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Langmuir analysis of the binding affinity and kinetics for surface tethered duplex DNA and a ligand-apoprotein complex

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ABSTRACT. In this work the hybridization and dehybridization of ssDNA with 20 bases at gold coated sensor surfaces modified with complementary 20 bases capture probe ssDNA was investigated at 18°C by quartz crystal microbalance measurements with dissipation monitoring (QCM-D). A sequence of 20 base pairs with a melting temperature of about 64 °C was chosen, since in many biosensor studies the target molecules are DNA or RNA oligomers of similar length. It turned out that at the applied experimental conditions the DNA hybridization was irreversible, and therefore the hybridization and dehybridization process could not be described by the *Langmuir* model of adsorption. Nevertheless, quantitative dehybridization could be achieved by rinsing the sensor surface thoroughly with pure water. When in contrast the hybridization of a target with only ten bases complementary to the outer most ten bases of the 20 bases capture probe was studied, binding and unbinding were reversible, and the hybridization/dehybridization process could be satisfactorily described by the *Langmuir* model. For the 10 base pairs sequence the melting temperature was about 36 °C. Apparently for *Langmuir* behavior it is important that the experiments are applied at a temperature sufficiently close to the melting temperature of the sequence under investigation to ensure that at least traces of the target molecules are unhybridized (*i.e*. there needs to be an equilibrium between hybridized and dehybridized target molecules). To validate the reliability of our experimental approach we also studied the reconstitution and disassembly of the flavoprotein dodecin at flavin-terminated DNA monolayers, as according to previous studies it is assumed that the apododecin-flavin system can be well described by the *Langmuir* model. As a result this assumption could be verified. Using three different approaches, K_D values were obtained that differ not more than by a factor of four.

Introduction

DNA hybridization at surfaces

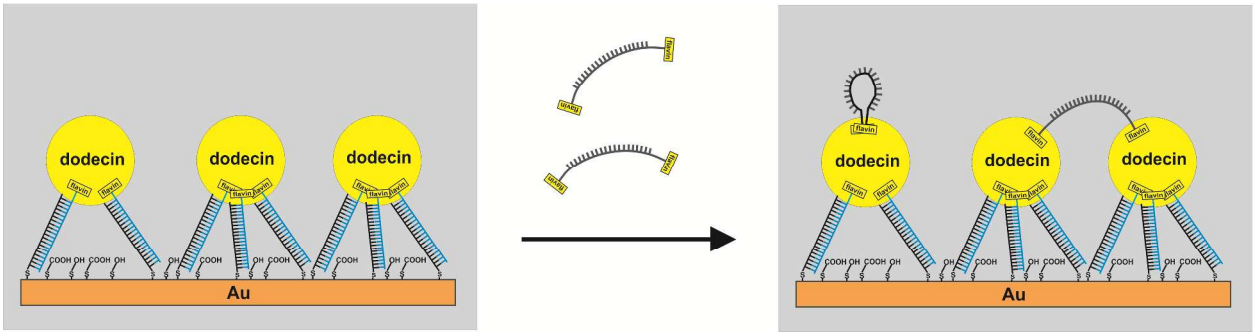
DNA hybridization at surfaces is the key reaction in many DNA biosensors. These sensors are developed to trace a specific target sequence *via* hybridization with a complementary capture probe single-stranded DNA (ssDNA) strand grafted to a surface. Most commonly the capture probe is chemisorbed to a gold surface by one or more thiol groups, and the signal transduction can be achieved by various techniques as for instance by an electrochemical technique such as cyclic voltammetry,[1-2] square wave voltammetry,[3] or impedance spectroscopy,[4-5] an optical technique, *e.g*. surface plasmon resonance (SPR),[6-7] surface plasmon fluorescence spectroscopy (SPFS),[8-9] or ellipsometry,[10] or by quartz crystal microbalance (QCM) measurements as an acoustic method.[11-13] Besides direct attachment of the capture probe to a gold surface also the streptavidin-biotin system has been widely employed.[6, 8] For this purpose, first biotin-terminated tethers are chemisorbed to gold *via* thiol anchors and employed to assemble a streptavidin layer at the surface.[14-15] In this case up to two binding sites of the homo-tetrameric streptavidin were used for surface attachment, and two binding sites remain, which can be used to bind biotinylated ssDNA capture probe strands in a sandwich type assembly. As an advantage of this method the capture probe strands are more evenly distributed over the surface, and the probability for direct interaction between two capture probe strands can be minimized.[16-17] Besides gold surfaces also glass surfaces have been modified with ssDNA capture probe strands covalently.[18-19] For many biosensing applications it is assumed that DNA hybridization at the surfaces follows, at least partially, the *Langmuir* model of adsorption.[15, 20-22] However, in only a few papers distinct experiments were carried out to prove this assumption.[15, 21-22] When we developed surface bound molecular beacons (MBs) in order to trace specific oligonucleotide

sequences at surfaces *via* SPFS we recognized that after hybridization with target almost none of the target molecules can be released by intense rinsing with the same buffer solution that was used also during the hybridization event.[9, 23] Also when we monitored DNA hybridization at surfaces by SPR, SPFS, or QCM we were not able to release a significant amount of target molecules by rinsing with buffer solution.[7, 24-25] Based on our results we began to question whether DNA hybridization at surfaces can be generally and unequivocally described by the *Langmuir* model. If in these experiments DNA hybridization/dehybridization can be described by the *Langmuir* model, the value for k_{off} has to be very low.

Protein-DNA nanostructures

On the other hand double-stranded DNA (dsDNA) monolayers at surfaces turned out to sufficiently stable to allow the formation of durable protein-DNA nanostructures. For instance we have shown that stable protein-DNA nanostructures can be formed by reconstitution of the multi-ligand binding flavoprotein dodecin at flavin-terminated DNA monolayers.[12-13, 26] Dodecin is a dodecameric flavoprotein with a diameter of 6-7 nm that comprises six flavin binding pockets. As in each pocket up to two flavin ligands can be incorporated, dodecin is able to bind up to twelve flavins in total.[27-28] Dodecin binds not only native, but also artificial flavins, as well as flavin-DNA ligands with high affinity, and therefore dodecin can be reconstituted on top of flavin-terminated DNA monolayers. Apododecin binds flavin ligands only with high affinity when they are oxidized, whereas flavin reduction induces the dissociation of the holoprotein complex.[12-13, 26-27] Based on these unusual redox properties we have shown that dodecin layers formed on top of flavin-terminated DNA monolayers can be disassembled (*via* flavin reduction) and reassembled in a reversible manner. This requires that dehybridization of the dsDNA does not occur within the time scale of the experiment. Furthermore, additional bidentate flavin-DNA

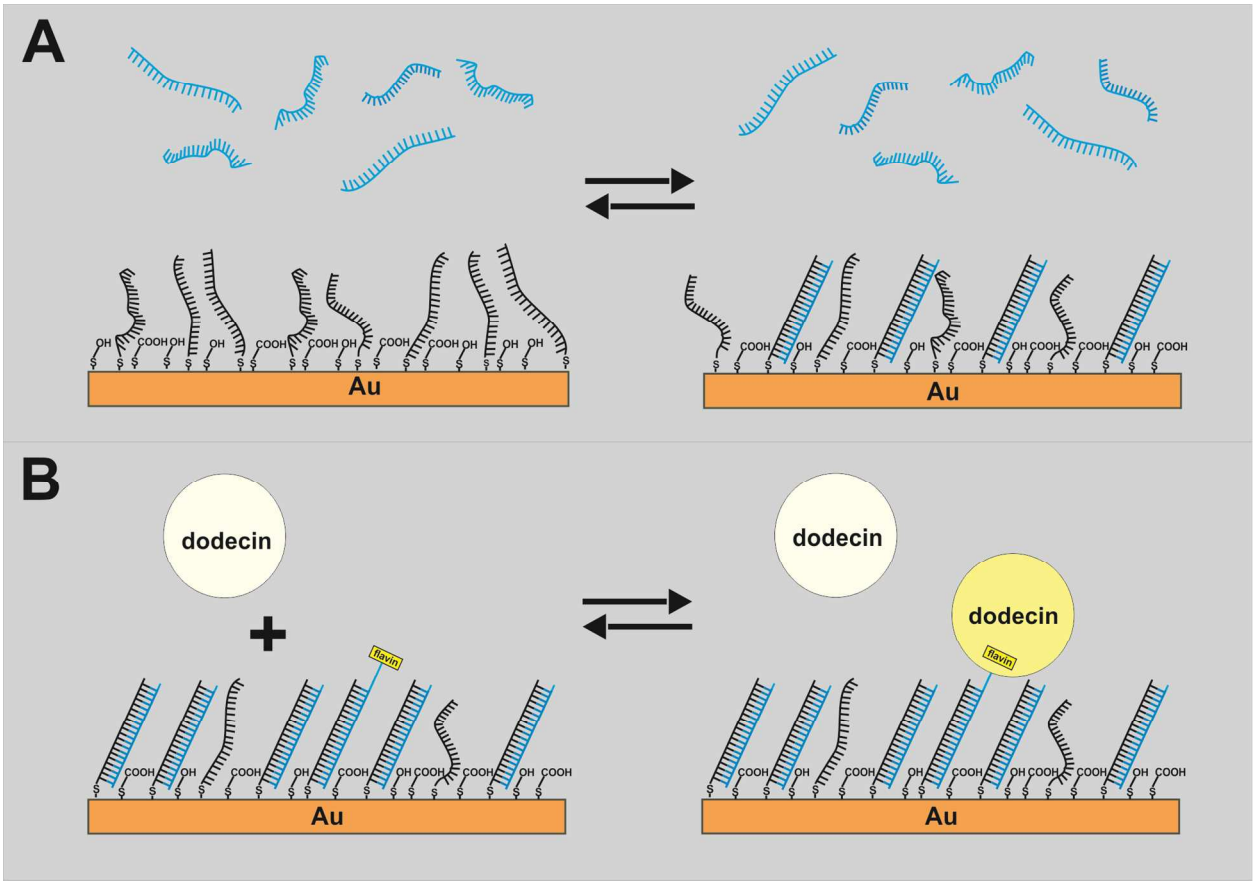
ligands can be bound on top of a dodecin-DNA layer leading to sandwich type DNA-protein-DNA layers as depicted in **Scheme 1**.[12] Previous studies revealed that in the case of rather bulky flavin-dsDNA ligands for steric reasons only one ligand is incorporated in each binding pocket, whereas two flavin-ssDNA ligands can be incorporated in the same binding pocket.[13] If multiple binding events (one apododecin may bind more than one surface-tethered flavin-DNA ligand) can be avoided, the apododecin-flavin binding system is assumed to follow the *Langmuir* model.[12-13]



Scheme 1. Stable dodecin monolayers can be formed on top of a flavin-terminated dsDNA layer with a relative flavin surface coverage of 100% (left hand side). To form a flavin-terminated dsDNA layer, surface grafted capture probe DNA (black) was hybridized with complementary flavin-terminated ssDNA (blue). The dodecin layer is stabilized by multi-ligand binding, *i.e.* one apododecin binds two or more flavins. Further binding of bidentate flavin-DNA ligands results in the formation of stable sandwich type DNA-protein-DNA layers (right hand side).

Does DNA hybridization and/or dodecin reconstitution follow the Langmuir model of adsorption?

To prove whether DNA hybridization can be described by the *Langmuir* model we have carried out a detailed QCM with dissipation mode (QCM-D) study of the hybridization of target DNA (20 bases) with surface-grafted complementary capture probe DNA, and for comparison we investigated the binding/unbinding of the apododecin variant DtE at a flavin-terminated DNA monolayer, for which *Langmuir* behavior is expected,[12-13] by the same experimental approach (see **Scheme 2 A** and **B**).



Scheme 2. In this work DNA hybridization using complementary capture probe and target ssDNA of 20 bases (**A**), as well as dodecin reconstitution (**B**), were studied at gold surfaces with respect to the *Langmuir* model. In (**B**) apododecin is reconstituted at a flavin-terminated dsDNA layer with a relative flavin surface coverage of 10% or 5% to avoid multi-ligand binding. One of

the boundary conditions for the *Langmuir* model is that by the adsorption/desorption process at the surface the concentration of the adsorbate in the solution does not change significantly. Thus, a constant number of dissolved adsorbate molecules in solution (as required for *Langmuir*) are drawn in the cartoons.

For the formation of the flavin-terminated DNA monolayer we use the same DNA sequences as for the study of DNA hybridization/dehybridization. To keep the flavin surface coverage low (*i.e.* to avoid multi-ligand binding) the surface grafted ssDNA capture probe was hybridized with a mixture of flavin-modified and flavin-free complementary ssDNA at ratios of 1 to 9 and 1 to 19 leading to a relative flavin surface coverage of 10% and 5%, respectively (with respect to the total amount of dsDNA). Both types of binding/unbinding events (DNA hybridization/dehybridization and dodecin reconstitution and disassembly) were analyzed according to the *Langmuir* model to prove whether by this model a reasonable description of the systems is possible. The apododecin-flavin binding can only be described by the *Langmuir* model if at the same time the flavin terminated dsDNA layer (with low relative flavin surface coverage to avoid multi-ligand binding) does not dehybridize within the time scale of the experiment (*i.e.* does not follow the *Langmuir* model).

Theoretical considerations

According to the *Langmuir* model the change in surface coverage with time can be described by equation 1 with θ as the fraction of adsorbed molecules (hybridized target DNA or reconstituted protein) with respect to the total number of binding sites, k_{on} and k_{off} are the kinetic rate constants

for binding and unbinding, and c is the concentration of adsorbate (*e.g.* target ssDNA or apoprotein in solution).[20, 29-30]

$$\frac{d\theta}{dt} = k_{on}c(1-\theta) - k_{off}\theta \qquad \text{equ. 1}$$

In equilibrium there is no net change in the surface coverage, and thus equation 2 holds true.

$$k_{off}\theta = k_{on}c(1-\theta) \qquad \text{equ. 2}$$

Taking in account that the *Langmuir* constant K_L can be expressed by $k_{on} \cdot k_{off}^{-1}$, equation 2 can be rearranged to equation 3, which describes the concentration dependent equilibrium surface coverage θ_{eq}.

$$\theta_{eq} = \frac{K_L c}{1 + K_L c} \qquad \text{equ. 3}$$

According to equation 3 the *Langmuir* constant K_L or the dissociation constant K_D ($K_D = K_L^{-1}$) can be determined experimentally by an equilibrium titration, *i.e.* different concentrations of adsorbate are added to the surface, and each time the experiment is carried out until the equilibrium surface coverage has been reached. As it can be deduced from equation 3, when θ_{eq} is $0.5 \times \theta_{eq}(\max)$, c equals K_D.

Alternatively K_D, as well as the values for k_{on} and k_{off}, can be determined by kinetic measurements. Solving equation 1 (which is a first order differential equation) with respect to the boundary conditions (binding: $\theta(t = 0) = 0$ and unbinding: $\theta(t = 0) = \theta_{eq}$ and $c = 0$) leads to exact solutions for the adsorption (binding) or desorption (unbinding) process. The adsorption kinetics can be described by equation 4.

$$\theta(t) = \theta_{eq}(1 - e^{-(c \cdot k_{on} + k_{off})t}) \qquad \text{equ. 4}$$

By analyzing an adsorption process only for a single concentration, which can be monitored experimentally, *e.g.* by SPR or QCM-D, only a value for the inverse of the binding time constant, $\tau^{-1} = (k_{on} \cdot c + k_{off})$, can be obtained. In order to determine distinct values for k_{on} and k_{off}, the adsorption process has to be investigated at different concentrations. Then a plot of τ^{-1} *vs* c reveals the individual values for k_{on} and k_{off}. Alternatively, the value for k_{off} (and thus also the value for k_{on}) can be determined from the desorption kinetics, which are described by equation 5.

$$\theta(t) = \theta_{eq} e^{-k_{off} t} \qquad \text{equ. 5}$$

For an adsorption/desorption process following the *Langmuir* model, some assumptions have to be fulfilled. In particular, all sites at the surface have an equal probability for adsorption, and each adsorption event happens independently. Furthermore, multivalent interactions as well as interactions between individual adsorbate molecules are excluded. Adsorption is restricted to monolayer formation, and the adsorption process has to be reversible. In addition the bulk concentration of the adsorbate in solution is assumed to be constant, *i.e.* the number of adsorbed molecules is negligible compared to the number of adsorbate molecules in solution. Also the adsorption reaction must not be diffusion limited.[31] If a process such as DNA hybridization or protein reconstitution at surfaces can be described by the *Langmuir* model, values for K_L or K_D determined from kinetic or thermodynamic (equilibrium data) should agree within the experimental error.

Materials and methods

QCM-D measurements were performed using a Q-Sense E1 (Biolin Scientific, Stockholm, Sweden) operated at a frequency of 4.95 ± 0.05 MHz using QSX 301 sensor slides equipped with a gold surface and a chromium adhesion layer underneath. The instrument was controlled by the Q-Soft Software (Biolin Scientific, Stockholm, Sweden). While all available overtones were measured, only a single overtone was used for data evaluation (for different QCM-D chips the signal of individual overtones may differ in intensity and noise). Mathematical fittings in this study were performed using the software Origin (OriginLab, Northhampton, USA). Since in previous studies it was shown that in QCM measurements the adsorbed mass is overestimated due to solvent molecules trapped in the ssDNA or dsDNA monolayer,[11, 32] it is not possible to determine the absolute surface coverage from QCM-D measurements (which is in contrast to SPR). Using the same and/or similar surface modification protocols and the same DNA sequence we have studied DNA hybridization by a combination of SPR, QCM-D and electrochemistry in our previous work.[7] From the SPR measurements the capture probe surface coverage and the hybridization efficiency could be determined.[7] Also for the reconstitution of dodecin on flavin-terminated DNA monolayers we have determined the dodecin surface coverage by SPR.[12]

This fact that in QCM-D measurements the adsorbed mass is overestimated should not have any influence on the kinetic data, if the number of trapped solvent molecules increases linearly with the amount of hybridized target DNA. If this effect does not increase (at least almost) linearly with the amount of bound target molecules and is thus significantly falsifying the kinetic data, a mono-exponential fit of the binding and unbinding curves should not match the experimental data satisfactorily. When we studied the kinetics of apododecin-flavin binding/unbinding in our previous work,[12-13] the values obtained by SPR, SPFS, and QCM-D were in agreement.

All DNA oligomers used were purchased from Eurogentec S.A. (Seraing, Belgium) or Metabion GmbH (Martinsried, Germany). All reagents for the preparation of buffers as well as for basic piranha were ordered from Carl Roth (Karlsruhe, Germany), whereas mercaptobutanol and tris-(carboxyethyl)phosphine were from Sigma-Aldrich (Darmstadt, Germany). Mercaptopropionic acid was supplied by AlfaAesar. Dodecin from *Halobacterium salinarum* was produced as described elsewhere[12, 33] and stored in buffer (1 M NaCl, 20 mM Tris, 5 mM $MgCl_2$, pH 7.5; in the following we will refer to this buffer as buffer B) at 4 °C. The protein was filtered through a 0.22 µm polyamide-6,6 syringe filter (ThermoFisher Scientific, Waltham, MA, USA) before use. All solutions in this study were prepared with purified water showing a minimum resistivity of 18 MΩ·cm @25 °C (MerckMillipore, Darmstadt, Germany).

For DNA hybridization/dehybridization experiments as well as for dodecin reconstitution, the gold coated QCM-D sensor chips were cleaned as described in earlier publications.[7, 12] In order to prepare clean gold surfaces, the QCM-D sensor crystals were set in an ozone chamber for 10 min (Bioforce, Ames, IA, USA) followed by immersion in hot basic piranha (1/1/5 ratio of 25% ammonium hydroxide solution, hydrogen peroxide and water; **CAUTION! This solution is highly corrosive and should be handled with special care**) for 5 min and a final treatment for additional 10 min in the ozone chamber. The chip was then mounted in the sensor platform (Q-Sense flow module, Biolin Scientific, Stockholm, Sweden) and left for equilibration in buffer until a stable frequency signal was recorded. For all experiments shown in this paper, the thermostat of the QCM-D instrument was set to 18 °C (291 K).

Experimental protocol for DNA hybridization studies

For DNA hybridization studies, measurements were performed with thiol modified DNA comprising 20 bases (sequence: 5'-HO-$(CH_2)_6$-S-S-$(CH_2)_6$-AAC-TAC-TGG-GCC-ATC-GTG-AC-3') used as a capture probe and unmodified complementary DNA with 20 bases (sequence: 5'-GTC-ACG-ATG-GCC-CAG-TAG-TT-3') and 10 bases (5'-GTC-ACG-ATG-G-3') as hybridizing target. The dithiol of the capture probe DNA was reductively cleaved prior to use by incubation with tris(2-carboxyethyl) phosphine overnight followed by size exclusion chromatography using Biospin P-6 spin columns (BioRad Laboratories, München, Germany) in order to remove the mercaptohexanol.[34] Briefly, 2 nmol (one aliquot) of capture probe DNA was dissolved in 90 µL phosphate buffer (buffer A as defined below) and incubated overnight at 10 °C in a thermomixer at 400 rpm with 10 µL of a tris (2-carboxyethyl)-phosphine hydrochloride solution (100 mM in water). Subsequently the capture probe DNA was purified by size exclusion chromatography and diluted to a final volume of 400 µL. This procedure may be advantageous, since in some studies it has been reported that free thiols exhibit faster adsorption kinetics than the corresponding disulfides.[27, 35-36] The mercaptohexanol is removed to avoid the reformation of the disulfide in the presence of oxygen. After deprotection the thiolated capture probe DNA should be used immediately to prevent disulfide formation of two capture probe strands.

As reported in earlier studies,[7] we were able to perform multiple hybridization/dehybridization cycles in 20 mM phosphate buffer, pH 7.0, also comprising 50 mM KCl and 5 mM $MgCl_2$. Hence, we used this buffer (in the following termed buffer A) for the binding and unbinding experiments of DNA described in this paper. Dehybridization was achieved by rinsing with pure water.[7, 37-38]

To prepare a capture probe DNA modified surface for hybridization studies, 400 µL of a 5 µM (deprotected) thiol-modified ssDNA capture probe solution in buffer A was added and incubated for approximately 1 h in the flow cell. In a successive step, the cell was rinsed with buffer A and deionized water followed by incubation of a mixture of short thiols in water (1 mM of mercaptobutanol and mercaptopropionic acid, each) for about 1 h. This step is necessary to passivate the surface, to displace nonspecifically adsorbed ss-DNA, and to orient the specifically adsorbed DNA toward the solution (by electrostatic repulsion between the OH and COO^- end groups of the thiols and the negatively charged phosphate backbone of the DNA) to enhance the hybridization efficiency. While often mercaptohexanol is used in this step, sometimes shorter thiols have been applied as well.[39-41] We prefer to apply a mixture of rather short thiols to ensure that these thiols may not hamper the hybridization event.[9] The flow cell was then rinsed with buffer A before complementary DNA solutions at concentrations between 0.1 and 5 µM were added. Target DNA solution volumes between 400 µL and 8 mL were evaluated to ensure that the amount of injected DNA was sufficient to establish hybridization without significant changes in the adsorbate concentration. These samples were left for incubation in the flow cell until a plateau was observed in the QCM-D kinetic scan curve, indicating the completion of the hybridization process. The cell was subsequently rinsed with buffer A for at least 15 minutes to prove whether DNA can be dehybridized at least to some extent. In some experiments rinsing with buffer was extended to five hours. If no dehybridization could be achieved by buffer rinsing, the cell was rinsed with deionized water extensively to regenerate the surface. After equilibration with buffer the next step of the titration could be carried out using a different concentration of the oligonucleotide. A second experimental protocol was carried out to obtain capture probe modified sensor surfaces with low surface coverage. For this purpose a solution of

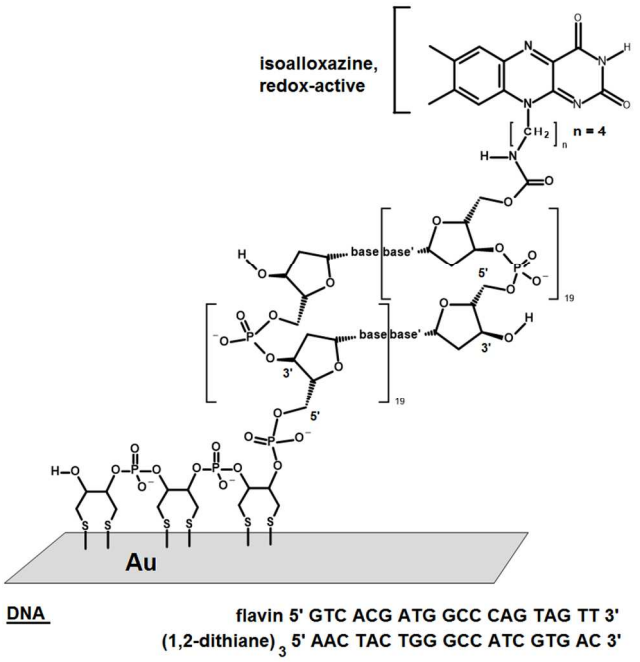
0.5 μM capture probe DNA (400 μL) also comprising 25 μM of mercaptobutanol (MCB) and mercaptopropionic acid (MPA) in buffer A was incubated on the sensor surface for 90 minutes. Then the flow cell was rinsed with buffer A and water before a mixture of MCB and MPA (0.5 mM each) was injected and incubated on the sensor surface for 60 minutes. Subsequently the surface was rinsed with buffer A. The corresponding DNA hybridization/dehybridization experiments are described in the supporting information.

A slightly modified protocol was used to prepare the capture probe modified surfaces for the hybridization with 10 bases complementary DNA. Here a solution of 1.0 μM 20 bases capture probe DNA (400 μL) in buffer A was incubated on the sensor surface for 30 minutes. Then the flow cell was rinsed with buffer A and water before it was incubated with a mixture of MCB and MPA (1.0 mM each) for 60 minutes. Subsequently the surface was rinsed with buffer A.

Experimental protocol for dodecin binding/release studies

For dodecin binding and release studies, we used a well-established set of oligomers with the same sequence as for DNA hybridization studies but slightly different modifications.[12-13] Capture probe DNA was chemisorbed to the gold surface by three dithiane rings at the 5'-end of the oligonucleotide whereas unmodified target strands as well as oligonucleotides with a N(10)-[4-(aminobutyl] flavin (CofactorC_4, CofC_4) modification at its 5'-end were used for hybridization. The synthesis of CofC_4 as well as the procedure to couple the flavin to the 5' end of the target DNA is described elsewhere.[27] While we have performed the coupling of the flavin CofC_4 to the 5' end of the target DNA previously by ourselves,[27] for the current study this synthesis step was carried out by the company *Eurogentec*. Three dithiane rings were used for surface grafting, since they are more stable at high salt concentration (as preferred by dodecin) than a single thiol

group.[42] The structure and sequence of the flavin- and disulfide modified DNA are shown in **Scheme 3**.



Scheme 3. Structure and sequence of the flavin- and disulfide modified DNA after chemisorption.

As dodecin originates from a halophilic organism, it exhibits optimum stability at high salt concentration. Therefore buffer B was used for the preparation of the sensor surface as well as for the binding and unbinding experiments performed with dodecin. At least 200 µL of 5 µM capture probe DNA with three dithiane rings were injected to the QCM-D cell and incubated for approx. 1 h. In a following step, the flow cell was rinsed with buffer B and water before a solution of mercaptobutanol and mercaptopropionic acid in water, 1 mM each, was incubated for 1 h followed by rinsing with buffer B. For the reconstitution of apododecin, a flavin-modified dsDNA layer with a relative flavin surface coverage of 10% and 5% (with respect to the total amount of dsDNA) was prepared to minimize the probability for multiple binding events (one

apododecin can bind multiple surface tethered flavins). For this purpose, the ssDNA capture probe modified surface was incubated with mixtures of flavin modified complementary DNA and unmodified complementary ssDNA in ratios between 1 to 9 and 1 to 19, respectively. For equilibrium titrations, 200 µL of the multivalent apododecin variant tE (DtE) with protein concentrations between 0.125 to 8 µM were injected into the QCM-D flow cell and incubated until the signal became constant. Subsequently, the flow cell was rinsed with buffer B until the baseline of the frequency signal was reached again. For the protein concentration (determined by the Bradford assay) an error of 10% was assumed.

Data evaluation

One approach to calculate the kinetic constants k_{on} and k_{off} as well as the resulting average K_D was to fit mono-exponential functions to the kinetic QCM scan curve describing the binding and unbinding process. For each binding and unbinding process, the inverse of a binding time constant τ^{-1} and a kinetic constant for the desorption process k_{off} could be obtained, respectively. As $\tau^{-1} = k_{on} \cdot c + k_{off}$ holds true if the *Langmuir* model is applicable, the k_{on} as well as the K_D value can be calculated for each individual titration step. The kinetic constants and K_D values obtained by fitting the binding and unbinding curves according to equations 4 and 5, respectively, are presented as average values of all titration steps with twice the standard deviation as error. In contrast, the evaluation of the equilibrium titrations (here the QCM frequency shifts were plotted *vs.* the adsorbate concentration c and fitting to equation 3) yielded solely the K_D value including its corresponding fit error. In a similar manner, the plot of the inverse of the binding time constant τ^{-1} *vs.* the adsorbate concentration c was fitted linearly yielding the corresponding k_{on}

and k_{off} values, respectively. These kinetic constants were used to calculate the K_D value with its error being estimated by the uncertainty propagation.

Results and discussion

Hybridization and dehybridization of DNA at capture probe modified surfaces

We started our investigation on the adsorption, *i.e.* hybridization and subsequent dehybridization of a 20 bases target DNA oligomer on QCM-D sensors modified with 20 bases capture probe DNA and short chain thiols serving as spacer. The preparation of the capture probe modified sensor chips was carried out as described in our previous work.[7] As mentioned before, the capture probe surface coverage cannot be estimated from QCM-D measurements, since in QCM-D measurements the adsorbed mass is overestimated due to solvent molecules being trapped in the DNA monolayer.[11] Therefore we have carried out SPR studies in our previous work using the same type of capture probe DNA. Following the same surface modification procedure as in the current work, a capture probe surface coverage of 1.9×10^{-11} mol·cm^{-2} has been determined from SPR studies.[7] For this relatively high capture probe surface coverage a hybridization efficiency of about 40% was reported.[7] In the current work we incubated for hybridization with a buffer solution containing 5 µM target DNA until the adsorption process was completed. Subsequently, the surface was rinsed with pure buffer solution for an extended period, *i.e.* for several minutes or even for a few hours (as during the experiment shown in **Figure 1**). If DNA hybridization/dehybridization follows *Langmuir* behavior, equilibrium between adsorption and desorption of the target DNA should be established, and thus, complete dehybridization should be achievable by continuous rinsing with buffer. However, as obvious from **Figure 1** we could not observe significant unbinding of complementary DNA upon rinsing

with buffer. This finding is in good agreement with previous studies by us and other groups.[7, 9, 23-25, 43-44]

Apparently either the DNA hybridization/dehybridization does not follow the *Langmuir* model, because one of the necessary boundary conditions, *i.e.* the reversibility of the adsorption and desorption process, is not fulfilled at the experimental conditions we applied (it is well known that temperature, ionic strength, pH, and the number of base pairs have an impact on DNA hybridization efficiency)[45-46] or the desorption process is too slow to achieve complete dehybridization in the time scale of the experiment, *i.e.* the corresponding k_{off} value is rather small.

In line with the latter assumption we fitted the kinetic binding and unbinding curves in **Figure 1** according to *Langmuir* to yield the kinetic constants k_{on} and $k_{off,}$ from which the dissociation constant K_D can be calculated. As kinetic constants $k_{on} = 2580 \pm 384\ M^{-1}\ s^{-1}$ (the mono-exponential fit of the adsorption process yielded $\tau^{-1} = 0.0129 \pm 0.0019\ s^{-1}$) and $k_{off} = 6.6 \pm 0.4 \cdot 10^{-7}\ s^{-1}$ were calculated, and consequently $K_D = 0.26 \pm 0.04$ nM was obtained. As it can be seen from **Figure 1**, a mono-exponential function does not fit the binding kinetics quite well. Furthermore, the fitted curve for the unbinding kinetics does not differ much from a straight line, and the corresponding value for k_{off} is even lower than that of the streptavidin-biotin system ($k_{off} = 3.8 \cdot 10^{-6}\ s^{-1}$), which is indeed rather low.[12, 47]

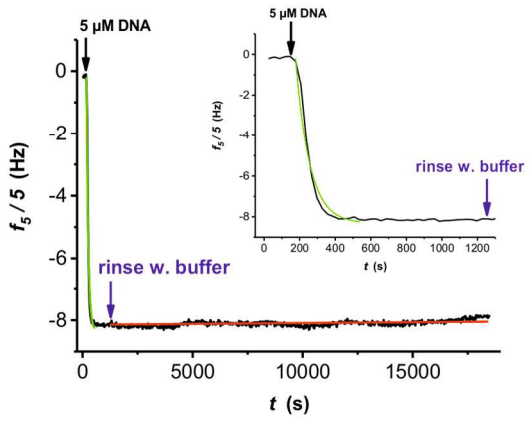


Figure 1. Hybridization of a 20 bases capture probe modified QCM-D sensor surface by incubation with 5 µM of 20 bases target DNA in 20 mM phosphate buffer, pH 7.0, also comprising 50 mM KCl and 5 mM $MgCl_2$. After completion of the hybridization process (a shorter time period is shown in the inset), the surface was rinsed with the same buffer for more than 4 hours. The experimental data was fitted according to equations 4 and 5, and the corresponding curves for adsorption (k_{on} = 2580 ± 384 M^{-1} s^{-1}, τ^{-1}=0.0129 ± 0.0019 s^{-1}) and desorption (k_{off}=6.64 ± 0.39 ·10^{-7} s^{-1}) are shown in green and red, respectively.

Nevertheless, the deviation between the fitted and experimental binding curve could also be explained by experimental parameters, *e.g.* by switching the peristaltic pump on or off. Therefore, we decided to determine the K_D value independently by a titration with target DNA at increasing concentrations. It was our intention to prove whether by this approach a K_D value can be obtained that is at least within the same order of magnitude as the value from the analysis of the kinetic curves. For this purpose three DNA equilibrium titrations were carried out independently on different QCM-D sensors. Representatively, one of these experimental runs is shown in **Figure 2A**, and the corresponding *Langmuir* isotherm (*i.e.* a plot of the QCM

frequency shift *vs*. concentration of target DNA, fitted according to equation 3) in **Figure 2B** (an enlargement of the individual titrations steps presented in **Figure 2A** and the corresponding fitted curves are shown in the Supporting Information file, **Figure S1**). The kinetic scan curves of two further DNA equilibrium titrations are shown in **Figure S2** and **Figure S3** in the Supporting Information File.

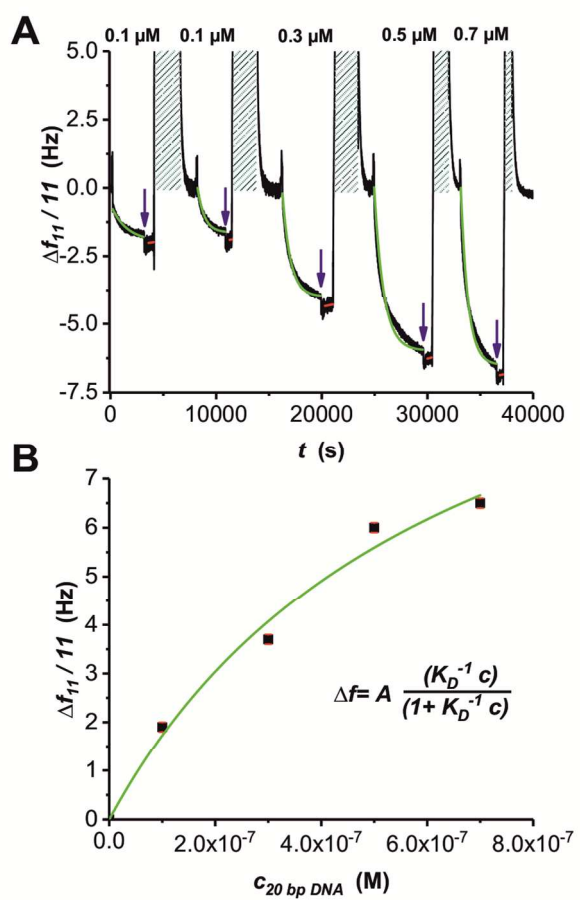


Figure 2. DNA equilibrium titration for target DNA comprising 20 bases concentrations of 0.1 µM (two times), 0.3 µM, 0.5 µM, and 0.7 µM (**A**) as well as the corresponding plot of frequency shift vs. concentration fitted to a *Langmuir* isotherm (**B**). The results of the 11th overtone are shown. The first titration step using 0.1 µM of target DNA was performed twice to prove that there is no ageing of the surface.

In these experiments a capture probe modified surface is incubated with different concentrations of complementary DNA followed by rinsing with buffer (indicated by blue arrows). Analogous to previous experiments, rinsing even for a few hours was not sufficient to achieve a significant ratio of dehybridization. Therefore we limited the periods for rinsing with buffer to a rather short time and performed more titration steps with different concentrations of target DNA instead. Between all titration steps, the surface was regenerated (*i.e.* dehybridization was achieved by extended rinsing with pure water indicated by the shaded background in **Figure 2A**). In all experiments, the surface could be fully regenerated multiple times without losses in performance (*i.e.* no frequency shift was observed for the ssDNA capture probe surface after individual dehybridization steps throughout the experiment).

The K_D value for target DNA determined by fitting the data in **Figure 2B** to the *Langmuir* isotherm is 637 ± 298 nM. As this result differs by more than three orders of magnitude from the K_D determined by analysis of the kinetic data in **Figure 1**, we also performed an analysis of the kinetic binding and unbinding curves, which were monitored in each titration step. From the analysis of the kinetics in **Figure 2A**, a mean K_D value (± 2σ) of 5 ± 4 nM was calculated (from the binding and unbinding curves of the individual titration steps in **Figure 2A** average k_{on} and k_{off} values (± 2σ) of 3736 ± 3494 M^{-1} s^{-1} and 1.95 ± 0.64 · 10^{-5} s^{-1} were calculated, respectively). The fact that the average K_D value (± 2σ) of 5 ± 4 nM determined from the kinetics of the experiment shown in **Figure 2A** differs by about three orders of magnitude from the K_D value of 637 ± 298 nM determined by fitting the data in **Figure 2B** (belonging to the same experiment) implies that here the *Langmuir* model is not applicable. Analogous results were obtained for the other two equilibrium titrations (depicted in the supporting information). One possible reason

why DNA hybridization/dehybridization cannot be described by the *Langmuir* model in these experiments might be that the surface coverage of the capture probe DNA is too large and therefore the boundary conditions for *Langmuir*, *i.e.* that each binding event has to be independent from another, is not fulfilled. We therefore also performed DNA equilibrium titrations on sensor surfaces prepared by a different protocol which is known to yield a lower surface coverage of capture probe DNA.[7] Following this procedure, which is also briefly described in the materials and methods section, in previous experiments hybridization efficiencies of 100% could be reached.[7] The results of this experiment are shown in **Figure S4** in the supporting information file. While for five titration steps (in the same experiment) the mean $K_D \pm 2\sigma$ determined by kinetic analysis of the data is 1.2 ± 1.1 nM (with average k_{on} and k_{off} values ($\pm 2\sigma$) of 6948 ± 5048 M^{-1} s^{-1} and $5.03 \pm 4.21 \cdot 10^{-6}$ s^{-1}), fitting of the concentration dependent frequency increments to the corresponding *Langmuir* isotherm yielded a K_D value of 1356 ± 617 nM.

It therefore seems that the *Langmuir* model is indeed not suitable to describe the DNA hybridization/dehybridization process at the experimental conditions we applied, and the reason for this is could be that a different boundary condition of the *Langmuir* model, *i.e*. that hybridization and dehybridization need to be reversible, is not fulfilled. The 20 base pairs dsDNA sequence used in the current study has a salt adjusted solution melting temperature of 64.4 °C (338 K) (calculated using the *OligoCalc* online tool)[48] indicating that not even fractions of the dsDNA might be dissociated at a temperature of 18 °C (291 K), at which the QCM-D measurements were performed. Thus, it seems to be consistent that dehybridization cannot be achieved by simple rinsing with buffer whereas it should be possible if the DNA melting temperature was much lower. We therefore performed additional measurements on a sensor

surface modified with the same 20 bases capture probe ssDNA as used before, but this time a slightly lower capture probe DNA concentration was used, when the sensor chips were prepared (the surface modification protocol is described in the materials and methods section). In this experimental series, we incubated with a ssDNA target comprising only 10 bases, that were complementary to the outermost 10 bases of the surface-grafted capture probe ssDNA. For this 10 base pairs sequence, a salt adjusted melting temperature of 35.9 °C (309 K) was calculated by the *OligoCalc* online tool[48] indicating that at 18 °C (291 K) at least a small fraction of the DNA are expected to be dehybridized, and therefore complete dehybridization by continuous rinsing with buffer might be possible.

Indeed this assumption could be confirmed in additional experiments depicted in **Figure 3** and in **Figure S5** in the supporting information. In **Figure 3** a titration with increasing concentrations of the 10 bases target DNA, as well as the corresponding *Langmuir* isotherm are shown.

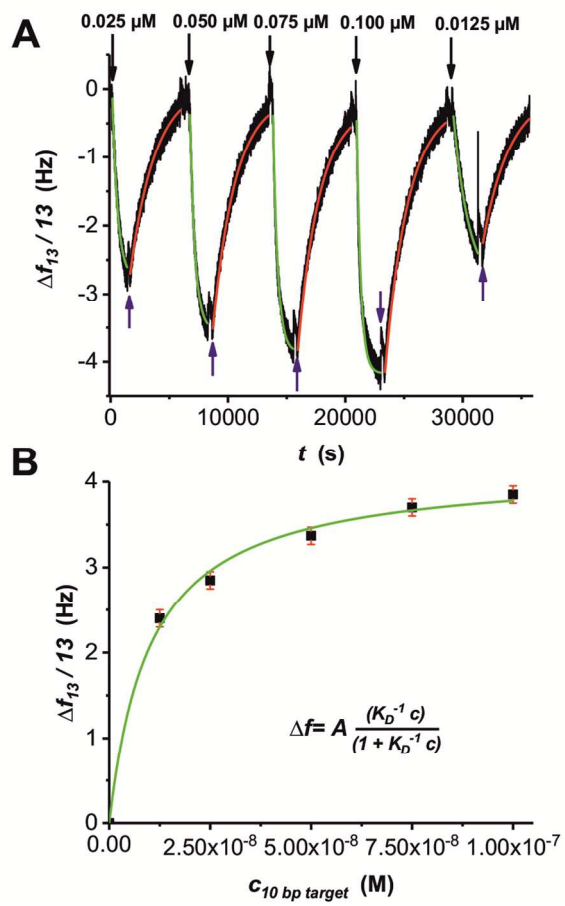


Figure 3. DNA equilibrium titration for 10 bases target DNA concentrations between 0.0125 µM and 0.100 µM (**A**) as well as the corresponding plot of frequency shift *vs.* concentration fitted to a *Langmuir* isotherm (**B**). The results of the 13th overtone are shown. After hybridization was completed, the cell was rinsed with buffer (indicated by blue arrows) to regenerate the surface. The DNA adsorption and desorption processes in (**A**) are fitted by mono-exponential functions shown in green and red, respectively. In (**B**), the maximum frequency shifts recorded for DNA hybridization are plotted against the corresponding target concentrations. The data is fitted to a *Langmuir* isotherm (equation 3) including an additional amplitude factor A to account for the maximum frequency shift expected for an infinite concentration of target DNA.

In contrast to hybridization with 20 base pairs complementary DNA, switching the peristaltic pump on or off had a significant influence on the QCM-D signal when experiments were performed with 10 bases complementary DNA. To avoid any influence of the peristaltic pump on the binding kinetics, the equilibrium titrations as shown in **Figure 3** were performed in a continuous flow of target DNA concentrations (which does not violate the *Langmuir* model as the constant concentration of adsorbate is one of the boundary conditions). Furthermore, by applying a continuous flow it can be assumed that the binding kinetics will not be diffusion limited.[31] After the QCM signal became constant, the pump was switched off followed by an equilibration period of several minutes. Then the inlet tube of the flow cell was dipped in buffer (resulting in a small spike in the QCM signal), and the surface was rinsed with buffer. When we performed an analysis of the binding and unbinding kinetics (shown in **Figure 3A**), we obtained a K_D value ($\pm 2\sigma$) of 16 ± 10 nM (for k_{on} and k_{off} average values ($\pm 2\sigma$) of 35593 ± 33241 M^{-1} s^{-1} and $4.94 \pm 0.81 \cdot 10^{-4}$ s^{-1} were calculated, respectively), which is in good accordance with the K_D value of 10 ± 2 nM obtained by the analysis of the *Langmuir* isotherm (shown in **Figure 3B**). It is also worth to note that the average k_{on} value of 35593 M^{-1} s^{-1} determined from the experimental run performed under continuous flow (shown in **Figure 3A**) does not differ significantly from that of k_{on} = 13652 M^{-1} s^{-1} determined on a different sensor chip without flow (see **Figure S5** in the supporting information). This implies that even without flow there is no significant diffusion limitation for the current system. It is advantageous to prove under continuous flow if the binding event may be diffusion limited, especially if the binding energy is very strong, and every molecule that makes contact with the surface is adsorbed.[31] Then (without flow) the adsorption rate is limited by the rate at which molecules can diffuse from the bulk region to the interface.[31] By having a look at the values determined from the binding and

unbinding kinetics shown in **Figure 3A** (K_D ($\pm 2\sigma$) = 16 ± 10 nM, k_{on} = 35593 ± 33241 M^{-1} s^{-1} and k_{off} = 4.94 ± 0.81 · 10^{-4} s^{-1}) especially the rather large error of the k_{on} value stands out. If the confidence interval is determined for 99.7% (*i.e.* the mean value ± 3σ instead of ± 2σ), even negative values for k_{on} are included (which makes physically no sense). Instead of the currently applied statistics based on a normal distribution, the mean and the confidence limits for 95% can also be calculated based on a log-normal distribution.[49] In fact it has been pointed out that especially for K_D values a log-normal distribution may be more appropriate.[50] From the K_D values determined for the individual titration steps a mean of K_D = 15 nM, with confidence limits for 95% from 9.8 nM to 23 nM was calculated based on a log-normal distribution. Furthermore, from individual k_{on} and k_{off} values, a mean of k_{on} = 32636 M^{-1} s^{-1} with confidence limits 95% from 18954 M^{-1} s^{-1} to 56195 M^{-1} s^{-1}, and a mean of k_{off} = 4.92 · 10^{-4} s^{-1} with confidence limits 95% from 4.38 · 10^{-4} s^{-1} to 5.53 · 10^{-4} s^{-1} were obtained based on a log-normal distribution. The mean values determined for a normal or a log-normal distribution do not differ much, but the error range determined based on a log-normal distribution seems to be more reasonable.

However, if the *Langmuir* model was indeed applicable for the description of the 10 base pairs DNA hybridization process, the hybridization kinetics should linearly depend on the concentration of the target strand as expressed by $\tau^{-1} = k_{on} \cdot c + k_{off}$. This analysis (as shown in **Figure 4**) yielded k_{on} = 11614 ± 4229 M^{-1} s^{-1}, k_{off} = 1.70 ± 0.29 · 10^{-3} s^{-1}, and consequently a K_D value of 146 ± 59 nM. As it is obvious from **Figure 4**, the linear fit does not match the experimental data quite well, and therefore the corresponding value for K_D, which differs by about one order of magnitude from the two other values, which are in good experimental agreement, should not be overestimated. When we tried to analyze a plot of τ^{-1} *vs*. the concentration of the 20 bases target DNA from the data depicted in **Figure 2**, no linear relation

could be found at all. Nevertheless, an exemplary plot including a (formal) linear regression is depicted in **Figure S6** in the supporting information file. As for the 10 bases target two independent experimental approaches, *i.e.* the analysis of the binding and unbinding kinetics and an equilibrium titration with increasing target concentrations, lead to the same K_D value, it seems that DNA hybridization/dehybridization can be described by the *Langmuir* model if the applied DNA sequence is sufficiently short, and the experimental conditions (temperature, buffer composition etc.) are eligible to allow the hybridization to be reversible.

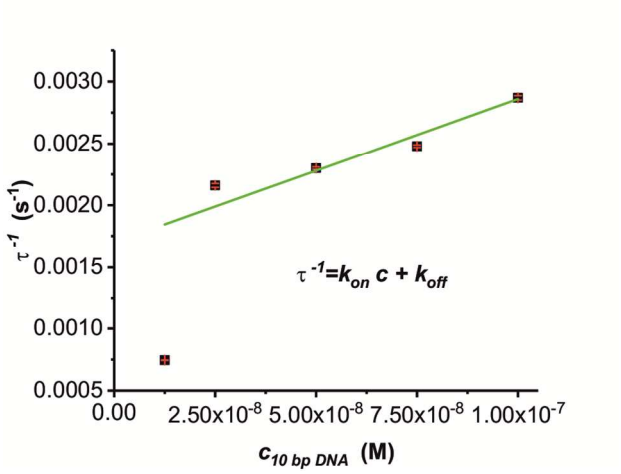


Figure 4. Plot of the kinetic constants τ^{-1} *vs.* the concentration of the 10 bases complementary DNA. With k_{on} =11614 ± 4229 M^{-1} s^{-1}, k_{off}=1.70 ± 0.29 · 10^{-3} s^{-1}, and consequently a K_D value of 146 ± 59 nM, the result differs by approximately one order of magnitude from the values obtained by kinetic analysis (16 ± 10 nM; **Figure 3A**) and fitting of the *Langmuir* isotherm (10 ± 2 nM; **Figure 3B**).

The fact that we did not obtain satisfying results by a plot of τ^{-1} vs. the target concentration could be explained by the limited number of experiments we performed with the shorter targets,

i.e. the experiments are not yet optimized. Alternatively, it might be a general tendency that the error of the latter evaluation method (plot of τ^{-1} vs. the target concentration) is larger than that of the first two approaches. To gain more experience in the determination of binding affinities according to *Langmuir* we decided to measure a second independent system (the apododecin-flavin system) for comparison, for which in previous experiments *Langmuir* behavior has been assumed.[12-13]

Reconstitution and disassembly of dodecin at flavin-terminated DNA monolayers

In addition to the experimental series on DNA hybridization, we studied the reconstitution of dodecin at flavin-terminated dsDNA layers. As known from previous studies,[12-13] a relatively high surface coverage of flavin-terminated dsDNA (≥ 10% flavin-modified oligonucleotides with respect to the total amount of surface grafted dsDNA) results in a significant number of multiple binding events, *i.e.* some apododecin molecules are bound by more than one surface tethered flavin-DNA ligand.[12-13] In this case the *Langmuir* model is not valid; and it is not possible to completely remove the apoprotein molecules bound *via* multiple flavins simply by rinsing with buffer solution. Therefore, we started our binding and unbinding studies with a relative flavin surface coverage of 10% (shown in the Supporting Information, **Figure S7**), but as first experiments revealed that at this surface coverage still a significant amount of apododecin molecules bind more than a single surface tethered flavin, we later continued our studies with a relative flavin surface coverage of 5%. A representative titration experiment with decreasing concentrations of apododecin from 8 µM to 0.5 µM is shown in **Figure 5A**, and the corresponding fit of the frequency shift *vs*. the apododecin concentration to the *Langmuir* isotherm (by equation 3) in **Figure 5B**. Apododecin in buffer solution was incubated at the

surface in various concentrations until the adsorption process reached equilibrium (at least almost). Subsequently, the flow cell was rinsed with buffer until the entire protein layer was removed. We performed three dodecin equilibrium titrations using different sensor chips modified with 5% of flavin-terminated dsDNA.

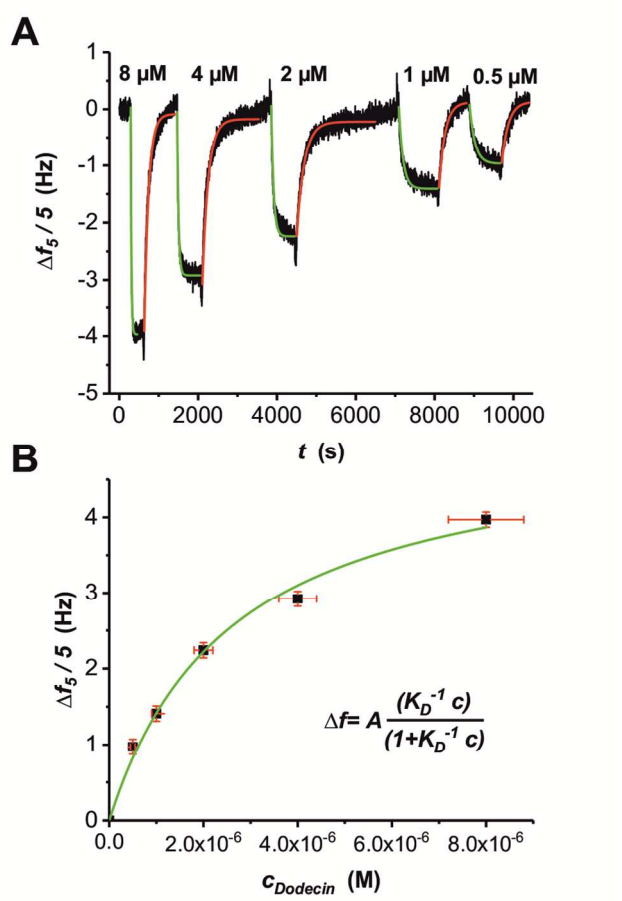


Figure 5. equilibrium titration performed with apododecin concentrations from 8 µM to 0.5 µM at flavin-terminated dsDNA surfaces with a flavin surface coverage of 5% with respect to the total amount of dsDNA (**A**) and the corresponding plot of the frequency shift *vs*. concentration including its fit to the *Langmuir* isotherm (**B**). In both panels, the results of the 5th overtone are shown.

For the titration shown in **Figure 5A** the kinetics of apododecin binding (reconstitution), and unbinding (removal by rinsing with buffer) were fitted to equations 4 and 5, respectively. In contrast to the DNA equilibrium titrations with a sequence of 20 base pairs, rinsing with buffer resulted in complete removal of the dodecin layer from the surface within a rather short time period. Furthermore, the binding as well as the unbinding processes could be described quite well by mono-exponential functions for all concentrations used in the experiment. While the K_D ($\pm 2\sigma$) value obtained from the kinetic data was determined to be 1.587 ± 1.030 µM (the average k_{on} and k_{off} values ($\pm 2\sigma$) were calculated to be 4589 ± 2793 M^{-1} s^{-1} and $6.62 \pm 2.56 \cdot 10^{-3}$ s^{-1}, respectively), the analysis of the corresponding titration curve (**Figure 5B**) yielded a K_D value of 2.628 ± 0.373 µM. Both K_D values agree within the experimental error. Alternatively, based on a log-normal distribution values of K_D = 1.5 µM, with confidence limits for 95% from 1.2 µM to 1.8 µM, k_{on} = 4350 M^{-1} s^{-1} with confidence limits 95% from 2722 M^{-1} s^{-1} to 4350 M^{-1} s^{-1}, and k_{off} = $6.5 \cdot 10^{-3}$ s^{-1} with confidence limits 95% from $5.0 \cdot 10^{-3}$ s^{-1} to $8.5 \cdot 10^{-3}$ s^{-1} were obtained from the kinetic data. These results confirm the assumption that the apododecin-flavin binding system follows the *Langmuir* model. Also for the experiment shown in **Figure 5A** we plotted the inverse of the binding time constant τ^{-1} *vs*. the protein concentration. As shown in **Figure 6**, this time linear fit matches the experimental data quite well. As a result of the linear fit values of $k_{on} = 6215 \pm 435$ M^{-1} s^{-1}, $k_{off} = 4.04 \pm 0.43 \cdot 10^{-3}$ s^{-1}, and $K_D = 0.650 \pm 0.083$ µM were obtained. The latter K_D value of 0.650 ± 0.083 µM agrees within the error with the K_D value of 1.587 ± 1.030 µM, determined from the analysis of the kinetic binding and unbinding curves but differs slightly from the K_D value of 2.628 ± 0.373 µM, derived from the *Langmuir* isotherm. However, the difference between 0.65 µM and 2.63 µM (a factor of 4) is not quite large, and here it seems that the real

error in these values is somewhat larger than calculated by us. Apparently the binding and unbinding of apododecin on a flavin terminated DNA layer can be well described by the *Langmuir* model if the relative flavin surface coverage is sufficiently low to avoid multiple binding events. Coincident results could also be obtained in two additional independent experimental runs, shown in **Figure S8** and **Figure S9** of the supporting information. The K_D values in the range between 0.65 µM and 2.63 µM determined in the current work are also in accordance with K_D values of 0.4 µM to 2.7 µM determined in earlier studies for the apododecin-flavin system.[12-13] The k_{on} (1040 M^{-1} s^{-1} to 6405 M^{-1} s^{-1}) as well as the k_{off} ($2.0 \cdot 10^{-3}$ s^{-1} to $9.0 \cdot 10^{-3}$ s^{-1}) values determined in this study are in the same order of magnitude as in earlier studies (k_{on} between 1300 M^{-1} s^{-1} and 7700 M^{-1} s^{-1}; k_{off} between $3.3 \cdot 10^{-3}$ s^{-1} and $4.5 \cdot 10^{-3}$ s^{-1}).

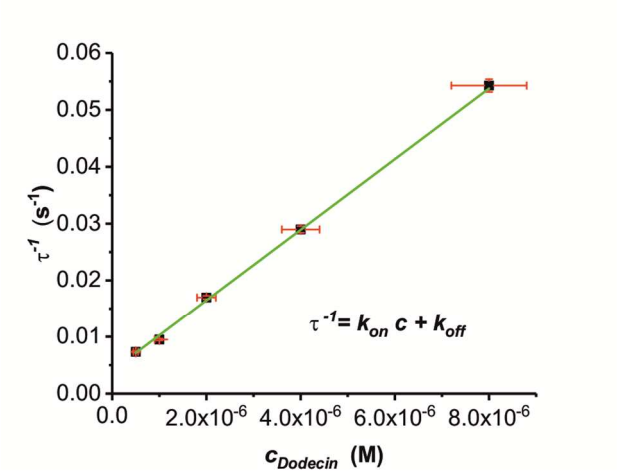


Figure 6. Plot of the inverse of the binding time constant τ^{-1} *vs*. the apododecin concentration, obtained from the kinetic analysis of the absorption process for the experimental data shown in **Figure 5A**. Since the apoprotein was filtered prior to use, an error of ±10% was considered for the apododecin concentration. As a result of the linear fit values of $k_{on} = 6215 \pm 435$ M^{-1} s^{-1}, $k_{off} = 4.04 \pm 0.43 \cdot 10^{-3}$ s^{-1}, and $K_D = 0.650 \pm 0.083$ µM were obtained.

Conclusions

In this work the hybridization and dehybridization of DNA with a length of 20 base pairs at surfaces was investigated. As a main result it turned out that the hybridization/dehybridization of DNA with 20 base pairs does not follow the *Langmuir* model of adsorption if the experiments are carried out at room temperature or slightly below (18 °C in the current study) because at temperatures far below the melting temperature the DNA hybridization is irreversible, and therefore an important boundary condition of the *Langmuir* model, *i.e.* the reversibility of the binding and unbinding process is violated. While the DNA hybridization is irreversible in buffer solution, we have shown that simple and quantitative dehybridization is possible by rinsing with pure water. This offers the possibility to bind a specific target at a capture probe modified surface, to purify the sample by rinsing with buffer solution, and finally to elute by rinsing with pure water.

As we could further show, whether DNA hybridization can be described by the *Langmuir* model or not, depends strongly on the experimental conditions. When we performed additional measurements on a sensor surface modified with the same 20 bases capture probe ssDNA as previously but incubated with a ssDNA target comprising only 10 bases (sequence with a melting temperature of about 36 °C), that were complementary to the outermost 10 bases of the capture probe, DNA hybridization/dehybridization was reversible (because when being closer than 20 °C to the DNA melting temperature at least traces of the DNA stay unhybridized), and the system can be described satisfactorily by the *Langmuir* model. To determine the dissociation constant K_D, three different approaches were compared, whereupon for the 10 bases target a K_D value of 16 ± 10 nM determined (the error was calculated with respect to a normal distribution) as $K_D = k_{off} \cdot k_{on}^{-1}$ from the k_{off} and k_{on} values that were determined by fitting the kinetic binding

and unbinding curves to equations 4 and 5, and the K_D value of 10 ± 2 nM determined from the *Langmuir* isotherm (using equation 3) were in agreement. When in contrast a plot of the inverse of the time constant τ^{-1} *vs*. the target concentration was used to determine the k_{on} and k_{off} values, there was significant deviation of the data from linear behavior. When the k_{on} and k_{off} values were determined nevertheless by a linear fit, values of k_{on} =11614 M^{-1} s^{-1} and k_{off} =$1.70 \pm 0.29 \cdot 10^{-3}$ s^{-1} resulting in a K_D value of 146 ± 59 nM were determined. This K_D value differs from the former two by about one order of magnitude (it is a little larger), but it is expected to be less accurate, because no good linear correlation between τ^{-1} and the target concentration could be obtained. The fact that all three values agree within one order of magnitude implies that in this case (with ten base pairs) the *Langmuir* model can be used to describe the DNA hybridization/dehybridization. When in contrast DNA hybridization with 20 base pairs was studied the K_D values determined by the individual approaches differed by about three orders of magnitude. Of course, additional studies at varying temperatures or using more different target and/or capture probe strands of different length could be carried out to confirm the results of this study more in detail, and besides QCM-D additional techniques such as SPR may be applied, but the main conclusion, *i.e.* whether DNA hybridization/dehybridization can be described by the *Langmuir* model or not, depends on the experimental conditions such as the number of base pairs, the temperature, and the buffer composition, is obvious.

To validate the reliability of our experimental approach we have studied the reconstitution and disassembly of the flavoprotein at flavin-terminated DNA monolayers, as according to previous studies, it is assumed that this system can be well described by the *Langmuir* model. When we calculated the K_D value as $K_D = k_{off} \cdot k_{on}^{-1}$ by fitting the binding and unbinding curves a value of $K_D =$ 1.587 ± 1.030 µM was obtained (the error was calculated with respect to a normal

distribution) which is in good agreement with the K_D value of 2.628 ± 0.373 µM derived by fitting the *Langmuir* isotherm. This time also for the plot of the inverse of the time constant τ^{-1} *vs*. the target concentration a linear relation was obtained, and from the corresponding values of k_{on} = 6215 ± 435 M^{-1} s^{-1} and k_{off} = 4.04 ± 0.43 s^{-1} a K_D value of 0.650 ± 0.083 µM was calculated. Again this value is not in good agreement with the former two (this time it is somewhat smaller). Nevertheless, as all three values are clearly in the same order of magnitude (they differ not more than by a factor of four), and they are also in accordance with the literature values (0.4 µM to 2.7 µM) that were obtained from different biological replicates. The fact that the values determined in the current study by different approaches differ slightly implies that K_D values cannot be determined much more accurately than within one order of magnitude. For the kinetic analysis of the binding and unbinding curves depicted in **Figures 3A** and **5A** we have shown that alternatively to a normal distribution the confidence limits can also be calculated based on a log-normal distribution, and that in the latter case the confidence limits seem to be more reasonable.

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