNA binding to PPy is little affected by ionic strength and shows a maximum in binding on varying the pH. The latter two results are consistent with the electrostatic adsorption characteristics of polyelectrolytes to an oppositely charged surface, where the polyelectrolyte possesses a specific solution conformation and array of functional groups [6].

Ionic interactions of DNA involving the negatively charged phosphate groups of the double helical backbone, and hydrogen bonding can occur both to the backbone phosphate oxygens and within the grooves of the DNA helix, where ligands can access both hydrogen bond donor and acceptor groups on the nucleotide bases [7-10]. Oxidized PPy monomer units contain a potentially hydrogen bonding nitrogen atom and the backbone contains positively charged defect structures, neutralized by negatively charged dopant molecules [11-13]. Therefore, PPy provides a unique surface for DNA binding. Due to its delocalized electronic structure, the positively charged defect structures of PPy are mobile along the chain axis. This mobility should allow for more flexibility towards the binding of DNA's fixed negative charge sites and subsequently higher affinities than for a surface of fixed positive charges. The rationale is strengthened by the fact that the positively charged PPy can exchange its negatively charged dopant easily with other negatively charged species, including biomolecules [14, 15]. We have demonstrated previously the electrostatic and reversible nature of DNA binding to electropolymerized PPy [2-4]. More recently, we have shown that the kinetics of a DNA adsorption process is consistent with a model of DNA penetration into interior channels, and with migration through the electropolymerized PPy matrix [6]. Full length DNA molecules were also clearly visualized on the PPY surface via TEM.

In the present study, we demonstrate the effect of the following electropolymerized PPy film properties on DNA binding: film pretreatment, film age, electrochemical oxidation level and the current density used during electrochemical

synthesis. The data all point to the critical role of the positive charged defects, responsible for electronic conduction of the films, in the largely electrostatic binding of DNA.

MATERIALS AND METHODS

PPy Film Preparation

The electrochemical syntheses of PPy films were performed in the absence of mechanical agitation in a two compartment cell designed with a graphite rod cathode, an anode compartment that held a smooth Pt plate (front side area of 32.5 cm^2) and a saturated calomel electrode, (SCE) as the reference electrode. The power supply was a Princeton Applied Research Model 231 potentiostat-galvanostat. The reaction vessel contained 200 mL of a 0.2 M solution of distilled pyrrole, (99%, Aldrich Chemical Co., Inc.), holding 0.2 M tetraethylammonium p-toluenesulfonate, (Aldrich) electrolyte in acetonitrile solvent, (Fisher Scientific, Optima grade), with 2% distilled water. Unless otherwise noted, chemicals were reagent grade. Pyrrole was distilled to a colorless purified form within 30 minutes prior to electropolymerization.

Except in Table 1 and Figure 1 experiments, the current density was held constant at 1.0 mA/cm^2 which stabilized at a voltage of about 0.75 volts vs. SCE. After 5 hours of reaction time, a film of approximately 50 m had grown. The film was first rinsed, then soaked for one hour in pure acetonitrile, then peeled off with a razor blade and tweezers. The free standing film was then soaked in 50 mL of pure acetonitrile for about 24 hours. Samples were allowed to dry on standing in air, cut into 0.28 cm^2 circular polypyrrole discs, placed on weighing paper and routinely stored in polystyrene petri dishes in the dark, except for those experiments where varying storage conditions were tested. The PPy disk dry densities were determined for individual disks by measuring the average thickness of individual disks at multiple points over their surface with a micrometer. The disk volumes were then calculated using the measured disk radius and finally disks were weighed, followed by calculation of the dry density.

Table 1. Electrooxidation Film Formation Conditions for Comparing the Effect of Current Density (C.D.) on DNA Binding

C.D. (mA/cm^2)	I (mA)	t (hours)	ixt (mAhours)	Voltage (V)	Density (g/cm3)	Rel. Slope
0.15	4.9	20	97.6	0.68	1.5	1.25
0.75	24.4	4	97.6	0.76	1.8	1.00
1.50	48.8	2	97.6	0.78	2.1	1.31

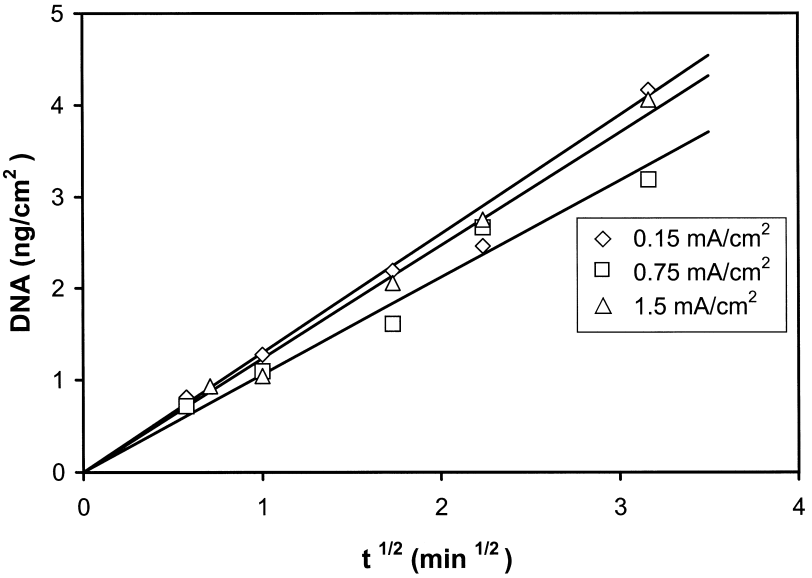

Figure 1. Comparison of DNA binding to PPy films electropolymerized at the different current densities shown.

DNA Radiolabelling and Binding to Polypyrrole

The ^{32}P end labeling of 1 g of Hha I digested ØX174 DNA was performed using a 3' end labeling kit (Du Pont NEN). The double stranded ØX174 DNA cleaved by HhaI contains 18 double stranded nucleotide fragments with an average length of 298.6 base pairs, or 2×10^5 g/mol. The radiolabeled DNA for each labeling reaction was dissolved in 500 L of a 1X TE buffer (1 mM EDTA and 10 mM Tris, at pH 8.0). The radiolabeled DNA was stored at 4°C in a silanated polypropylene eppendorf centrifuge tube. Sample droplets containing 0.2 g/mL of ^{32}P labeled DNA in 1X TE buffer were placed in droplet form on a clean polypropylene surface. The droplet was further placed within a covered petri dish such that a 1X TE buffer pool surrounded the polypropylene in order to prevent droplet evaporation. The disc shaped PPy substrate was then set upon the DNA solution droplet with the rough side, the electrochemical growth face of the disk, downward on the droplet for varying amounts of time at 23°C. The substrate was subsequently washed in 1X TE buffer for 20 minutes.

ß-rays emitted from the ^{32}P labeled DNA were detected using a high voltage gas flow proportional counter. Radiation was detected in the ß-ray plateau region at 1,980 volts using a Tennelec high voltage power supply. The anodic detector chamber was purged with a constant airflow of 60 cm^3/ min with an argon gas mixture containing 10% methane. The detector had approximately a 40% efficiency. Samples were counted over 10 minute periods as a standard. All of the raw

sample counts were adjusted for background counts (40 cpm) and detector efficiency, then normalized to the same time with the 14.3 day ^{32}P half-life and converted to ng of DNA per unit surface area.

RESULTS AND DISCUSSION

Effect of Current Density of Film Formation on DNA Binding

The conductivity of PPy films can be affected by the current density or, by default, the voltage at which the synthesis was performed [16-21]. Therefore, such differences in the film synthesis conditions affect availability of positively charged defect sites. Also, along with the conductivity, differences in other film properties have been observed as a function of current density, including differences in chemical functionalities or orientations, surface morphology and film strength [16-28]. A noteworthy observation in p-toluenesulfonate doped PPy films is that synthesis conditions can impart property differences due to the orientation of the dopant [22-28]. For these reasons, we varied the synthesis current density to study its effect on DNA binding.

In experiments where the effect of the current density was studied, the oxidation potential was changed slightly for each experiment in order to maintain an equivalent amount of charge passed (ixt)for the growth of each film as is shown in Table 1. Current densities of 0.15, 0.75 and 1.5 mA/cm^2 were used to grow films varying in thickness from 25 to 30 m. Although the current density was varied greatly during the synthesis, the DNA binding to these films was affected only marginally, as is evident from the DNA binding data in Figure 1. In all cases, a $t^{1/2}$ dependence was observed. This may be attributed to the fact that although the current ranged from 4.9 to 48.8 mA/cm^2, the oxidation potential used to produce each of these films only differed by about 0.1 volts and the final electroxidation levels (ixt) were the same. In Table 1, we compare the film synthesis conditions to the relative DNA binding slopes taken from Figure 1. This comparison reveals little difference in binding rate for disks prepared at the three current densities.

All of the reports involving film conductivity as a function of the synthesis current density or voltage show that a maximum conductivity is produced at medium values [17-20]. There is a question as to which current density or voltage produces the highest conductivity, as this quantity may depend on several factors, such as the solvent, electrode or dopant. However, in most cases, the region of maximum conductivity has ranged from 0.4 to 1.0 mA/cm^2 and 0.5 to 0.6 volts vs. the SCE. Overall, it is difficult to compare the data presented here to the literature data since in most of the cases no relationship between the current density and voltage for their experimental setup is given. Given the lack of a significant current density dependence to the DNA binding kinetics, in all subsequent experiments we utilized the 1.0 mA/cm^2 current density condition to form PPy films, as is discussed in the Methods section.

An overview of some of the film properties shows the thicknesses of the three films to be very similar (all within 25-30 m). However, the dry film densities increased sequentially from 1.5 to 1.8 to 2.1 g/cm^3 with increasing current density, resembling the trend seen by Satoh, *et al*. [20]. It was observed that the film grown at 0.75 mA/cm^2 was mechanically more flexible, whereas the other two films were much more brittle. These two observations suggest that some variation in the chemical structure or orientation/packing of the PPy film may exist.

At some oxidation potentials, different chemical functionalities are produced depending on the solvent system that is used. For example, electropolymerizations of pyrrole at 0.125 mA/cm^2 produce deprotonated nitrogens, whereas syntheses at 1.5 mA/cm^2 formed hydroxyl groups on the nitrogen [16]. Also, overoxidation, which results from holding PPy films electrochemically at >0.7 V for extended periods of time, allows the formation of oxygen functionalities, thought to exist in hydrophilic channels interspersed with hydrophobic bulk phase PPy [29, 30]. We have presented a model for DNA binding and internal migration through PPy channels that is in basic agreement with this overoxidation concept and that is in agreement with the results presented here [6]. Since our oxidation potential only varied by 0.1 volts around 0.7 V, it can be argued that while overoxidation occurred, variations in chemical structure between the three films prepared at different current densities are not likely. In the current study, we show a time dependent increasing level of DNA binding to the rough face of the PPy disk, an observation quantitatively similar to what we observed in the previous reference [4, 6]. In that study, we also demonstrated that DNA uptake on the rough surface was nearly twice the level observed on the smooth electrode facing surface of the PPy disk.

Film Pretreatment/Storage Conditions Effect on DNA Binding

It has previously been reported that PPy films, stored over three months time, could absorb up to 5% water by weight from air [31]. Also, both water and oxygen are known to affect film conductivity with time [32]. Since we previously demonstrated a correlation between PPy film conductivity and DNA binding, due presumably to the presence of charged defect structures in the electroxidized films, we decided to investigate this further by performing experiments on DNA binding levels to PPy films stored under different environments.

For these pretreatment studies, PPy substrates were exposed to one of five environmental conditions during the 15 hour period prior to DNA uptake and following the standard pretreatment of all electropolymerized PPy substrates which includes rinsing in acetonitrile and air drying. After the 15 hour environmental exposure, all of these samples were washed in 1X TE buffer for 20 minutes. The five conditions, listed in Table 2, varied PPy substrate exposure to water, oxygen, light and buffer ions. It is clear that the DNA adsorbed under the conditions of dessication in a vacuum oven at 30 mm Hg and 30°C, is relatively the same as

Table 2. Dependence of DNA Uptake by PPy on Different PPy Storage Conditions

Storage Condition (15 hr)	Avg. DNA uptake[a] (ng)/10 min	S.D.[a]
Vacuum oven (30 mm Hg) With dessicant at 30 °C	2.8	0.3
Standard treatment; Petri dish in dark	2.9	0.3
Open to air and light, With dust cover	2.6	0.1
Soaked in distilled water	1.4	0.5
Soaked in 1X TE buffer	0.5	0.1

[a]Average and standard deviation (S.D.) of eight samples at each condition.

under our standard storage conditions, and marginally greater than the DNA uptake for the samples that were left open to air. DNA adsorption at either of these three pretreatment conditions was about twice the amount adsorbed by samples that were pretreated by soaking in distilled water, and 5 to 6 times the amount adsorbed when soaked in 1X TE buffer.

The minor difference observed in the binding between those samples dried in the vacuum oven and those stored in a covered petri dish in the dark suggests that the presence of oxygen or minor amounts of water absorbed from the atmosphere does not significantly affect the DNA binding ability of PPy films. This observation may parallel the finding that the initial PPy conductivity was not affected by absorbed CH_3CN or H_2O [31].

The samples that were stored in distilled water and 1X TE buffer were able to bind significantly less DNA during the 10 minute period. This behavior suggests that the presence of bulk water impedes the DNA absorption process and is consistent with our previous observation that electropolymerized PPy adsorbs DNA into internal channels, allowing migration through the polymer matrix [6]. In the case of the 1X TE buffer storage, the solute anions may occupy potential DNA binding sites and thereby decrease the level of DNA binding due to the necessity of a slow ligand exchange process, a process we have previously observed to be slow experimentally [4]. For this reason, the observed uptake level for 1X TE buffer storage is lower than that of distilled water storage. Based upon the results of the storage experiment described above, we chose dry storage in covered petri dishes in the dark at room temperature as our standard storage condition.

Film Age Effect on DNA Binding

In order to investigate the effect of film aging on DNA binding, all disks were stored in the standard manner-covered polystyrene petri dishes in the dark. These samples varied in age from 2 to 136 days, measured from their date of syn-

thesis. Each sample was exposed to radiolabeled DNA droplets for 10 minutes and washed in 1X TE buffer for 20 minutes. Figure 2 plots the decrease in the ability of the PPy films to bind DNA upon aging. These results clearly demonstrate that the DNA bound more readily to the most freshly prepared films. We were able to fit the time dependent loss of DNA binding with a logarithmic function having a resonable goodness of fit. Taken together with our previous investigations these data suggest that the time dependent loss of DNA binding is due to the loss of positive charged defects in the film.

There is a decay in the conductivity of PPy with time [17, 31, 32]. The loss of conductivity has two decay components. Moss, *et al*. [32], have shown that an initial increase up to 112% of the original conductivity during the first 15 days is followed by a logarithmic decay which becomes slower with time. In the above reference, it was suggested that the presence of absorbed water slows down the degradation of conductivity at room temperature, whereas atmospheric oxygen can decrease the conductivity by removing charged groups in the polymer, in agreement with our Table 2 film storage data. We decided to compare the loss of DNA binding to the decrease in relative conductivity of PPy with time from the results of Moss, *et al.* [32]. This comparison is presented in Figure 3. For this comparison, the DNA binding data have been normalized and are taken as values from the logarithmic fit in Figure 2. In developing the relative conductivity data in Figure 3, the data points at 15 days and less (conductivity increasing) were removed since these values may have been largely affected by the initial uptake of water from the air in their study. To compare the trend with the DNA binding data,

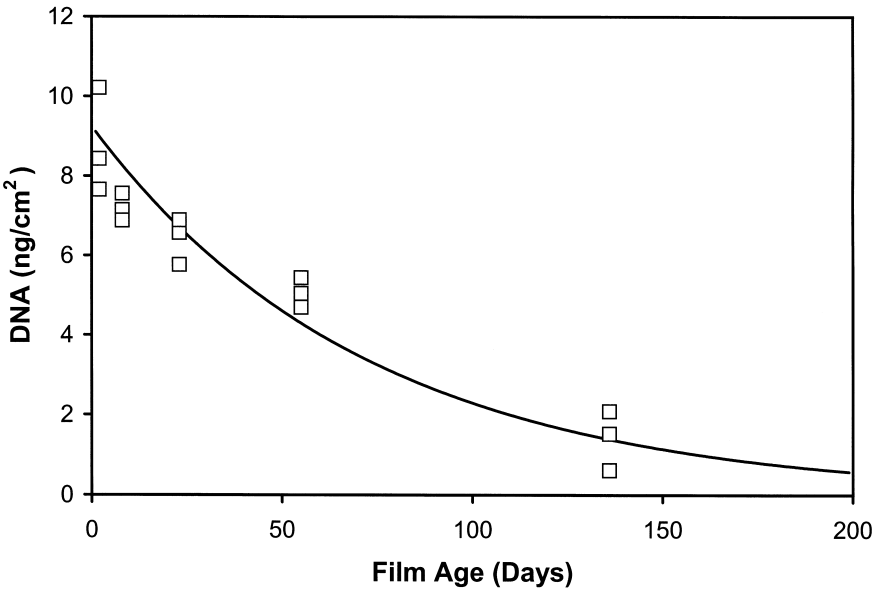

Figure 2. The dependence of DNA binding to PPy films on film age. The data were fit to the exponential curve: $y = 9.24259(10^{-0.006062x})$ shown.

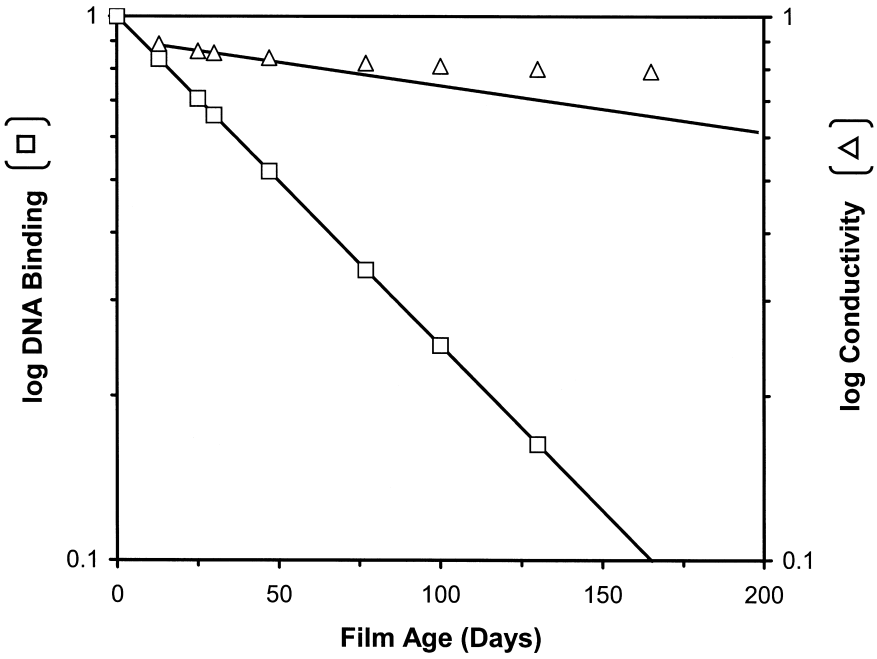

Figure 3. The log DNA binding (expressed in ng/cm^2: the fit function from Figure 2) and log conductivity (expressed in S/cm: data from Moss, *et al.* [34]) of PPy films as a function of film age, for PPy/p-toluene sulfonate films stored in the dark at 23°C. The time zero point for the conductivity data is not displayed on the graph since it lies directly atop the DNA binding point.

the remaining data points were normalized to the 15 day (day 0) point value. Our DNA binding data points were not shifted by 15 days on the premise that the experimental design places the PPy samples into water saturation as they are introduced to the DNA droplet. This is substantiated by the fact that the DNA binding results do not parallel the expected initial 15 day transient increase in conductivity discussed above.

The logarithmic decrease in the conductivity levels with time are qualitatively similar to the measured lowering of DNA binding of PPy films upon aging, although it is a far slower process. In fact, there appears to be more than a single logarithmic decay component for conductivity. This is evident from the best log fit line shown in Figure 3, computed from the first four low time conductivity points. This fit line clearly lies below the longer time conductivity values, demonstrating the presence of more than a single decay component, in agreement with the assertion by Moss, *et al.* [32] about their time dependent loss of conductivity. The loss of DNA binding observed here, however, behaves as the single logarithmic decay shown. This film aging pattern provides evidence suggesting that the driving force for DNA binding is due to the same features that cause PPy to be a conducting polymer. It has been shown that PPy film conductivity correlates with the presence of polaron or bipolaron positive charge defects [11, 33]. The decrease in

DNA binding is much more rapid than the decrease in conductivity of the PPy/p-TS film. Calculated half-times for the two processes are 49.7 days for the loss of DNA binding and 771 days for the loss of conductivity, based upon a linear fit to all of the conductivity datapoints. However, this difference is to be expected since DNA binding is due entirely to surface functional groups while conductivity is expressed through the bulk phase density of functional groups. Therefore, conductivity would be expected to decay much slower than DNA surface binding, since positive charged defects should be preferentially removed from the PPy surface with time.

Electrochemical Film Reduction Effect on DNA Binding

To test directly whether the binding of DNA to PPy is brought about by the presence of the positive charged defects, the following experiment was performed. The binding of DNA was compared between freshly oxidized PPy and to a portion of that film subsequently reduced. Electrochemical reduction of oxidized PPy is known to form a film with significantly lowered conductivity [34]. As charges are removed reductively, dopant ions simultaneously diffuse out of the film [22]. The electrochemical reversibility of PPy has been shown by repeated cycling, indicating that the film structure and integrity is not altered [11]. The above reference presents electron spin resonance studies showing that the chemical reversibility is marked by the removal of positive charged defects on reduction and their replacement upon reoxidation.

Substrates for studying the effect of film reduction on DNA binding were produced by removing one half of a fully oxidized PPy film and reducing the other half by switching the electrode polarity and passing the current in the opposite direction (at -1.25 V) for 30 minutes. This was performed in 200 mL of 0.2 M tetraethylammonium p-toluenesulfonate electrolyte in acetonitrile solvent containing 2% distilled water. During the reduction, the current through the PPy film dropped from 3.5 to 1.0 mA, indicating a simultaneous loss in the film conductivity.

Figure 4 demonstrates that DNA has a lower binding rate with the partially reduced PPy film compared to the fully oxidized film. The reduced film retained the characteristic $t^{1/2}$ uptake dependence, suggesting the same diffusion limited binding seen in all of the fully oxidized film experiments. Since film reduction results in a reduction in positive charged defect structures, the four-fold decrease in the rate of DNA binding strongly suggests that the binding interaction directly involves these positive charged defects on the oxidized PPy. It is clear that not all of the DNA binding sites have been removed by PPy film reduction. Together with the results of the Figure 3 film aging experiments, these results strongly suggest that DNA binding to PPy involves the positive charged defect surface sites on PPy and is largely ionic in nature.

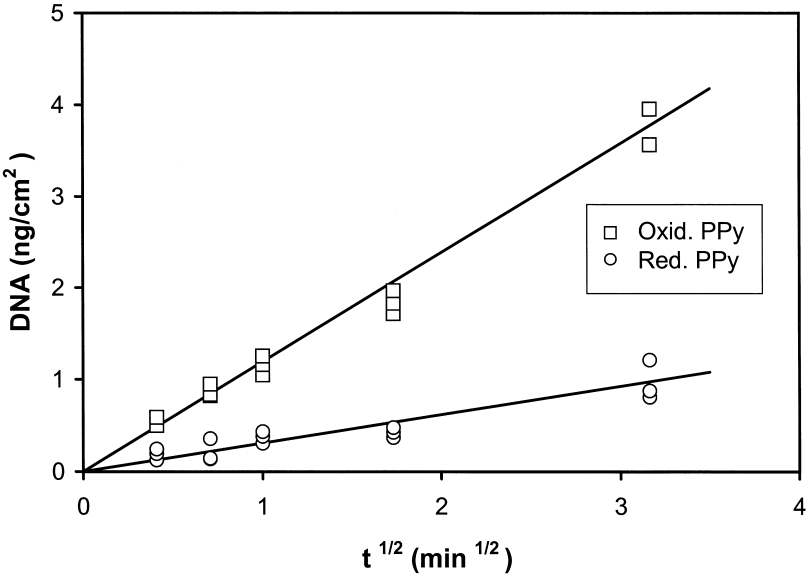

Figure 4. DNA binding to fully oxidized PPy compared with DNA binding to partially reduced PPy.

CONCLUSION

DNA binding to PPy, as a representative conducting polymer, is an important area of investigation. A number of recent reports have appeared studying DNA-PPy based sensors [37-42]. The interaction of the underlying PPy signal transduction matrix with the DNA biosensor element in those studies is clearly of great importance in the operation of such devices. This represented in part the motivation for the research that we focused on in this report.

In summary, the results of our DNA binding experiments suggest that diffusion limited DNA binding to the PPy surface originates from largely ionic interactions with the positively charged defects produced by electroxidation. The DNA binding to films grown over a range of current densities showed similar values. A first-order decay in PPy DNA binding level follows a qualitative pattern of decay similar to that of the slower loss of bulk conductivity with time, supporting the idea that both phenomena are caused by the same functional group feature of electroxidized films. Further support for this notion comes from partial film electrochemical reduction, which resulted in about a four-fold decrease in the rate of DNA binding.

ACKNOWLEDGMENTS

Support is acknowledged from grants DAAL03-91-G-0064 and DAAH04-94-0003.

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