Optical Study of DNA Surface Hybridization Reveals DNA Surface Density as a Key Parameter for Microarray Hybridization Kinetics

Wolfgang Michel, Timo Mai, Thomas Naiser, and Albrecht Ott
Chair of Experimental Physics 1, University of Bayreuth, Bayreuth, Germany

ABSTRACT We investigate the kinetics of DNA hybridization reactions on glass substrates, where one 22 mer strand (bound-DNA) is immobilized via phenylene-diisothiocyanate linker molecule on the substrate, the dye-labeled (Cy3) complementary strand (free-DNA) is in solution in a reaction chamber. We use total internal reflection fluorescence for surface detection of hybridization. As a new feature we perform a simultaneous real-time measurement of the change of free-DNA concentration in bulk parallel to the total internal reflection fluorescence measurement. We observe that the free-DNA concentration decreases considerably during hybridization. We show how the standard Langmuir kinetics needs to be extended to take into account the change in bulk concentration and explain our experimental results. Connecting both measurements we can estimate the surface density of accessible, immobilized bound-DNA. We discuss the implications with respect to DNA microarray detection.

INTRODUCTION

The relevance of quantitative detection of DNA-strands is increasing rapidly. Microarrays, where several thousands of different DNA-sequences can be identified simultaneously are becoming a standard tool in investigation of gene expressions profiles, thus a major tool for pharmacogenomics and clinical pathology. The basic mechanism of microarrays is the hybridization of complementary strands of DNA, where the bound-strand is immobilized on a substrate and the free-strand is in solution. Knowledge of the kinetic and thermodynamic properties of this reaction is important for working efficiently with microarrays. Recently, many different methods like surface Plasmon spectroscopy (1,2), total internal reflection measurements (3–5), mechanical and impedance-based techniques were used to study specific adsorption of DNA onto modified substrates (6,7). We apply the total internal reflection method, enhanced by the possibility of measuring the change of free-DNA concentration in solution in real time. Aim of this work is to get more detailed insight into the kinetics of the hybridization, characterizing our substrate-sequence system.

We show that under standard hybridization conditions the change in bulk concentration has an important impact on hybridization kinetics. We present an extended framework compared to the standard Langmuir kinetics, which considers changes in both bound- as well as free-DNA concentrations. We conclude about the limits of DNA microarray detection.

MATERIALS AND METHODS

Oligonucleotides are purchased from MWG Biotech (Ebersberg, Germany). The sequence and type of modification is given in Table 1.

Functionalized glass slides with 3-aminopropyltrimethoxysilane with 1,4-phenylenediisothiocyanate (SAL-slides) are purchased from Asper Biotech (Tartu, Estonia).

Immobilization procedures

We immobilize bound-DNA using two different methods.

A. Aminated oligonucleotides (i22) are diluted 1:1 with 200 mM carbonate buffer at pH 9.0 to a final concentration of 5 μM. 20 μl are spotted onto the surface with a pipette. The slides are incubated at 37°C for 1 h in a humidified chamber.
B. SAL-slides are immersed in 30 ml solution 100 mM carbonate buffer at pH 9.0 with 10 nM aminated oligos (i22) at 37°C for 1 h. After immobilization the slides are treated with a 1% (v/v) ammonia solution for 10 min., and washed twice with water, 3 min. per wash. They are dried under a stream of nitrogen and subsequently used for experiments. In the experiments we use SAL-slides, prepared following protocol A, unless stated otherwise. The reproducibility of the surface density following protocol B is better.

Hybridization experiments

For hybridization oligonucleotides (p22, m22) are diluted to appropriate concentration in a 3 × SSC hybridization buffer containing 0.01% SDS (w/v) and 1 mM EDTA. In all experiments the temperature is maintained constant at 40°C, unless stated otherwise.

Regeneration of substrates

For reuse, the substrates are treated with aqueous 10 mM NaOH solution. They are subsequently rinsed with hybridization buffer (3 × SSC, 0.01% SDS w/v, 1 mM EDTA).

Carbonate buffer

200 mM Na_2CO_3/$NaHCO_3$ (1:1), adjust to pH 9.0 with glacial acetic acid.

Instrumental setup

For experiments one strand (bound-DNA) is immobilized on the glass surface. The complementary strand (free-DNA), which is labeled with the fluorescent dye Cy3, is in solution in the reaction chamber (Fig. 1).

The reaction chamber consists of two polydimethylsiloxane (PDMS, Sylgard 184, Dow Corning) rings squeezed between two glass plates: the upper glass plate, where the DNA is immobilized, and a bottom glass plate. The surface area of the reaction chamber is ~40 mm^2 and the distance

Submitted June 23, 2006, and accepted for publication October 11, 2006.

Address reprint requests to Wolfgang Michel, E-mail: woife@ep1.uni-bayreuth.de.

0006-3495/07/02/999/06 $2.00

doi: 10.1529/biophysj.106.092064

TABLE 1 Sequences and modifications used in the experiments

i22	5′-NH_2-C6-TTT-TTT-TTT-TTT-TTT-TGA-TAG-GGT-GGT-GCT-TGC-GAG-T-3′
p22	5′-Cy3-ACT-CGC-AAG-CAC-CAC-CCT-ATC-A-3′
m22	5′-Cy3-TGA-GCG-TTC-GTG-GTG-GGA-TAG-T-3′

p22 and m22 were labeled with the dye Cy3 at the 5′-end. i22 was labeled with a amino-group at the 5′-end.

between the two glass plates is ~1.2 mm, so the volume of the reaction chamber is ~50 μl.

A diode pumped frequency-doubled (Nd:Yag) (532 nm) laser serves as a light source. The beam is divided into two parts. One beam penetrates into the glass slide via a glass half cylinder; it is totally internally reflected from the glass-liquid interface. This causes an evanescent wave penetrating into the liquid. The evanescent field excites the fluorescent molecules, which are tagged to the free-DNA near the glass surface. The penetration depth of the evanescent field is ~100 nm. The other part of the beam is coupled into a fiber, which is directed into the reaction chamber parallel to the surface to excite the fluorescent molecules in the bulk. The fiber is sandwiched between two PDMS-layers. The divergence of the beam leaving the fiber is 0.3, the core diameter of the fiber is 3.5 μm. We can detect a minimal bulk concentration of dye-labeled DNA of ~100 pM.

Both beams are blocked with shutters S1 and S2, which can be opened separately. During measurements the shutters are opened for only a few seconds for each data point to minimize bleaching of the dye molecules. The emission of the dye molecules from the surface (termed ''surface-signal'' in the following) and from the bulk (termed ''bulk-signal'' in the following) is collected with an objective and led through a filter to a photomultiplier (Hamamatsu H7732-01). The signal was preamplified (not shown) and then led to a lock-in amplifier.

RESULTS AND DISCUSSION

Using an evanescent wave for measuring DNA-hybridization

The evanescent wave excites fluorophores which are close to the surface (~100 nm). With this setup there is no direct way to distinguish whether the free-DNA is hybridized or just nonspecifically adsorbed to the surface. For that reason the nonspecific adsorption is assessed with the sequence m22, which is not complementary to the immobilized strands.

We find that the surface signal of the nonspecific adsorption of the noncomplementary strand (m22) is one to two orders of magnitude lower than the surface signal of hybridization of the complementary strand (p22) (see Fig. 2). We therefore neglect the contribution of the nonspecific substrate adsorption to the surface-signal.

Using a fiber to measure free-DNA concentration in the bulk

To measure by how much the concentration of dye-labeled free-DNA in the flow chamber varies during the experiment, we excite the fluorophores in bulk with an optical fiber. We measure the effect of the nonspecific adsorption of free-DNA to the reaction chamber walls. We use a commercial glass slide without any surface functionalization, and we find that the nonspecific adsorption of free-DNA in the bulk to either the reaction chamber walls or to the nonfunctionalized glass slide is negligible (Fig. 3). The concentration of free-DNA in the bulk detected with the fiber remains constant during the whole measurement. The bulk signal is proportional to the free-DNA concentration in the bulk. On functionalized glass substrates we observed a stronger nonspecific adsorption. In 3 h, ~10% of the initial noncomplementary free-DNA adsorbed nonspecifically from the bulk to the substrate.

Combining bulk and surface measurement

Fig. 4 shows the relation between surface signal and bulk signal during experiment. The first few data points equal the

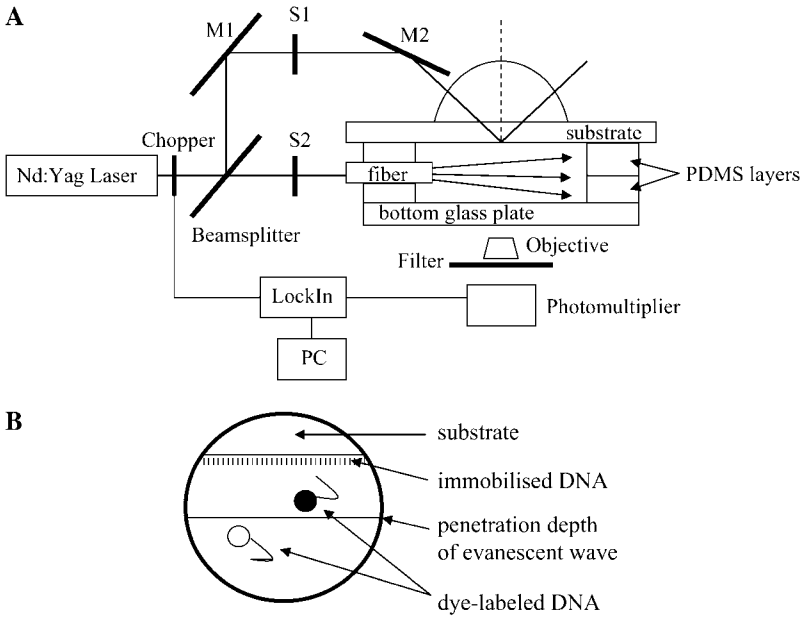

FIGURE 1 (*A*) Schematic of the measurement setup. The laser beam is chopped and split. One part illuminates the reaction chamber through an optical fiber. The second beam penetrates the substrate via two mirrors (*M1* and *M2*) and a glass half cylinder (lenses not shown). Both beams are blocked with shutters (*S1* and *S2*), which are controlled by the PC. The reaction chamber consists of two PDMS ring-shaped layers as walls and a bottom glass plate with inlet and outlet for changing solutions (not shown); the height of chamber is ~1.2 mm, the volume ~50 μl. The illumination area of the fiber and the penetration depth of the evanescent wave are schematized. The objective is 10× micro-plan objective (numerical aperture 0.25, Edmund Optics, Karlsruhe, Germany). (*B*) The drawing shows schematically the excitation of the dye-labeled DNA with the evanescent wave close to the substrate surface.

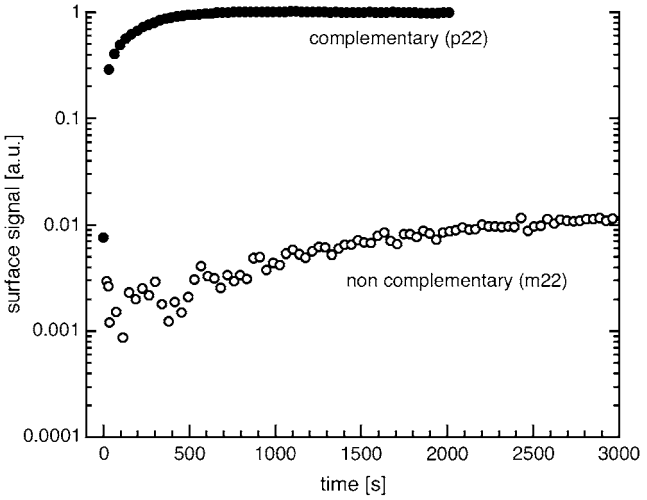

FIGURE 2 Comparative measurements of the not matching sequence (m22) and perfect matching sequence (p22). In both measurements, the initial free DNA concentration was 100 nM. At $t = 0$, the chamber was filled with hybridization solution.

known initial free-DNA concentration in bulk. From that the current free-DNA concentration in bulk can be estimated. As nonspecific adsorption of free-DNA to chamber walls can be neglected (as shown above) it is safe to assume that the loss of the free-DNA molecules in bulk (termed L in the following) is equal to the increase of the hybridized molecules on the surface. This is used to estimate the surface density of accessible bound-DNA. For this we carry out several hybridization experiments with different initial free-DNA concentrations. The loss of the concentration of free-DNA in the bulk is estimated for each experiment. We find that the loss stays constant above a certain initial free-DNA concentration (Fig. 5), hence the surface must be saturated.

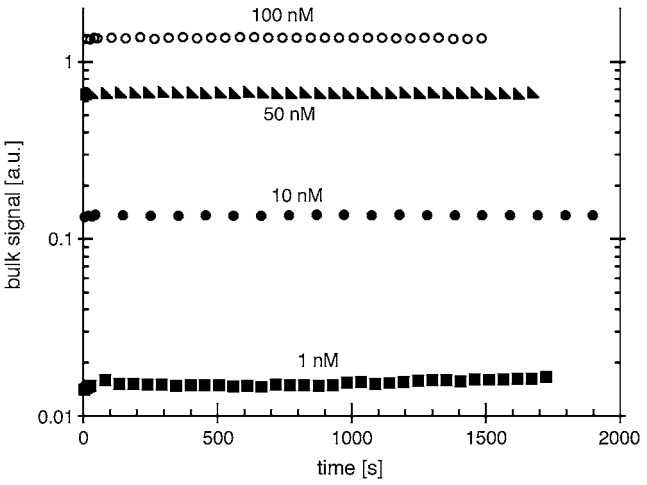

FIGURE 3 Measurement with a nonfunctionalized glass substrate shows that there is negligible nonspecific adsorption onto the glass surfaces and onto the PDMS walls at different free-DNA concentrations in bulk (1 nM, 10 nM, 50 nM, 100 nM). At $t = 0$, the reaction chamber was filled.

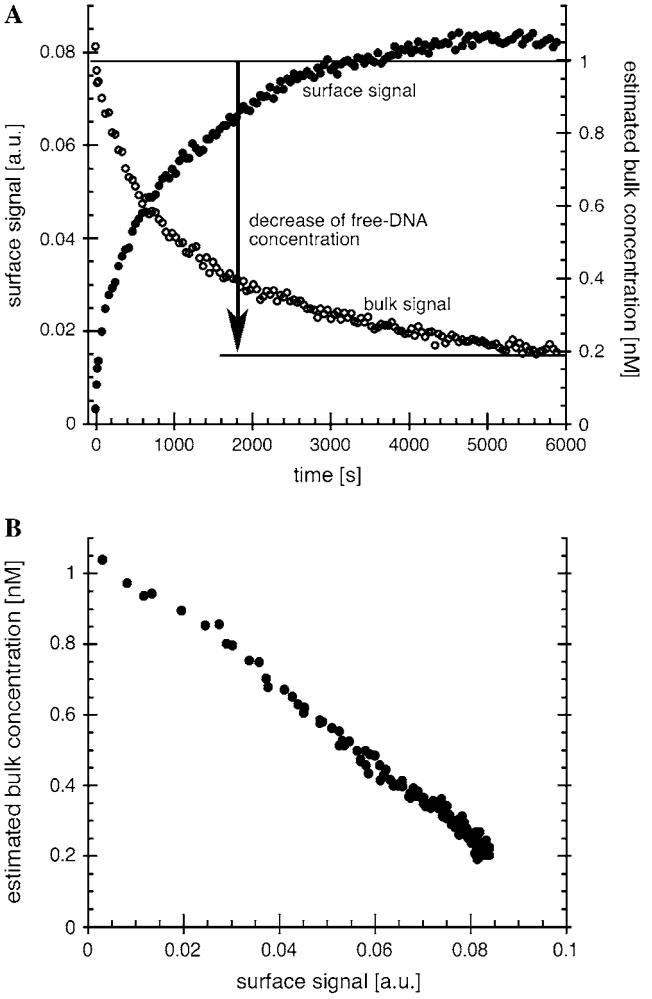

FIGURE 4 (*A*) Connection between surface signal and estimated bulk concentration of free DNA from a hybridization experiment, (initial free-DNA concentration 1 nM). The lines give the estimated value for the initial bulk value and the final bulk value, respectively. We estimated the decrease of free-DNA concentration in bulk from these two values (for details, see text). (*B*) Estimated bulk concentration of free DNA plotted against surface signal.

With the parameters of our experiment (volume of the reaction chamber 50 μl, surface area with immobilized surface-DNA 0.25 cm^2) the surface density of accessible bound-DNA can be estimated as $\sim 5 \times 10^{11}$ molecules/cm^2 for a substrate prepared following protocol A.

At higher surface densities of DNA the equilibrium constant may become different due to electrostatic repulsion between the DNA molecules (13). However, the ionic strength of our buffer is 0.72 M leading to a Debye screening length of $\sim$0.4 nm. The intermolecular distance on the surface is $\sim$15 nm, so electrostatic interactions between the DNA molecules can be supposed to be negligible.

To decrease the surface density of bound-DNA molecules we change the immobilization procedure to the protocol (B). With protocol B, the surface area with immobilized DNA is

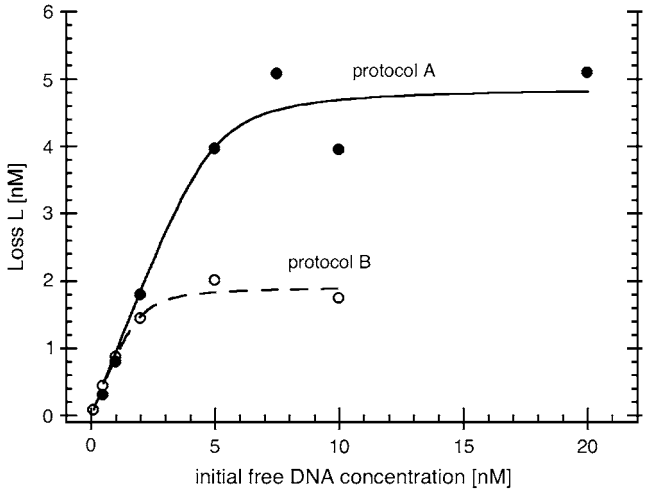

FIGURE 5 Decrease of the bulk concentration of free-DNA as a function of the initial bulk concentration for protocols A and B. The maximal decrease of the free-DNA concentration in the bulk here is ~5 nM with substrates prepared following protocol A (*solid circles, solid line*) and ~2 nM with substrates prepared following protocol B (*open circles, dashed line*). The lines represent a fit with Eq. 8.

~0.42 cm^2. There we achieve a surface density of accessible bound-DNA of ~1.4×10^{11} molecules/cm^2 (Fig. 5).

Extension of the Langmuir formalism

As a first approximation, the kinetics of hybridization can be characterized as follows, with k_+ and k_- as the rate constants for specific adsorption (hybridization) and desorption, respectively.

$$\mathrm{B} + \mathrm{F} \underset{k_-}{\overset{k_+}{\rightleftharpoons}} D,$$

where B stands for bound-DNA, F for free-DNA, and D for Duplex-DNA.

For simplification it is assumed that the concentration of the free-DNA in solution does not change while the reaction is going on (F = const). Assuming first-order kinetics, this leads to the following differential equation:

$$\dot{D}(t) = k_+ F_0[B_0 - D(t)] - k_- D(t), \tag{1}$$

where F_0 and B_0 denote the initial concentrations of free-DNA and bound-DNA, respectively. Introducing $\Theta(t) = D(t)/B_0$ as ratio of hybridized molecules to total number of immobilized bound-DNA molecules leads to

$$\Theta(t) = \Theta_\infty(1 - e^{-\gamma t}), \tag{2}$$

where

$$\gamma = k_+ F_0 + k_- \quad \text{and} \quad \Theta_\infty(B) = \frac{k_+ F_0}{\gamma}. \tag{3}$$

We find that in our experiments B_0 is ~5 nM as we determined above, and F_0 is in the range from 100 pM to 20 nM, so $B_0 \approx F_0$. The assumption of the Langmuir-model $B_0 \ll F_0$ is not justified in our experiments. Still the surface-signal can be fitted with the Langmuir-model, but one cannot extract any useful information from the parameters. The time constant versus initial free-DNA concentration does not show the expected linear behavior. Assigning the equilibrium constant K from $\Theta_\infty(F_0)$ leads to erroneous results. There are other reasons why the Langmuir model may not be applicable. To describe the kinetics of mismatched DNA hybridization, Peterson et al. (9) instead of the Langmuir model propose the Sips model (14), where a distribution of binding energies is considered.

Another theoretical work suggests that the hybridization reaction becomes diffusion-limited under certain conditions (15). Although some deviations from our extended Langmuir description as developed below exist, so far we cannot attribute them to a well-defined origin. The extended Langmuir description allows us to adjust our data and extract rate constant with reasonable agreement. Therefore, in the following we consider only the depletion of free-DNA molecules in the bulk.

The modified Eq. 1 then reads

$$\dot{D}(t) = k_+[F_0 - D(t)][B_0 - D(t)] - k_- D(t). \tag{4}$$

This results in an extended description for the kinetics

$$D(t) = \left(\frac{g}{2}\right)\left[\frac{1 - f\exp(gk_+t)}{1 + f\exp(gk_+t)}\right] + \frac{b}{2}, \tag{5}$$

where

$$g = \sqrt{b^2 - 4B_0F_0} \quad \text{with} \quad b = B_0 + F_0 + k_-k_+^{-1} \quad \text{and}$$
$$f = \frac{g + b}{g - b},$$

with the equilibrium value for D,

$$D_\infty = \frac{1}{2}(b - g). \tag{6}$$

With this description, the values of the equilibrium constant K and the rate constants k_+ and k_- can be assigned from our measurement. One drawback is that in principle the number of hybridized molecules on the surface needs to be known. With the bulk signal we can estimate $D(t)$ as we have already shown in the experimental section.

Another drawback of the extended kinetic description is the difficulty of fitting experimental data, because as many as three parameters B_0, k_-, and k_+ are free. By looking at the equilibrium values of a set of hybridization experiments at different initial free-DNA concentrations F_0 we can determine B_0. The definition of the equilibrium constant gives

$$K = \frac{D_{eq}}{B_{eq}F_{eq}} = \frac{L}{(B_0 - L)(F_0 - L)}, \tag{7}$$

where L denotes the loss of the concentration of free-DNA in the bulk and the suffix ''eq'' means the equilibrium values. We can solve the equation for $L(B_0,K)$, which gives

$$L(B_0,K) = 0.5\Big\{(B_0+F_0)+K^{-1} - \sqrt{[(B_0+F_0)+K^{-1}]^2 - 4B_0F_0}\Big\}. \quad (8)$$

Fitting the data $L(F_0)$ (Fig. 4) with this expression, we obtain both the number of bound-DNA molecules B_0 and the equilibrium constant K. Inserting B_0 in the extended kinetics equation (Eq. 5) we can fit the kinetic surface signal curves for individual initial free-DNA concentration F_0.

Assigning rate constants

From the loss in bulk, we conclude that on the substrates following protocol A, the average surface density (of 11 substrates) of accessible bound-DNA is $5.2 \pm 0.5 \times 10^{11}$ molecules/cm^2, on substrates following protocol B, the surface density is $1.38 \pm 0.10 \times 10^{11}$ molecules/cm^2 (of three substrates). This is lower than the reported surface density of immobilized molecules of $\sim 10^{12}$ molecules/cm^2 (9) to $\sim 10^{13}$ molecules/cm^2 (10,11), where different immobilizing chemistry was used.

To assign the hybridization rate constants from our measurements, the first step is to determine the surface density of accessible bound-DNA molecules and the equilibrium constant K with Eq. 8. The rate constants are determined in a two-parameter fit according to Eq. 5. The values are given in Table 2. The two ways of determining the equilibrium constant give different values. With the curve fit of Eq. 8, one can only estimate the approximate value for the equilibrium constant.

It should be mentioned that the kinetic model cannot be applied to all surface-signal curves (Fig. 6). For the substrates (A), it worked for low initial free-DNA concentrations. The curves with high initial free-DNA concentrations where $F_0 > B_0$ cannot be described, because there the signal went abruptly into saturation. For the substrates (B), the model can be applied for almost all curves. The assessed rate constants are of the same order of magnitude as reported elsewhere (8) although the experimental conditions are different. In Lehr et al. (8), the length of the strand is 30 bases, the substrates are epoxy-functionalized, the hybridization buffer has a concentration of 0.8 M monovalent cations (compared to 0.59 M in our case) and the hybridization temperature is 25°C (compared to 40°C in our case), surface density of immobilized molecules is not given. The assessed rate constant k_+ in solution (12) is of the same order of magnitude as reported here. In Henry et al. (12), the hybridization buffer has a concentration of 0.1 M monovalent cations, the concentrations of the two complementary 22 mer strands are 4.8 nM and 7.7 nM, respectively, and the hybridization temperature is about room temperature.

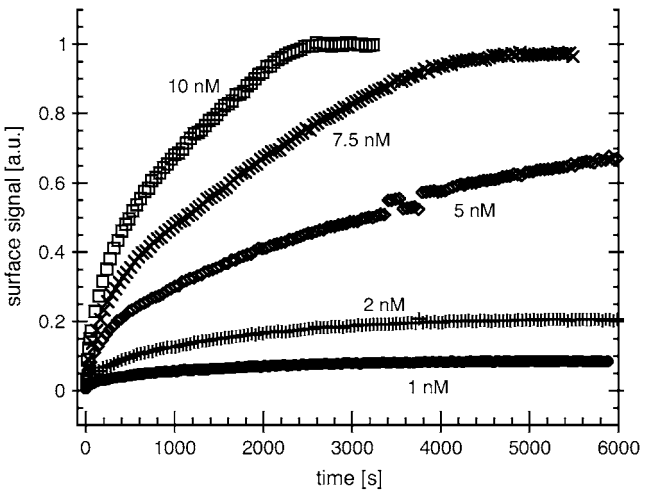

FIGURE 6 Hybridization experiment with different initial free-DNA concentrations (1 nM, 2nM, 5 nM, 7.5 nM, 10 nM) on a substrate prepared following protocol A. The surface signal of the curve with 10 nM initial free-DNA concentration saturates abruptly.

With the equilibrium constant $K = 10^9$ M^{-1} and a surface density of $\sim 10^{12}$ molecules/cm^2, we can estimate the sensitivity of substrates with immobilized DNA. We neglect diffusion and suppose equilibrium. With a spot size of immobilized DNA of 50 μm we get 25×10^6 immobilized molecules per spot. Using Eq. 8, we can estimate how many molecules hybridize at a given initial free-DNA concentration with a reaction chamber volume of 50 μl (see Fig. 7). The range where the number of hybridized molecules is roughly proportional to the initial free-DNA concentration is here from 1 fM to 100 pM. In practice the lower limit is given by the sensitivity of the detector, the upper limit depends on the system geometry such as spot size and reaction chamber volume. One has to consider that one may conclude from the

TABLE 2 Assigned rate constants and the equilibrium constant K from the fit with Eq. 8 of different experiments and directly calculated from the rate constants

	k_+ [M^{-1}s^{-1}]	k_- [s^{-1}]	K [M^{-1}] from Eq. 8	K [M^{-1}] from k_+, k_-
Protocol A	$9.01 \pm 0.11 \times 10^4$	$2.2 \pm 0.6 \times 10^{-4}$	$2.2 \pm 1.4 \times 10^9$	$4.1 \pm 1.1 \times 10^8$
Protocol B	$4.3 \pm 0.4 \times 10^5$	$2.9 \pm 0.7 \times 10^{-4}$	$8 \pm 2 \times 10^9$	$1.5 \pm 0.4 \times 10^9$
Lehr et al. (8)	$4.48 \pm 0.07 \times 10^5$	$7.75 \pm 1.83 \times 10^{-3}$	–	0.58×10^8
Henry et al. (12)	5.06×10^5	–	–	–

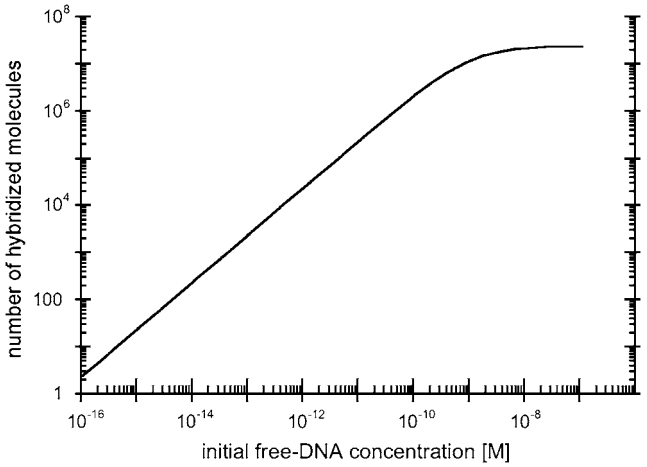

FIGURE 7 Estimation of the number of hybridized molecules at given initial free-DNA concentrations. We use the surface density and the equilibrium constant from our results and assume a spot size of 50 μM and a reaction chamber volume of 50 μl. The number of hybridized molecules is roughly proportional to the initial free-DNA concentration only in the range from 1 fM to 100 pM initial free-DNA concentrations.

number of hybridized molecules to the initial free-DNA concentration only in a limited range. This agrees well with results of theoretical work reported elsewhere (13). However, for longer, different free-DNA strands with different equilibrium constants this may no longer be true.

CONCLUSION

We investigate the kinetics of hybridization reactions on commercial phenylene-diisothiocyanate-functionalized (SAL) substrates with bound-DNA surface densities typical for microarray experiments. The measurement of the bulk concentration of free-DNA during hybridization experiments is useful for surface adsorption experiments, where Langmuir model cannot be applied. With the extended Langmuir kinetic model, where both the change of free-DNA and bound-DNA is considered, most of the observed hybridization kinetics can be explained. With the results for the equilibrium constant and the surface density of immobilized molecules we estimate the range where the number of hybridized molecules is proportional to the initial free-DNA concentration for microarray conditions. This shows that quantitative analysis of microarrays is possible albeit in a limited range of initial free-DNA concentration. The DNA surface density turns out to be the crucial design parameter for microarrays. It needs to be sufficiently low that hybridization is not hindered by the high surface density (13), but high enough to provide the highest range with the number of surface hybridized molecules proportional to the initial free-DNA concentration.

REFERENCES

1. Liebermann, T., W. Knoll, P. Sluka, and R. Herrmann. 2000. Complement hybridization from solution to surface-attached probe-oligonucleotides observed by surface-Plasmon-field-enhanced fluorescence spectroscopy. *Colloids Surf. A.* 169:337–350.
2. Peterson, A. W., R. J. Heaton, and R. Georgiadis. 2000. Kinetic control of hybridization in surface immobilized DNA monolayer films. *J. Am. Chem. Soc.* 122:7837–7838.
3. Chan, V., D. J. Graves, P. Fortina, and S. E. McKenzie. 1997. Adsorption and surface diffusion of DNA oligonucleotides at liquid/solid interfaces. *Langmuir.* 13:320–329.
4. Watts, H., D. Yeung, and H. Parkes. 1995. Real-time detection and quantification of DNA hybridization by an optical biosensor. *Anal. Chem.* 67:4283–4289.
5. Watterson, J. H., P. A. E. Piunno, C. C. Wust, and U. J. Krull. 2001. Controlling the density of nucleic acid oligomers on fiber optic sensors or enhancement of selectivity and sensitivity. *Sensor. Actuat. B.* 74: 27–36.
6. Larrson, C., M. Rodahl, and F. Höök. 2003. Characterization of DNA immobilization and subsequent hybridization on a 2D arrangement of Streptavidin on a biotin-modified lipid bilayer supported on SiO_2. *Anal. Chem.* 75:5080–5087.
7. Marquette, C. A., I. Lawrence, C. Polychronakos, and M. F. Lawrence. 2002. Impedance based DNA chip for direct T_m measurement. *Talanta.* 56:763–768.
8. Lehr, H.-P., M. Reimann, A. Brandenburg, G. Sulz, and H. Klapproth. 2003. Real-time detection of nucleic acid interactions by total internal reflection fluorescence. *Anal. Chem.* 75:2412–2420.
9. Peterson, A. W., L. K. Wolf and R. M. Georgiadis. 2002. Hybridization of mismatched or partially matched DNA at surfaces. *J. Am. Chem. Soc.* 124:14601–14607.
10. Hong, B. J., V. Sunkara, and J. W. Park. 2005. DNA microarrays on nanoscale-controlled surface. *Nucleic Acids Res.* 33:e106.
11. Watterson, J. H., P. A. E. Piunno, C. C. Wust and U. J. Krull. 2000. Effects of oligonucleotide immobilization density on selectivity of quantitative transduction of hybridization of immobilized DNA. *Langmuir.* 16:4984–4992.
12. Henry, M. R., P. W. Stevens, J. Sun and D. M. Kelso. 1999. Real-time measurements of DNA hybridization on microparticles with fluorescence resonance energy transfer. *Anal. Biochem.* 276:204–214.
13. Vainrub, A., and B. M. Pettitt. 2003. Sensitive quantitative nucleic acid detection using oligonucleotide microarrays. *J. Am. Chem. Soc.* 125: 7798–7799.
14. Sips, R. 1948. On the structure of a catalyst surface. *J. Chem. Phys.* 16:490–495.
15. Pappaert, K., P. Van Hummelen, J. Vanderhoeven, G. V. Baron and G. Desmet. 2003. Diffusion-reaction modeling of DNA hybridization kinetics on biochips. *Chem. Eng. Sci.* 58:4921–4930.