Chapter 31

Surface Acoustic Wave (SAW) Biosensors: Coupling of Sensing Layers and Measurement

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Abstract

Surface acoustic wave (SAW) devices based on horizontally polarized surface shear waves enable direct and label-free detection of proteins in real time. Signal response changes result mainly from mass increase and viscoelasticity changes on the device surface. With an appropriate sensor configuration all types of binding reactions can be detected by determining resonant frequency changes of an oscillator. To create a biosensor, SAW devices have to be coated with a sensing layer binding specifically to the analyte. Intermediate hydrogel layers used within the coating have been proven to be very suitable to easily immobilize capture molecules or ligands corresponding to the analyte. However, aside from mass increase due to analyte binding, the SAW signal response in a subsequent binding experiment strongly depends on the morphology of the sensing layer, as this may lead to different relative changes of viscoelasticity. Bearing these points in mind, we present two basic biosensor coating procedures, one with immobilized capture molecule and a second with immobilized ligand, allowing reliable SAW biosensor signal responses in subsequent binding assays.

Key words: Biosensor, Surface acoustic wave, SAW, SAW resonator, Protein coupling, Protein detection, Surface modification, Dextran, Polyethylene glycol, Carbodiimide chemistry

1. Introduction

1.1. SAW-Based Biosensors

Special types of surface acoustic wave (SAW) devices providing horizontally polarized surface shear waves allow the design of SAW-based biosensors for direct and label-free detection of biomolecules in real time (1–3). A SAW device typically consists of a piezoelectric substrate, such as lithium tantalate ($LiTaO_3$) or quartz, with interdigital transducers (IDT) as a planar electrode structure. The SAW is generated on the substrate by applying a high-frequency alternating voltage via the IDTs. For the detection of proteins the device surface has to be coated with capture

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molecules or ligands binding specifically to the analyte of interest (3). Binding reactions on the biosensor surface are detected by determining changes in surface wave velocity caused mainly by mass adsorption or viscosity changes in the sensing layer (4–6). In the literature two different IDT designs are typically used: the SAW resonator (3, 7) and the SAW delay line design (3, 8, 9). The latter provides a free sensor surface without electrode structures allowing any coating including metallization. In the case of a gold coating, standard procedures based on alkane thiols (10) forming self-assembled monolayers (SAM) are typically used to compose the sensing layer. In contrast to this, the SAW resonator design does not permit any conductive layer because the sensor surface is completely covered with IDT electrodes. As we work exclusively with SAW resonators, the procedures described in this chapter use nonconductive coatings only. However, they are basically suitable for both types of SAW biosensor designs. In addition, these procedures can be applied on other acoustic sensing devices such as Lamb wave or Love wave devices and even on quartz crystal microbalances.

Generally, direct adsorption of the layer components on the SAW device surface would be the easiest way; however, as this method usually suffers from a lack of reproducibility, other immobilization methods, such as covalent coupling, are preferred (11). Prior to the application of biospecific layers the SAW resonators should be coated with a suitable polymer to chemically homogenize the device surface. A suitable material for this purpose is parylene C (poly-(2-chloro-*p*-xylylene)), which can be deposited by chemical vapor deposition methods (CVD) at room temperature (12). Parylene layers provide a chemically stable and homogeneous surface with almost no intrinsic mechanical stress and low intrinsic attenuation for the surface acoustic waves. Covalent binding on the inert parylene surface is possible by means of activation via plasma treatment and subsequent silanization of the parylene (13). However, for the detection of proteins it is useful to couple the corresponding capture molecules not directly on the parylene layer but via an intermediate hydrogel layer (7, 13). This approach enables mild reaction conditions so that capture molecules, e.g., antibodies, will keep their functionality (14). Furthermore, hydrogels have been shown to be good shielding layers against nonspecific interactions (15).

1.2. Effect of the Intermediate Hydrogel Layer on the SAW Signal Response

For our biosensor coatings we use two types of hydrogels, dextrans or polyethylene glycols (PEG), for coupling of capture molecules or ligands (6). Dextrans immobilized on the sensor surface typically represent a three-dimensional (3D) hydrogel scaffold, because functional groups occur not only on the top but also within the hydrogel layer. On the other hand, PEGs immobilized in an end-on configuration (i.e., the polymer chains are aligned perpendicularly

to the device surface) represent a two-dimensional (2D) hydrogel scaffold since functional groups occur only on the top of the hydrogel layer. In principle, hydrogels based on 3D scaffolds should allow a greater number of binding sites in the sensing layer, implying a higher binding capacity than hydrogels based on 2D scaffolds.

Basically, any analyte binding in these hydrogel layers leads to mass loading and thus to a frequency shift of the sensor response. However, mass increase is not the only effect influencing the SAW biosensor signal. Another important parameter is the change of viscoelasticity of the sensing layer due to analyte binding. Changes of viscoelasticity are, amongst other factors, greatly influenced by the initial layer thickness with regard to the penetration depth of the SAW as well as changes of other viscoelastic properties based on the morphology of the complete layer setup (16). Hence, this effect may be promoted by sensing layers based on 3D scaffolds allowing analyte binding within the layer. A change of viscoelasticity during analyte binding might counteract or add to the effect of mass loading, i.e., leading to reduced or increased SAW signal responses (5, 6, 16, 17).

Morphology and thickness of the intermediate hydrogel layer influence the signal response of SAW biosensors, depending on the type of applied affinity assay and on the amount of binding sites in the sensing layer; the latter provided by capture molecules or ligands corresponding to the analyte. Only a limited amount of capture molecules can usually be immobilized in the sensing layer due to size and availability. In this case, a relatively thin 3D hydrogel layer such as dextran with a low molar mass implying low chain length, or a 2D hydrogel layer like PEG should be used. On the other hand, ligands, i.e., small molecules interacting specifically with the analyte, may allow a large number of binding sites in the sensing layer. In this case, a relatively thick 3D hydrogel layer such as dextran with a higher molar mass implying higher chain length should be used. In consequence, depending on the amount of the immobilized binding partner desired, the hydrogel has to be chosen carefully considering layer thickness and morphology to get an optimal signal response (5, 6).

1.3. Immobilization of Capture Molecule or Ligand

As previously mentioned, it is advantageous to couple capture molecules or ligands corresponding to the analyte via an intermediate hydrogel layer. In particular, hydrogels providing carboxyl groups allow the covalent coupling of capture molecules or linker proteins, as well as ligands, under mild reaction conditions by means of carbodiimide chemistry (Fig. 1). In this case, the carboxyl groups are activated by a carbodiimide, e.g., 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), and converted into an active ester, usually by means of *N*-hydroxysuccinimide (NHS). The NHS-ester reacts with the amino groups of proteins. The

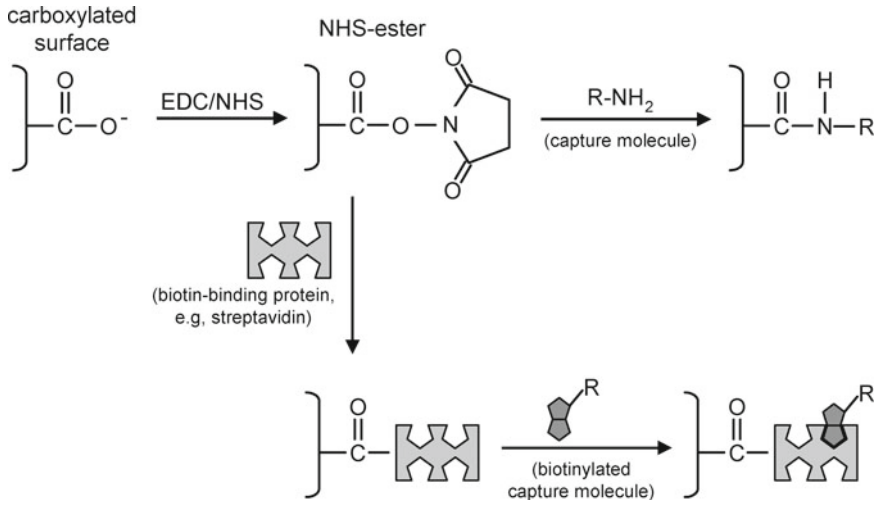

Fig. 1. Coupling procedures for capture molecules on carboxylated surfaces.

EDC/NHS coupling procedure is the most common and currently most used method (11). If the intermediate hydrogel provides amino groups, they can be converted into carboxyl groups by using dicarboxylic acid anhydrides to allow the same coupling scheme. However, the reaction conditions of the conversion reaction may also influence the morphology of the sensing layer and have to be considered carefully (18).

A disadvantage of the carbodiimide-based coupling procedure results from the randomized distribution of amino groups all over the protein molecule. This may lead to a blocking of binding sites after the coupling step, resulting in loss of signal response. For this reason a directed coupling of capture molecules via linker proteins may be preferred. This could be achieved, for example, by binding the corresponding antibody via Fc receptors such as protein A or G, or if biotinylated capture molecules are available by using biotin-binding proteins, such as streptavidin (11). However, it has to be taken into account that additionally introduced linker molecules may lead to a higher thickness of the sensing layer. This could be a problem for detection methods that imply a transduction mechanism near the surface, such as evanescent field techniques (11) or SAW biosensors (5, 6). In consequence, layer composition and concentrations of the single components have to be adjusted for each application to obtain optimal results in a subsequent assay, but approximate values valid for a group of sensing layers and applications can easily be given.

In this chapter we present general procedures to prepare SAW biosensors for affinity assays with either immobilized capture

molecules or immobilized ligands corresponding to the analyte. Hydrogels providing carboxyl groups are used. Capture molecules are immobilized randomly oriented via a thin 3D dextran layer or via a 2D PEG layer. Additionally, capture molecules are immobilized in an oriented manner by means of linker molecules, which are also immobilized via a thin 3D dextran layer or via a 2D PEG layer. Ligands are immobilized via a thick 3D dextran layer. The decision on which of these basic procedures should be used for a given application, can be made with the considerations mentioned above.

2. Materials

2.1. SAW Biosensor Measurement Setup

1. SAW device: A shear horizontal SAW resonator based on a small (4×4 mm^2) 36° YX-$LiTaO_3$ device with gold transducers and a frequency of operation in aqueous media of 428.5 MHz (see Note 1) is used.
2. Electronic setup: SAW measurements are performed in an oscillator circuit developed in-house with the SAW resonator as the frequency-determining element. Output signals are obtained as difference frequencies relative to a permanently oscillating reference oscillator, featuring a constant frequency in the range of 434 MHz. Difference frequency changes (see Note 2) are monitored continuously with a time resolution of 1 s. The frequency resolution of the data acquisition is 1 Hz. The short-term noise is approximately 40 Hz.
3. SAW device integration: A flow cell was designed in-house, in which the SAW device is mounted upside down onto isolated contact pads of a circuit board and coupled capacitively. A flow channel located between the contact pads allows the fluid to pass along the SAW path. The dimensions of the flow channel at the sensor position are $0.6 \times 0.6 \times 4$ mm^3, corresponding to an effective sample volume of 1.44 µl. Stainless steel capillaries, inner diameter 0.8 mm, are used as connections to the fluidics.
4. Fluidic setup: A flow injection analysis (FIA) system equipped with two peristaltic pumps, Ismatec (Wertheim/Germany) and an injection valve, Besta-Technik (Wilhelmsfeld/Germany), is used. Polytetrafluoroethylene (PTFE) tubes serve as connections between single components and as sample loop. The volume of the sample loop (including connecting tubes) is 220 µl. Volumes of sample and reagent solutions are to be 250 µl each. A schematic of the FIA system and its settings is shown in Fig. 2.

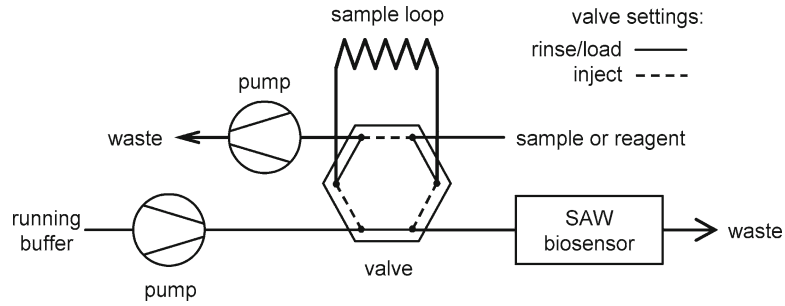

Fig. 2. Fluidic setup and system settings.

2.2. Surface Homogenization and Activation

1. Polymer: Parylene C dimer (di(2-chloro-*p*-xylylene)), Specialty Coating Systems (Indianapolis/USA).
2. Silane: (3-Glycidyloxypropyl)trimethoxysilane (GOPTS).
3. Rinsing solution: Acetone.

2.3. Coating with Hydrogel Layer

1. Hydrogel 1: Dicarboxy polyethylene glycol (DC-PEG), M_r 2,000, Rapp Polymere (Tübingen/Germany).
2. Hydrogel 2: Aminodextran (AMD), M_r 3,000, Life Technologies, Karlsruhe, Germany.
3. Hydrogel 3: AMD, M_r 70,000, Life Technologies, Karlsruhe, Germany.
4. Glutaric anhydride (GA).
5. Solvents: Dichloromethane (DCM), bidistilled water, dimethyl formamide (DMF).
6. Rinsing solutions: Bidistilled water, DMF, acetone.

2.4. Coupling of Proteins (i.e., Capture Molecule or Linker)

1. Running buffer: Phosphate buffered saline (PBS), pH 7.4, containing 10 mM phosphate buffer, 137 mM NaCl, and 2.7 mM KCl.
2. Immobilization buffer: Sodium acetate is diluted in bidistilled water to a concentration of 10 mM. The pH value of the solution is adjusted with KOH in such a way that the pH value of the resulting mixture containing capture molecule or linker will lie within the range of 5–6 (see Note 3). The immobilization buffer can be divided into working aliquots and stored at –20°C.
3. Activation solution: *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride (EDC) is rapidly weighed and dissolved in water at 0.4 M. *N*-hydroxysuccinimide (NHS) is weighed and dissolved in water at 0.1 M. Freshly prepared solutions of EDC and NHS are mixed in a ratio of 1:1 immediately before use (see Note 4) to obtain a mixture containing 0.2 M EDC and 0.05 M NHS. Non-dissolved quantities of EDC or NHS can be stored at –20°C.

4. Capture molecule (e.g., antibody) binding specifically to the corresponding analyte (e.g., antigen) is dissolved in immobilization buffer. The concentration depends on the application (see Note 5). A reasonable concentration to start is 1.5 μM.
5. Linker (e.g., biotin-binding protein, such as streptavidin) binding specifically to the corresponding capture molecule (e.g., biotinylated antibody) is dissolved in immobilization buffer. For the subsequent coupling step capture molecule is dissolved in running buffer. Both concentrations depend on the application (see Note 5). A reasonable linker concentration to start is 1.8 μM. A reasonable capture molecule concentration to start is 0.05 μM.
6. Deactivation solution: Ethanolamine hydrochloride is diluted in bidistilled water at 1 M. The pH value of the solution is adjusted with HCl to a final pH value of 8.5. The solution can be divided into working aliquots and stored at –20°C.

2.5. Coupling of Small Molecules (i.e., Ligand)

1. Activation solution contains 1 M NHS and 1.2 M diisopropylcarbodiimide (DIC) dissolved in DMF. The solution must be prepared immediately before use (see Note 4).
2. Rinsing solutions: DMF, bidistilled water.
3. Ligand (small molecule) binding specifically to the corresponding analyte is dissolved in DMF. The concentration depends on the application (see Note 5). A reasonable concentration to start is 1 mM.

2.6. Measurements with the SAW Biosensor

1. Running buffer: PBS, pH 7.4 (see item 1 in Subheading 2.4).
2. Analyte buffer: PBS, pH 7.4 (see item 1 in Subheading 2.4, Note 6).
3. Blocking solution: Bovine serum albumin (BSA) is dissolved in running buffer at 1 mg/ml.
4. Rinsing solutions for cleaning sample loop in between the measurements: 2-propanol, bidistilled water.

3. Methods

3.1 Homogenization and Activation of the SAW Device Surface

1. Homogenization with parylene C:

   Coat all SAW devices used in binding experiments with 0.1 μm parylene C using a commercial parylene deposition system, Labcoater 1, PDS 2010, Speedline Technologies (Indianapolis/USA), to obtain a chemically homogeneous surface (see Note 7).
2. Activation of parylene surface:

Activate the parylene C-coated SAW devices by a two-step process: oxidation via plasma treatment and subsequent silanization. For this, treat the SAW devices 15 s in a plasma cleaner (PDC-32G, Harrick Plasma, NY, USA) at 0.2 mbar air pressure and 50 W power. Immediately after the oxidation step, apply GOPTS on the device surfaces (5 µl per SAW device). After 1 h silanization reaction, rinse the devices with dry acetone and dry them with nitrogen. The activated SAW devices have to be introduced to the next step (i.e., coating with hydrogel) immediately.

3.2. Coating of the SAW Device Surface with Hydrogel Layer

1. Coating with hydrogel 1, DC-PEG, $M_r = 2{,}000$:

   Apply a solution of 1 mM DC-PEG in DCM on the activated parylene surfaces (10 µl per SAW device). After evaporation of the solvent, heat the devices to 70°C for 20 h. After that, rinse the SAW devices thoroughly with hot bidistilled water, $T = 60$°C, and with room temperature bidistilled water (see Note 8) and dry them.

2. Coating with hydrogel 2, AMD, M_r 3,000, or hydrogel 3, AMD, M_r 70,000, and subsequent carboxylation:

   Apply an aqueous solution of AMD on the activated parylene surfaces (10 µl per SAW device). The concentration depends on the application (see Note 9). Reasonable concentrations to start are 1 mM for AMD, M_r 3,000, and 0.1 mM for AMD, M_r 70,000. After 20 h reaction, rinse the SAW devices thoroughly with bidistilled water and dry them. Convert the amino groups to carboxyl groups by means of GA in DMF, $c = 2$ mg/µl (10 µl per SAW device). After 30 h reaction, rinse the SAW devices with DMF and acetone and dry them.

3.3. Coupling of Proteins (i.e., Capture Molecule or Linker) with FIA System

1. Use a SAW device coated with DC-PEG, M_r 2,000, or a SAW device coated with carboxylated AMD, M_r 3,000 (see Subheading 3.2). Insert the SAW device in the flow cell and integrate it in the measurement setup as described in item 3 of Subheading 2.1.
2. Monitor the SAW sensor signal during the coupling procedure. An overview of the FIA system settings involved is given in Table 1.
3. Switch the FIA system to rinse/load mode and set the flow rate to 0.03 ml/min.
4. Rinse the carboxylated SAW device with running buffer until a stable baseline can be observed.
5. If prepared in advance, defrost vials of EDC, NHS, immobilization buffer and deactivation solution. Otherwise, use freshly prepared solutions.

Table 1
Coupling of proteins with FIA system: solutions and system settings

Time (min)	Mode	Reagents
Before start	Load	Activation solution: EDC/NHS
0–1	Flow rate: 0.03 ml/min rinse	Running buffer
1–9	Inject	Activation solution: EDC/NHS
9–17	Rinse	Running buffer
13–14 17–25	Load Inject	Protein solution: capture molecule or linker
25–33	Rinse	Running buffer
29–30 33–41	Load Inject	Deactivation solution: ethanolamine
45–60	Flow rate: 0.05 ml/min rinse	Running buffer
60	End of measurement	

6. Prepare the EDC/NHS activation solution immediately before use. Load the solution into the sample loop.
7. Activate the carboxylated SAW device surfaces by injecting the EDC/NHS mixture in the running buffer for 8 min (see Note 10).
8. Rinse with running buffer for 8 min.
9. During the rinsing step, load the solution of the capture molecule (e.g., antibody) or linker (e.g., biotin-binding protein, such as streptavidin) dissolved in immobilization buffer into the sample loop.
10. Couple the capture molecule or linker on the activated surface by injecting the mixture in the running buffer for 8 min.
11. Rinse with running buffer for 8 min.
12. During the rinsing step, load the deactivation solution into the sample loop.
13. Deactivate potentially remaining NHS-ester groups by injecting the deactivation solution in the running buffer for 8 min (see Note 11).
14. Rinse with running buffer for 4 min and switch the flow rate to 0.05 ml/min (see Note 10). Wash for further 15 min. Finish the measurement.

Table 2
Binding experiments with FIA system: solutions and system settings

Time (min)	Mode	Reagents
Before start	Load	Protein solution
0–1	Flow rate: 0.05 ml/min rinse	Running buffer
1–5	Inject	Protein solution
5–9	Rinse	Running buffer
9	End of measurement	

15. In the case of immobilized capture molecule, such as antibody, the SAW biosensor is ready for measurements.
16. In the case of immobilized linker (e.g., biotin-binding protein) a subsequent binding of capture molecule (e.g., biotinylated antibody) is required. Monitor the SAW biosensor signal during the binding procedure. An overview of the FIA system settings involved is given in Table 2. The flow rate is maintained at 0.05 ml/min. Load the solution of capture molecule dissolved in running buffer into the sample loop. Inject the solution in the running buffer for 4 min. Rinse with running buffer for 4 min. Finish the measurement. Now the SAW biosensor is ready for measurements.
17. Perform measurements with the SAW biosensor using the FIA system (see Subheadings 3.5 and 3.6) immediately after the SAW biosensor is ready.

3.4. Coupling of Small Molecules (Ligand)

1. Use SAW devices coated with carboxylated AMD, M_r 70,000 (see step 2 in Subheading 3.2). Insert the SAW devices in a glass chamber containing DMF vapor.
2. Prepare the activation solution as described in step 1 of Subheading 2.5 immediately before use.
3. Activate the carboxylated SAW device surfaces by applying the activation solution (15 µl per SAW device) and 2 h reaction at room temperature.
4. Rinse the SAW devices with DMF and dry them with nitrogen.
5. Couple the ligand on the activated surfaces by applying the ligand solution (15 µl per SAW device) and 20 h reaction at room temperature.
6. Rinse the SAW devices with DMF and bidistilled water and dry them.

7. SAW biosensor surfaces are now ready for measurements (see Note 12). Perform measurements with the SAW biosensor using the FIA system (see Subheadings 3.5 and 3.6).

3.5. Measurement with SAW Biosensor: Blocking Step/Quality Testing

1. Use a SAW biosensor with immobilized capture molecule or ligand corresponding to the analyte (see Subheadings 3.3 and 3.4). If the SAW biosensor is already inserted in the flow cell and integrated in the FIA system due to previous preparation steps, proceed with the next step. If not, insert the SAW biosensor in the flow cell and integrate it in the measurement setup as described in Subheading 2.1.
2. Monitor the SAW sensor signal during the testing procedure. An overview of the FIA system settings involved is given in Table 2.
3. Switch the FIA system to rinse/load mode and set the flow rate to 0.05 ml/min.
4. Rinse the SAW biosensor with running buffer until a stable baseline can be observed.
5. Load the blocking solution into the sample loop.
6. Inject the blocking solution in the running buffer for 4 min.
7. Rinse with running buffer for 4 min.
8. Finish the measurement. Signal heights can be determined by calculating the mean of the signal response curves in a defined range of 30 s after the injection interval (see Fig. 3). If the signal height obtained is 1 kHz or higher, the SAW biosensor is not suited for binding experiments and has to be discarded (see Note 13). If the signal height obtained is below 1 kHz, the SAW biosensor is ready for binding experiments. Perform

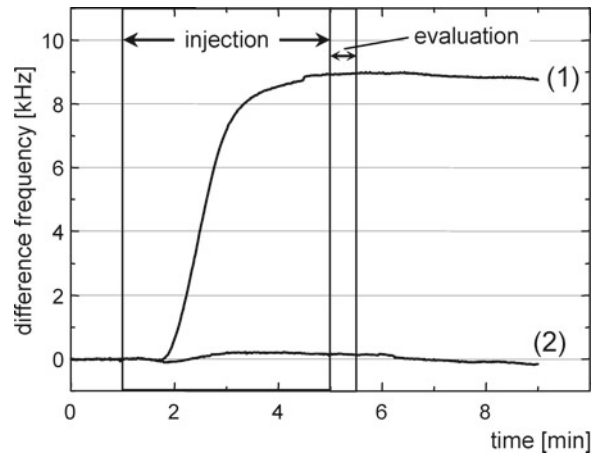

Fig. 3. SAW signal responses for analyte (1) and non-analyte (2) samples injected in the running buffer. Injection interval: 1–5 min, evaluation interval: 5.0–5.5 min.

binding experiments with the SAW biosensor using the FIA system (see Subheading 3.6) immediately after the SAW biosensor is ready.

3.6. Measurement with SAW Biosensor: Binding Experiment

1. Use a SAW biosensor with immobilized capture molecule or ligand corresponding to the analyte which passed the quality test (see Subheading 3.5). Due to the latter it is already inserted in the flow cell and integrated in the measurement setup.
2. Monitor the SAW sensor signal during the binding experiment. An overview of the FIA system settings involved is given in Table 2.
3. Switch the FIA system to rinse/load mode and set the flow rate to 0.05 ml/min.
4. Rinse the SAW biosensor with running buffer until a stable baseline can be observed.
5. Load the analyte solution into the sample loop.
6. Inject the analyte solution in the running buffer for 4 min.
7. Rinse with running buffer for 4 min.
8. Finish the measurement. Signal heights can be determined by calculating the mean of the signal response curves in a defined range of 30 s after the injection interval (see Fig. 3).
9. Clean the sample loop with rinsing solutions before starting a new binding experiment (see Note 14).

4. Notes

1. The operation frequency of the SAW device is one of the parameters determining the magnitude of the signal response. In principle, the higher the frequency, the higher the SAW signal response. However, the penetration depth of the surface acoustic wave decreases with increasing operation frequency, which could lead to reduced signal responses (see also Note 5). Therefore, the chosen frequency must neither be too high nor too low. We think best results should be obtained with a similar SAW resonator configuration as used here. However, if another operation frequency has to be used, then all coating-relevant data affecting the thickness of the sensing layer should be reconsidered. If a higher frequency is used it might be necessary to adjust all coating parameters towards a lower resulting layer thickness. Conversely, if a lower frequency is used it could be advantageous to adjust all coating parameters towards a higher resulting layer thickness to improve the sensor performance. In case of a delay line device all considerations concerning the

layer composition most likely must be reconsidered regardless of its operation frequency.

2. Difference frequencies below 20 MHz are more easily countable by a commercially available frequency counter than the absolute frequency of several hundreds of MHz of a SAW device. As difference frequencies (relative to the reference oscillator) were used as signal output, and as the frequency of the reference oscillator is higher than that of the SAW biosensor, processes leading to frequency decrease in the oscillator circuit, such as mass increase, result in increasing frequencies on the monitor. In some cases changes of viscoelasticity dominate, which may result in decreasing frequencies on the monitor. For the sake of clarity, signal response curves were plotted to start at 0 Hz (see Fig. 3).
3. The pH value of the coupling reaction has to be chosen regarding two facts. First it has to ensure that the amino groups of the protein molecules to be coupled are positively charged while maintaining a negative charge of the carboxyl groups on the surface to allow electrostatic attraction. Second, it has to allow the reaction with the active ester.
4. Rapid weighing of EDC is recommended due to the hygroscopic nature of EDC. Fresh preparation of carbodiimide mixtures is recommended due to the reduced stability of those solutions.
5. The optimal amount (and hence the concentration of the reagent solution) of capture molecule, linker or ligand depends, among others, on the morphology of the hydrogel layer. If, for instance, the thickness of the sensing layer, including immobilized capture molecules or ligands, is too high, the subsequent interaction of analyte and corresponding binding partner might not be detected due to the limited penetration depth of the surface acoustic wave, which would lead to a loss of signal response. As the coupling efficiency is one of the parameters determining the morphology of the sensing layer, the concentrations of the reagent solutions have to be optimized for each application and only approximate values can be given here.
6. Ideally, running buffer and analyte buffer are the same to prevent effects of the sample medium on the SAW signal response. If this is not possible, e.g., due to the sample medium in which the analyte is delivered, analyte signals still can be determined by the difference of the signal response before and after the injection interval, both monitored while rinsing with running buffer and thus avoiding medium effects.
7. The surface of the SAW resonator consists of two materials: $LiTaO_3$ and gold. As chemical coupling procedures can typically be optimized for one of the materials only, starting from

the device surface might result in inhomogenities of the subsequently prepared sensing layer. The initial coating with a polymer provides a chemically homogeneous surface resulting in a sensing layer without inhomogenities.

8. High temperature variations can damage the SAW device. Besides, it is easier to rinse off the remaining molten DC-PEG with hot water than with cold water.
9. The thickness (and hence the concentration of the reagent solution) of the 3D hydrogel AMD has to be chosen regarding two facts. First, the density of the AMD layer must be sufficient to prevent nonspecific adsorption of the sample medium in the subsequent assay. Second, in case of potential disadvantageous effects resulting from changes of viscoelasticity of the sensing layer, the thickness must not be too high, which in turn depends mainly on the morphology of the sensing layer. Therefore, the concentration of the hydrogel solution has to be optimized for each application and only approximate values can be given here.
10. The incubation time is one parameter to control the EDC/NHS coupling efficiency. It has been proven to be useful to chose the reaction times (and hence injection intervals) to be longer during the coupling reaction (8 min) than during the analyte binding (4 min). Therefore, in order to avoid increased reagent consumption, the flow rate is reduced to 0.03 ml/min during coupling procedure compared to 0.05 ml/min during the SAW biosensor binding experiments. When the EDC/NHS coupling procedure is finished, the flow rate is set to that of the binding experiments (0.05 ml/min) to increase the rinsing efficiency and to allow a first evaluation of the signal stability considering subsequent SAW biosensor measurements.
11. When performing the EDC/NHS coupling procedure, it has to be guaranteed that after the reaction there are no free NHS-ester groups remaining, because any protein used in a subsequent sample could couple to these active ester groups leading to false positive results. Therefore, a deactivation step is performed using a primary amine, such as ethanolamine, at a slightly alkaline pH to react with potentially remaining NHS-ester groups and prevent them from other coupling reactions in subsequent binding experiments.
12. The shelf life of SAW biosensors with immobilized ligands depends on the application. In our experiments, such biosensors could be stored up to 2 weeks at 4°C without loss of functionality.
13. It is not recommended to perform more blocking steps until the BSA signal meets the requirements, because proteins contained

in the sample of a subsequent binding experiment could bind nonspecifically to the BSA, leading to false positive results.

14. After each sample injection, the sample loop should be cleaned with the rinsing solutions to avoid carryover effects.

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