Chapter 13

Aptamers and Biosensors

Thomas M. A. Gronewold

Abstract

The immobilization procedure to a biosensor surface has a major influence on the measurement results. To characterize the immobilization onto various biolayers, the interaction of DNA anti-thrombin aptamer with the protein thrombin was used as a model system. The aptamer was immobilized to a two-dimensional alkanethiol SAM via carboxylamide bonds and to a three-dimensional dextran matrix via streptavidin–biotin interaction. The calculated K_D values of about 260 and 267 nM, respectively, were comparable, while the amount of bound analyte varied by a factor of 2, depending on the accessibility of the immobilized aptamer. Differences in the specificity were shown by use of the similar protein elastase.

Key words: Surface acoustic wave sensor, DNA anti-thrombin aptamer, immobilization, alkanethiol SAM, dextran.

1. Introduction

Biosensors are devices for measuring the concentration or interaction of biological molecules. A versatile biosensor converts the molecular recognition processes of specific binders such as antibodies or aptamers, to mobile proteins or even whole cells into detectable, preferably electrical, signals. For on-line, real-time, label-free detection of binding effects and in situ measurements in the liquid phase, a whole range of biosensors is in use. Most widespread are optical sensors based on the Surface Plasmon Resonance (SPR) technology. As an alternative emerge Surface Acoustic Wave (SAW) sensors. The sensing technologies differ in their basic principles, but the produced curves resulting from the measurements are very similar, and the typical limit of detection is in the range of 1–20 pg/cm^2 for proteins binding to the sensor

Günter Mayer (ed.), *Nucleic Acid and Peptide Aptamers: Methods and Protocols, vol. 535*

DOI 10.1007/978-1-59745-557-2_13 Springerprotocols.com

surface. The biosensor principle is based on the detection of small mass changes that result from binding of a mobile molecule, the analyte, to an immobilized binding partner coupled to the active sensor surface, the ligand. Binding events are transduced into an electrical signal proportional to the additional mass loading on the sensor surface. Advanced sensors have several channels, enabling parallel measurements. Such sensor signals can be compared directly and reference sensors can be used to distinguish signals of varying fluid parameters as are variations in viscosity or ion concentrations, or unspecific binding events from sensor signals due to specific binding events. Depending on the binding events detected, reliable determination of kinetic constants as are the on-rate k_{on}, the off-rate k_{off}, and the equilibrium dissociation constant $K_D = k_{off}/k_{on}$ is enabled. The K_D value is a measure of how readily analytes sorb to the surface, how tightly they bind to the aptamer surface and how long the analytes remain to be bound.

In our approach, the S-sens® K5 system (Biosensor GmbH, Bonn, Germany) is used, which is based on a physical transducer of the Love-wave surface acoustic wave (SAW) sensor type, measuring at two fixed excitation frequencies *(1)* working in delay-line geometry. The propagation velocity of acoustic shear waves travelling through a guiding layer at the sensor surface is very sensitive to additional mass loading. The sensitivity for small mass depositions depends on changes in the phase velocity in the guiding layer *(1, 2)*. Advantage compared to other sensor types is the elimination of cross-sensitivities even under high damping conditions (fluids with high viscosity or with varying salt contents). Additionally can mass be discriminated from viscoelastic effects by use of both the phase shift and the amplitude shift of the surface acoustic wave *(1)*.

The surface of the SAW sensor chips can be modified by the customer. The sensitivity is determined by the sensor setup. The specificity of a sensor element for a certain analyte strongly depends on the modification of the sensor chip surface. Limiting are the specificity of the coupled ligands, but also the binding method used. Ligands can be coupled to the active sensor area using standard biochemical protocols, providing specific binding-sites for their analytes. Immobilization methods are based on (i) adsorption needing very little preparatory efforts; (ii) fixation to a membrane; (iii) covalent bonds via an interface layer of a spacer molecule with two functional ends; (iv) fixation in a matrix of linked molecules or in a gel coat. One end of such a spacer molecule (iii) binds covalently to the surface. Currently, almost all coupling methods onto the sensor surface are based on the extremely stable sulfur–gold bond *(3)*. Sulfur-containing alkanethiols form two-dimensional self-assembled monolayers (SAMs) on gold or other noble surfaces. The other end of the spacer molecule presents a functional group (*see* **Table 13.1**), which comes in contact with the sensor liquid, enabling chemical

Table 13.1
Bonds formed with commonly used alkanethiols. Alkanethiols with the formula $R–(CH_2)_n–SH$, $n \geq 10$ form stable SAMs with quasi-crystalline domains. Large head groups of alkanethiols as is the carboxyl terminus hinder the development of such quasi-crystalline structures

Alkanethiols with n=10	End group, R	Ligand	Bond
1-Decanthiol	–H	Hydrophobic groups, membranes	Unspecific interactions
1,9-Decane-dithiol	–SH	–SH	Disulfide
11-Mercapto-1-undecanol	–OH	$–PO_4$	Phosphate
11-Mercaptoundecanoic acid	–COOH	$–NH_2$	Carboxylamide

Source: Schreiber, *(17)*.

reactions with a ligand. The head groups determine the enabled binding chemistries and the characteristics of the surface formed. Hydroxy alkanethiols can be used as a basis for the formation of dextran layers (iv), forming a hydrophilic polymer hydrogel, which is highly flexible, non-crosslinked and extends 100–200 nm from the coupling surface under physiological conditions *(4)*. The three-dimensional matrix formed has a much higher immobilization capacity compared to two-dimensional surfaces. Diffusion of small molecules is fast enough not to be hindered. On 3-D matrices, the bound ligands have more degrees of freedom, enabling better binding of their analytes. Both the alkanethiols and the dextran surface are biocompatible, similar to separation media used in protein purification, are long-term durable, and stabilize even sensitive proteins. Dextran surfaces mostly do not require blocking agents and, by nature, show low unspecific binding, which significantly enhances the specificity. The embedding of ligands in a gel-matrix often enhances the biostability of the surface.

To demonstrate the differences in binding, two coupling chemistries are applied as an example. Used are two of the most common binding chemistries. Both are based on surfaces containing carboxyl groups, as alkanethiol head groups (as in iii), and as carboxymethyl-dextran (as in iv). The well-known DNA aptamer against thrombin *(5)* is used as the biological test system. Thrombin is multifunctional with hormone-like properties. As the last protease in the clotting cascade, thrombin plays a central role in thrombosis, haemostasis, blood-coagulation and in platelet activation *(6)*. Elastase is a related serine protease, thus utilized as a reference

protein. It has been shown that linkage of the DNA aptamer to the sensor surface has no effect on the binding properties compared with filter binding experiments. The modified surface can be regenerated several times without loss of functionality *(7)*. Amino-functionalized ligands are coupled via carbodiimide chemistry to the sensor surface, forming carboxylamide bonds *(8)*.

2. Materials

2.1. Surface Cleaning

1. Precleaning solutions: 100% ddH_2O, 100% acetone and 100% isopropanol.
2. Piranha solution: 3:1 mixture of sulfuric acid and 30% hydrogen peroxide, either mixed before application, or the sulfuric acid is applied to the surface first, followed by the peroxide. Be careful, piranha solutions are harmful and have to be handled with extreme caution!
3. Ammonia–peroxide solution: A 5:5:1 mixture of ddH_2O, ammonia (32%) and hydrogen peroxide (30%).

2.2. Surface Preparation

1. Prepare a 2 mM stock solution of alkanethiols (**Table 13.1**). With each 200 µl of a 2 M alkanethiol stock solution, 10 ml of a 200 mM ethanolic solution can be prepared (dilution 1:50 v/v). Some alkanethiols are of low solubility.
2. Activation solution: 0.6 M epichlorhydrin in 0.4 N NaOH. Prepare 10 ml 0.4 N NaOH and add 1 ml epichlorohydrin. Add 10 ml diglyme (diethylene glycol dimethyl ether; *see* **Note 1**).
3. Dextran with a molecular weight of about 400,000–500,000 Da (from *Leuconostoc mesenteroides*, Sigma D1037) (Shorter dextran molecules might be applied, e.g. when the penetration depth of the sensor is reduced).
4. Dextran solution: 1 g dextran (**Step 3** above) in 3.3 ml 0.1 N NaOH.
5. Carboxylation solution: 5 g bromoacetic acid in 7.7 g 2 N NaOH.

2.3. Immobilization Procedures

1. Carboxyl activation solution: 400 mM 1-[*3*-(dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride (EDC, 8.00907.0005, Merck) and 100 mM *N*-hydroxysuccinimide (NHS, H-7377, Sigma). Mix 1:1 immediately before use, resulting in a mixture containing 200 mM EDC and 50 mM NHS. Prepare solutions fresh as required.
2. 2 µM aqueous solution of anti-thrombin aptamer carrying a 5′-amino linker with the sequence 5′-NH_2-$(CH_2)_3$-GGT TGG TGT GGT TGG-3′, synthesized by Operon. The ligand might

be diluted in water, or in buffer at a pH close to the isoelectrical point.

3. 200 µg/ml Streptavidin (Molecular Probes, S888).
4. 2 µM aqueous solution of anti-thrombin aptamer with the sequence 5′-BioTEG- GGT TGG TGT GGT TGG-3′. A 5′-biotin moiety was attached via a 15-atom spacer through the 4-carboxy group of the aptamer. It was synthesized by Operon.
5. Phosphate buffered saline with $MgCl_2$ ($MgCl_2$-PBS): 140 mM NaCl, 2.7 mM KCl, 1 mM $MgCl_2$, 10 mM Na_2HPO_4, pH 7.4.
6. Capping solution: 1 mM ethanolamine-hydrochloride, pH 8.5. Important for a successful deactivation is the use of a redistilled ethanolamine (Aldrich, 41.100-0).

2.4. Protein Binding

1. Binding buffer: 20 mM Tris–HCl, pH 7.4, 140 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$.
2. Human α-thrombin (Haematologic Technologies, Essex Junction, VT, USA), molecular weight 33,600 g/mol. Dilutions of 1–1,000 nM were performed in binding buffer **Section 2.4**, **Step 1**.
3. Elastase type I from porcine pancreas (E-1250, Sigma), molecular weight 25,900 g/mol. Dilutions of 1–1,000 nM were performed in binding buffer **Section 2.4**, **Step 1**.
4. Regeneration solution 0.1 NaOH in running buffer.

3. Methods

The functionalities of the aptamers and the specificity of the binding of analytes to the aptamer-modified sensor surface mostly depend on the stringency of the conditions applied to the SELEX procedure. When applied to sensors, aptamers bind directly to the gold surface, but without a proper orientation. An orientation is achieved by use of thiol-derivatized oligonucleotides coupled via carbodiimide chemistry to alkanethiol surfaces, or by binding to dextran surfaces. The amount of background noise from unspecific binding to dextran surfaces is significantly lower than to alkanethiol surfaces. Unspecific or unwanted binding can have a number of reasons. It can be attributed to increasing unspecificity of aptamers at excessive concentrations. Often bind very short aptamers, as is the 15-mer used, not only their cognate analyte, but also other molecules with similar patches. The modern techniques fabricate longer, but also highly specific aptamers.

The immobilization procedure might also affect the binding specificity to the surface itself. Basic principles for biosensors apply, e.g. the size ratio of ligand to analyte. To prefer is coupling of the smaller molecule. For very large ligands it becomes increasingly difficult to measure binding of very small molecules. Low amounts of small molecules result in very small signals, also influenced by the choice of the surface (–chemistry).

In general, the sensor chip surface is composed of gold. Its preparation with a carboxymethyl dextran layer is a process which allows forming hydrogels of different thicknesses, depending on the molecular weight of the dextran material used *(9)*. Since the S-sens K5 technology does not require gold as the sensitive surface, alternative coupling methods might be applied, e.g. binding of organosilanes to SiO_2 surfaces. The lifetime of a modified chip is limited. The first factor is the regeneration. For most oligonucleotides, the binding capacity only changes after the first few injections to remain stable afterwards. Antibody-modified surfaces are more challenging, since they are continuously decreasing in their binding capacity when regenerated. Second factor is the growth of bacteria and yeast. Dextran, for example and other organic materials bound to the surface are subject to decay (fouling) if stored in aqueous solutions outside the constant flow of the sensor. The sensor chips equipped with a dextran surface are to be stored against air at 4°C, even when oligonucleotides are bound. The three-dimensional structure of most proteins would be destroyed. After usage, the sensor chip surface can be stripped from all bound materials and especially organic residues by harsh methods as are chemical or plasma etching. This regenerates the surface completely down to the bare sensor surface without destroying the sensor chips.

3.1. Cleaning of the Sensor Chip Surface

1. The sensor chips are precleaned by three 3-min washing steps in an ultrasonic bath sonifier with precleaning solutions (*see* **Section 2.1**) removing salts and organic residues.
2. The washes are followed by etching in O_2 plasma formed in a TePla etcher at 300 W. Alternatively used are chemical etching procedures (**Steps 3** and **4** below).
3. Chemical stripping using piranha solution (*see* **Section 2.1**) for 1 min.
4. Chemical stripping by heating the chips in ammonia–peroxide solution (e.g. H_2O_2, 30%: H_2O: ammonia, 32% at 1:5:1) to a temperature of >65°C for 5 min.
5. After chemical stripping, the chips are carefully cleaned with ddH_2O. Dry with a stream of nitrogen or argon and store at 4°C.

3.2. Reuse of the Sensor Chips and Storage

1. Sensor Chips: The sensor chip surface is very resistant. The methods mentioned in **Section 2.1** are not harmful to the sensor chip. But frequent usage might mechanically wear off the layers on the chip and produce scratches. About 50 usages per quartz chip are feasible by careful usage.
2. Biological layers: When a biologically active surface is created in the S-sens K5 fluidics system, the sensor chip can be reused hundreds of times. This is only limited by the biosurface created. Aptamer surfaces are simply regenerated by pH changes, e.g. by use of 0.1 N NaOH solution. In between, the chips are storable outside the sensor machine in plain water at 4°C for reuse. Aptamer surfaces might even be stored against air at 4°C for several years.
3. Aptamers: Stored at –20°C stable for many years without any loss in activity.

3.3. Preparation of Alkanethiols Monolayers

1. Freshly clean the sensor chip surface with solutions **Section 2.1, Step 1**. Each washing step is performed for 3 min in a sonifier.
2. Remove all remains off of the chip surface by chemical or plasma etching according to **Section 3.1**. Place the chip into an inert (glass) container.
3. Self-assembled monolayers are formed by adsorption of solution listed in **Section 2.2**. Cover the chip surface with about 2–5 ml of the solution (*see* **Note 2**). Store dark for at least 12 h and allow the formation of a SAM over night. Longer incubation times are required to ensure proper, densely packed SAMs.
4. Remove unbound alkanethiols: Sonicate for 3 min. Remove the thiol solution and wash the coated sensor chip three times with pure solvent ethanol. Dry the sensor chip with a stream of dry nitrogen or argon.
5. Store at 4°C. SAMs are very stable, but might oxidize over time at long storage.

3.4. Preparation of Dextran-Based Hydrogels

1. Prepare a SAM of 11-mercapto-1-undecanol (Sigma) (according to **Section 3.3**). Place each sensor chip into a small glass container. The total time to prepare the chips is 4 days depending on the long incubation times.
2. Activate the hydroxyl groups of the SAM with activation solution. Add 2.5 ml to each chip and incubate at room temperature for 4–5 h.
3. Wash three times with ddH_2O, then twice with 100% ethanol and three times with ddH_2O.
4. Prepare dextran solution and drip onto the sensitive surface. Incubate at room temperature over night. The thickness of the

resulting dextran layer depends on the molecular weight of the dextran. The dextran used forms a layer with a thickness of about 200 nm.

5. Wash thoroughly with 50°C ddH_2O (about 15 times).
6. Add 500 µl of carboxylation solution (*see* **Section 2.2, Step 5**) to each chip. Incubate at room temperature for about 4 h. The hydroxyl groups in the dextran layer form carboxymethyl groups.
7. Repeat **Section 3.4**, **Step 5**, and add 2.5 ml of carboxylation solution (*see* **Section 2.2, Step 5**) Incubate at room temperature over night. Repeat **Section 3.4, Step 5**.
8. Dry the sensor chips with dry N_2 or argon and store at 4°C.

3.5. Measurement Using the S-Sens® K5 Sensor

1. These instructions assume the use of an S-sens® K5 biosensor. Place the sensor chip with the interface into the sensor.
2. Start the continuous buffer flow at about 30–40 µl/min. The flow rate is limited to 10–300 µl/min (*see* **Note 3**). The higher the flow rate, the higher is the shear stress applied.
3. Take a spectrum between frequencies 145 and 155 MHz and choose two frequencies so that the phase difference is at about 180°. This results in maximal sensitivity.
4. Start measurement. Recorded as output signals are phase shift and amplitude of the propagating wave. The baseline stabilizes according to the surface within minutes. Dextran might be subject to swelling, increasing the time for stabilization.
5. At a constant baseline, inject samples into the buffer flow using the connected autosampler. A volume of up to 500 µl can be injected from an injection loop. The size of the flow chamber is about 2.4 µl per sensor element. The total contact time is the coefficient of amount of substance injected and flow rate, e.g. for 200 µl injected at 40 µl/min is the contact time about 300 s.
6. After injection, running buffer is pumped over the surface. Excess, non-hybridized analyte molecules are expelled from the cell. The next injection cycle can start.
7. From the recorded phase and amplitude signals, the amount of binding and viscosity changes, respectively, can be extracted. By use of a reference solution, the mass signal might be separated from the viscosity signal. An injection of 5% glycerol gives a viscosity signal in the range of most biological measurements.

3.6. Immobilization of Aptamers to a SAM

1. Insert a sensor chip with a 11-mercaptoundecanoic acid SAM into the S-sens® K5 and start measurement with ddH_2O as running buffer.

2. Inject carboxyl activation solution **Section 2.3, Step 1** (*see* **Note 4**).
3. Inject aptamer bearing a primary amine **Section 2.3, Step 2** (*see* **Fig. 13.1**). Carbodiimide chemistry is commonly used to couple molecules presenting a primary amine to sensor surfaces presenting carboxyl groups, forming carboxylamides (*see* **Note 5**).
4. Inject capping solution **Section 2.3**, **Step 6** (*see* **Note 6**).

3.7. Immobilization of Aptamers to a Dextran Surface Forming a Biotin–Streptavidin Complex

1. Insert a sensor chip with a carboxymethyl-dextran layer (**Section 3.4**) into the S-sens® K5 and start measurement with ddH_2O as running buffer (*see* **Note 7**).
2. Inject carboxyl activation solution.
3. Inject streptavidin solution.
4. Inject capping solution.
5. Exchange buffer to $MgCl_2$–PBS buffer.
6. Inject biotinylated aptamers (*see* **Fig. 13.1**).

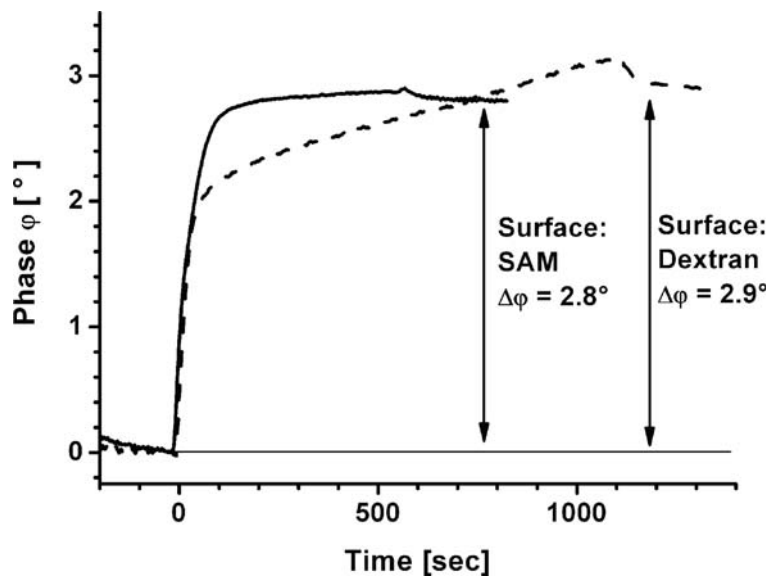

Fig. 13.1. Immobilization of ligand 2 μM DNA anti-thrombin aptamer at a flow rate of 40 μl/min in running buffer PBS-$MgCl_2$. The *dashed line* shows coupling of 200 μl of NH_2–$(CH_2)_3$–aptamer (**Section 2.3**, **Step 2**) to a COOH SAM via carbodiimide chemistry and the solid line 300 μl of biotinylated aptamer (**Section 2.3**, **Step 4**) to a streptavidin-modified dextran surface (**Section 3.7**). Both binding curves show an immediate increase. At 60 s after begin of injection, the SAM was saturated, while the amount of binding to the dextran surface was progressing until the injection stopped, indicating the larger binding capacity of the 3-D surface. The capacity was not saturated afterwards, but was limited by the amount presented. For the 2-D alkanethiol surface, it is necessary to adjust the amount of ligand. In general, higher total amounts can be immobilized to the 3-D surface.

3.8. Specific Binding of Proteins to a Biosensor Surface

1. Immobilize the aptamer according to **Section 3.6** or **3.7**.
2. Exchange running buffer to binding buffer, favouring specific binding of the cognate analyte in high amounts.
3. Inject sample fluid containing, or suspected of containing, analyte at desired concentrations. The mobile proteins elastase as a reference and analyte thrombin were injected.
4. Running buffer automatically was flowed over the surface to determine the off-rate of analytes detaching from the immobilized aptamers.
5. Regeneration solution is injected to preferably regenerate the sensor surface completely, detectable at the return of the signal down to baseline.
6. New cycles of binding and regeneration can follow starting with **Section 3.7**. **Step 5** (*see* **Fig. 13.2**).

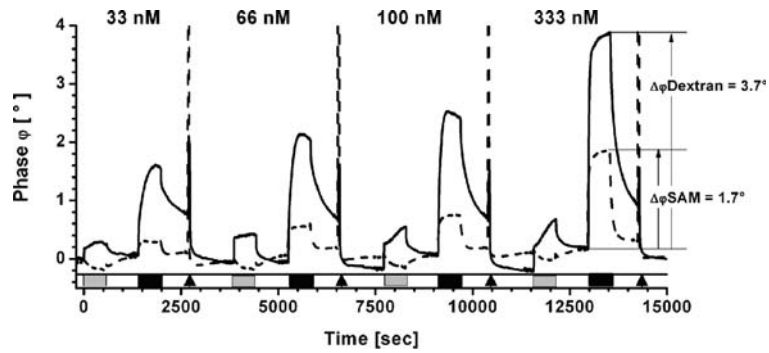

Fig. 13.2. Binding to SAM surfaces (*dashed line*) and dextran (*solid line*) modified by anti-thrombin aptamer (*see* **Fig. 13.1**). Buffer was exchanged to binding buffer. Elastase (*grey bars*) and thrombin (*black bars*) were injected at increasing concentrations from 1 to 1,000 nM. Subsequently, buffer was flowed over the surface to dissociate unbound protein and the surface was regenerated to baseline by an injection of 0.1 N NaOH (indicated by *triangles*). For clarity, injections at medium concentrations of 33, 66, 100 and 333 nM are displayed. The system is based on specific binding of the protein in the sample to surface-immobilized aptamers. In each injection, a specifically bound protein–aptamer complex is formed according to the available and reactive aptamers and to the K_D value of the ligand. Each analyte molecule binds according to the association constant, depending on the space spared by the previously bound analyte molecules. The surface was regenerated and the next round of binding was started. The binding experiments to the dextran surface resulted in a phase shift of 0.3° for 1 μM elastase. With a sensitivity of 515 [° cm^2 / μg], this equals to about 0.58 ng/cm^2 or 14 fmol/cm^2. The injection of 1 μM thrombin resulted in $\Delta\varphi = 3.7°$. This equals to about 7.2 ng/cm^2 and 196 fmol/cm^2. The same experiment on a SAM resulted in a phase shift of 1.7° for 1 μM thrombin, equalling to about 3.3 ng/cm^2 and 90 fmol/cm^2. The amount of thrombin bound to the SAM surface was only about 45% of the dextran surface. The total amount of immobilized aptamer differed by only about 4%. Thus, the additional amounts of bound thrombin to the 3-D dextran surface result from the approachability of immobilized aptamers from more sides compared to the flat, 2-D SAM. The fraction of elastase which equals to about 5–7% of the bound thrombin also is detectable in filter binding experiments and was already shown in previous sensor experiments *(7)*.

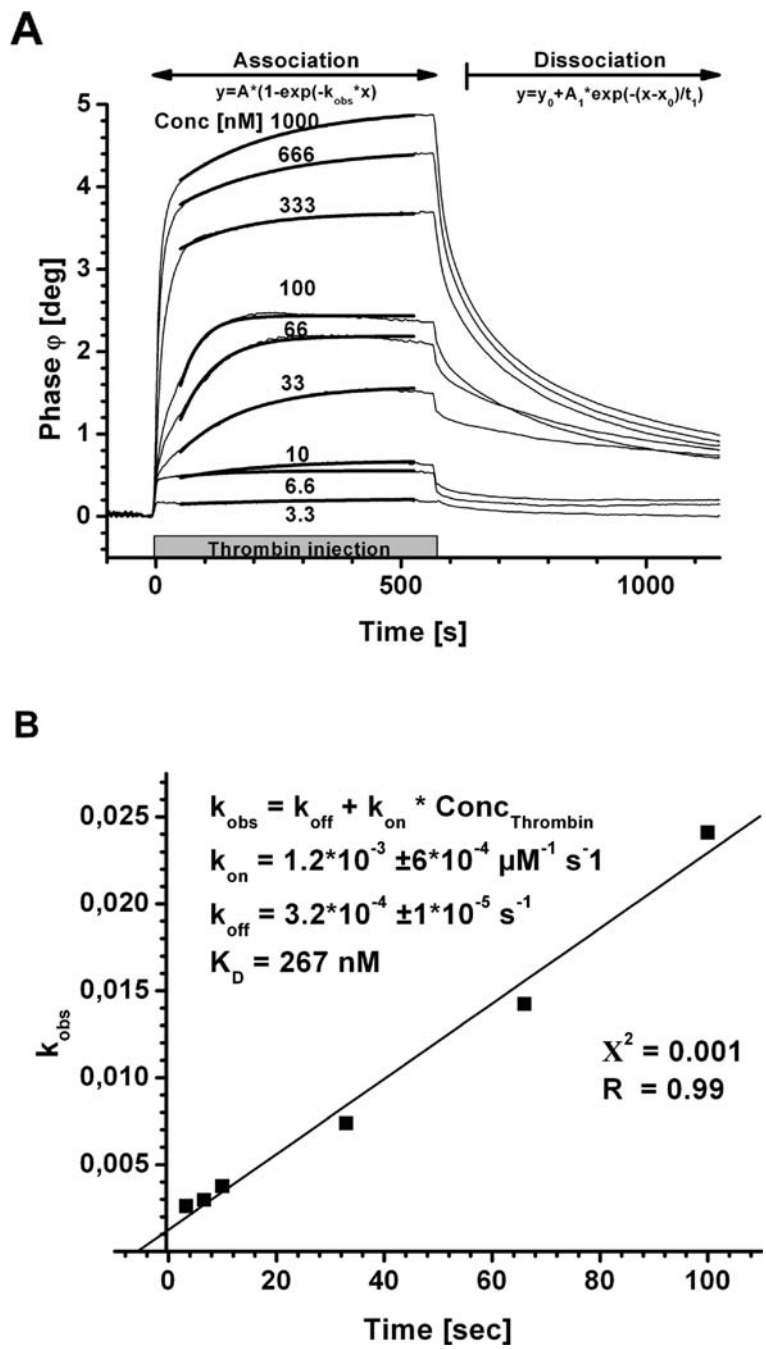

Fig. 13.3. Overlay plot and analysis of injections at different concentrations of thrombin taken from **Fig. 13.2**. (**A**)**.** Overlay plot with combined signals of injections to a aptamer-modified dextran surface. Fits for association of thrombin were applied under the assumption of a 1:1 binding model with $A =$ association signal, maximum binding extrapolated to infinite long injections, and $k_{obs} =$ pseudo-first-order kinetic constant. Fits for dissociation of thrombin were applied, with $y_0 =$ off-set, $x_0 =$ end of injection and begin of buffer injection, $A_1 =$ dissociation signal, and $t_1 =$ decay constant (half-life

3.9. Evaluation of Data

1. Evaluation of bound masses (*see* **Fig. 13.2**) *(7)*: Differences in phase and amplitude are measured as an interval, e.g. at the starting and end point of an injection (*see* **Fig. 13.2**). The phase shift is assumed to be directly proportional to mass or fluid loading on the sensor surface and is identical for all five sensor elements. For proteins, after subtraction of the reference, bound masses can be calculated using a sensitivity of the S-sens® K5 515 ° $cm^2/\mu g$ assumed for proteins (determined analogous to *(10)*). With the molecular weight, the concentrations can be calculated.
2. Extraction of kinetic data: The program Fitmaster (Biosensor GmbH, Bonn, Germany) was used, based on the Origin Software 7.5G SR6 to overlay the binding events into a single plot and to extract kinetic data *(11)*. Binding of elastase and thrombin at concentrations from 1 to 1,000 nM were monitored (*see* **Fig. 13.3A**) and calculated k_{obs} values were plotted versus concentration of the injected fragments (*see* **Fig. 13.3B**) to extract kinetic data (*see* **Fig. 13.3**).

4. Notes

1. Diglyme is volatile and has a low surface tension.
2. In each step, enough material should be prepared for all sensor chips to ensure a constant concentration. Chip-to-chip variations make it more difficult to compare different measurements.
3. The speed at which the running buffer is pumped over the surface equals the product transport. It determines the contact time of analyte and aptamer. At increased speed, the length of contact time is decreased, and the shear force applied to surface-bound analytes and ligands is increased. Based on the shear force, strong, specific binding events are preferred, while weaker binding is reduced. This might be applied to small analytes, since large objects are affected stronger. Simultaneously, the amount of injected sample might increase, too.

Fig. 13.3. (continued) of complex). (**B**) The k_{obs} values extracted from the sensor signals in (**A**) plotted versus concentration of injected thrombin. A linear best fit was applied to the data using the equation shown with k_{on} = association rate constant (on-rate) and k_{off} = dissociation rate constant (off-rate). $K_D = k_{off}/k_{on}$ = dissociation constant. The fits applied resulted in a $k_{on} = 1.2*10^{-3} \pm 6*10^{-4}\ \mu M^{-1}\ s^{-1}$, a $k_{off} = 3.2*10^{-4} \pm 3*10^{-4}\ s^{-1}$, resulting in a $K_D = 267$ nM for the dextran surface. For the SAM, a $k_{on} = 2.8*10^{-3} \pm 1*10^{-3}\ \mu M^{-1}\ s^{-1}$, a $k_{off} = 7.3*10^{-4} \pm 4*10^{-5}\ s^{-1}$ were calculated, resulting in a $K_D = 260$ nM. The resulting K_D values of about 260 and 267 nM agree well with the previously obtained value in the range of 300–400 nM obtained with a preliminary model to the S-sens K5 sensor and with a number of alternative methods *(7)*.

Low amounts of an expensive, delicate or hard to obtain analyte are to be injected at reduced flow rates.

4. The fast reaction of EDC with carboxyl containing molecules forms an *O*-acylisourea as an amine-reactive intermediate. The intermediate is stabilized by the NHS, forming an amine reactive NHS–ester *(12, 13)*. The NHS increases the efficiency of EDC-mediated reactions.
5. The amount of bound ligand is directly related to the concentration of ligand applied and to the number of accessible activated esters on the sensor surface. Therefore, for 2-D surfaces as are SAMs, lower amounts are bound than to three-dimensional surfaces. The concentration of aptamers can be in the high nanomolar to low-micromolar range. Lower concentrations are used for larger ligands or analytes to avoid steric hindrance.
6. Small blocking agents can be added after conjugation to terminate the chemical reaction and to quench any unreacted primary amines. The compounds containing primary amines will result in modified carboxyl groups on the surface ("capping"). Examples for primary amines used are 1–50 mM of hydroxylamine or substances containing a primary amine such as lysine, glycine, ethanolamine, or Tris. Most commonly used is ethanolamine, *see* **Section 2.3, Step 6**.
7. Mostly, proteins are bound directly. But both the carboxymethyl-dextran layer and the oligonucleotide-based aptamers are negatively charged, resulting in an electrostatic repulsion. Salt will reduce the repulsion between them. But to bind high amounts of oligonucleotides, biotinylated aptamers are coupled to a CM-dextran chip surface with covalently immobilized streptavidin. The affinity of 244 Da vitamin H biotin for the bacterial streptavidin is very strong *(14, 15)*. Each tetrameric streptavidin molecule can bind up to four single biotin molecules with positive cooperativity between the subunits *(16)*. Advantage of this attachment system are (i) biotinylated compounds are bound with the correct orientation. (ii) Non-specific binding is reduced, since streptavidin has no carbohydrate group and an isoelectric point of 5. (iii) The complexes formed are extremely stable over a wide range of temperature and pH, unaffected by most organic solvents and even denaturing agents. (iv) Various biomolecules including proteins and antibodies can be biotinylated. Oligonucleotides can be synthesized with biotin moieties at almost every position and in any number. The position of linkage determines the efficiency of biological material interacting with an analyte. (v) The length of the tethering arms covalently attached to biotin determines the binding capacity, the accessibility and flexibility at solid–fluid interfaces and the binding kinetics for analytes.

Acknowledgments

I would like to thank Antje Baumgartner for technical assistance and Ulrich Schlecht for helpful discussions.

References

1. Perpeet, M., Glass, S., Gronewold, T., Kiwitz, A., Malavé, A., Stoyanov, I., Tewes, M. and Quandt, E. (2006) SAW sensor system for marker-free molecular interaction analysis. *Anal. Lett.* **39**, 1747–1757.
2. Du, J.K., Jin, X.Y., Wang, J. and Xian, K. Love-wave propagation in functionally graded piezoelectric material layer. *Ultrasonics* **46**(1) (2007):13–22.
3. Dubois, L.H. and Nuzzo, R.G. (1992) Synthesis, structure, and properties of model organic surfaces. *Annu. Rev. Phys. Chem.* **43**, 437–463.
4. Schäferling, M. and Kambhampati, D. (2004) Protein microarray surface chemistry and coupling schemes. In: Kambhampati, D. (ed.), Protein Microarray Technology. Wiley-VCH Verlag, Weinheim, pp. 11–37.
5. Bock, L.C., Griffin, L.C., Latham, J.A., Vermaas, E.H. and Toole, J.J. (1992) Selection of single-stranded DNA molecules that bind and inhibit human thrombin. *Nature* **355**, 564–566.
6. Stubbs, M.T. and Bode, W. (1993) A player of many parts: the spotlight falls on thrombiń s structure. *Thrombosis Res.* **69**, 1–58.
7. Gronewold, T.M. A., Glass, S., Quandt, E. and Famulok, M. (2005) Monitoring complex formation in the blood-coagulation cascade using aptamer-coated SAW sensors. *Biosens. Bioelectron.* **20**, 2044–2052.
8. Duevel, R.V. and Corn, R.M. (1992) Amide and ester surface attachment reactions for alkanethiol monolayers at gold surfaces as studied by polariziation modulation FTIR. *Anal. Chem.* **64**, 337–342.
9. Löfås, S. and Johnnson, B. (1990) A novel hydrogel matrix on gold surfaces in surface plasmon resonance sensors for fast and efficient covalent immobilization of ligands. *J. Chem. Soc. Chem. Commun.* **21**, 1537–1544.
10. Schlensog, M., Gronewold, T., Tewes, M., Famulok, M. and Quandt, E. (2004) A love-wave biosensor using nucleic acids as ligands. *Biosens. Actuators.* **101**, 308–315.
11. Gronewold, T. Baumgartner, A., Quandt, E. and Famulok, M. (2006) Discrimination of single mutations in cancer-related gene fragments with a surface acoustic wave sensor. *Anal. Chem.* **78**, 4865–4871.
12. Grabarck, Z. and Gergely, J. (1990) Zero-length crosslinking procedure with the use of active esters. *Anal. Biochem.* **185**, 131–135.
13. Staros, J.W., Wright, R.W. and Swingle, D.M. (1986) Enhancement by N-hydroxysulfosuccinimide of water-soluble carbodiimide-mediated coupling reactions. *Anal. Biochem.* **156**, 220–222.
14. Kuntz, I.D., Chen, K., Sharp, K.A. and Kollmann, P.A. (1999) The maximal affinity of ligands, *Proc. Natl. Acad. Sci. USA*, **96**, 9997–10002.
15. Böhm, H.-J. (1994) The development of a simple scoring function to estimate the binding constant for a protein ligand complex of known 3-dimensional structure. *J. Comput. Aided Mol. Des.* **8**, 243–256.
16. Williams, D.H., Stephens, E. and Zhou, M. (2003) Ligand binding energy and catalytic efficiency from improved packing with receptors and enzymes. *J. Mol. Biol.* **329**, 389–399.
17. Schreiber, F. (2000) Structure and growth of self-assembling monolayers. *Prog. Surf. Sci.* 65, 151–256.