Influence of Preparative Carboxylation Steps on the Analyte Response of an Acoustic Biosensor

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***Abstract*—Biosensors typically work with analyte specific biomolecules coupled on a transducer surface. In many cases, biomolecule coupling requires intermediate carboxylation steps. We performed several experimental procedures for the carboxylation of an aminodextran (AMD) coated surface acoustic wave (SAW) biosensor prior to biomolecule coupling and investigated their impact on the analyte signal in a subsequent binding assay. The carboxylation procedures were based on glutaric anhydride (GA) solutions and molten GA, where molten GA led to lower analyte signals than GA solutions. This demonstrates that the mere experimental procedure to fulfill a certain immobilization strategy may play a critical role in the final performance of an acoustic biosensor.**

***Index Terms*—Biosensor, chemistry, surface acoustic wave (SAW) resonators, surface treatment.**

I. Introduction

BIOSENSORS typically require the immobilization of analyte specific capture molecules, such as antibodies or enzymes, on a transducer device. These proteins are often immobilized via an intermediate hydrogel layer to prevent interfering unspecific binding on the biosensor surface. Hydrogels commonly used are dextran derivatives providing carboxyl or amino groups. Carboxyl groups are able to react with the proteins' amino groups in the presence of a condensing reagent, e.g., carbodiimide, forming peptide bonds. This coupling method is stated to be easy to use, robust and effective and hence is the method most frequently applied [1]. Therefore, if only aminated hydrogels are available, for protein coupling it may be advantageous to convert the amino groups to carboxyl groups. This is usually carried out by means of dicarboxylic acid anhydrides, such as succinic anhydride and glutaric anhydride (GA). In most of the published experimental procedures, aminated support material is exposed to a concentrated solution of anhydride. If anhydride solutions based on aqueous solvents are used, e.g., buffer, it is strongly recommended that the pH value of the solution is maintained in the range 6–9 throughout the reaction [2]. The adjustment of the pH value becomes redundant using anhydride solutions based on organic solvents, e.g., dimethyl formamide (DMF) [3]; however, it has to be verified that those parts of transducer and surrounding material coming in contact with the reaction solution are solvent resistant. Typically, reactions using anhydride solutions are carried out at room temperature. Reaction times range from few hours to overnight, where the latter is preferred to ensure the complete modification of all surface bound amino groups [2]. Shorter reaction times of a few hours only are reported, when the reaction was performed using molten anhydride as this implies higher reaction temperatures, depending mainly on the melting point of the respective anhydride [4].

Naturally, the anhydride based carboxylation procedures follow the same underlying immobilization principle. Using the example of carboxylation via GA (melting point: 56.5 °C), we investigated whether those procedures also are equivalent with regard to the analyte signal in a subsequent binding experiment using a surface acoustic wave (SAW) biosensor or whether the reaction conditions applied in the particular procedures have an effect on the final biosensor response.

Manuscript received August 17, 2009; revised September 11, 2009; accepted September 14, 2009. Current version published November 06, 2009. The associate editor coordinating the review of this paper and approving it for publication was Prof. E. H. Yang.

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Digital Object Identifier 10.1109/JSEN.2009.2033027

II. Experimental

Shear horizontal SAW resonators based on 36°YX-$LiTaO_3$ substrates and a frequency of operation of 428.5 MHz were used. SAW measurements were performed by means of an oscillator circuit developed in-house with the SAW resonator as frequency determining element. Difference frequencies relative to a permanently oscillating reference oscillator featuring a constant frequency in the range of 434 MHz were used as signal output. For the sake of clarity, signal response curves were plotted to start at 0 Hz. All SAW resonators were coated with parylene C [poly(2-chloro-p-xylylene)] and aminodextran (AMD), M_r 3000, as previously described [5].

The amino groups of the AMD layer were converted into carboxyl groups by means of GA following four procedures: 1) GA/H_2O: 10 μl of a solution of GA in 8 mol/l aqueous NaOH solution, $c = 0.5\ \mathrm{mg}/\mu\mathrm{l}$, were applied on each sensor surface. The pH-value of the GA solution was approximately 7. After reaction overnight, the sensors were rinsed with bidistilled water and dried. 2) GA/DMF: 10 μl of a solution of GA in DMF, $c = 2\ \mathrm{mg}/\mu\mathrm{l}$, were applied on each sensor surface. After reaction overnight, the sensors were rinsed with DMF and dry acetone, then dried. 3) GA/melt (30 min): 8 μl of molten GA (corresponding to 10 mg GA) were applied on each sensor surface. After 30 min reaction time at 60 °C, the sensors were rinsed with bidistilled water and dried. 4) GA/melt (2 h): This procedure was performed similarly to 3), but the reaction time was extended to 2 h.

Streptavidin was coupled on the carboxylated SAW resonators via carbodiimide chemistry. Streptavidin coupling as well as the subsequent binding experiments using nonbiotinylated or biotinylated bovine serum albumin (BSA) were performed by means of a flow injection analysis system as previously described [5]. Phosphate buffered saline (PBS), pH 7.4, was used as carrier stream. Shielding against unspecific

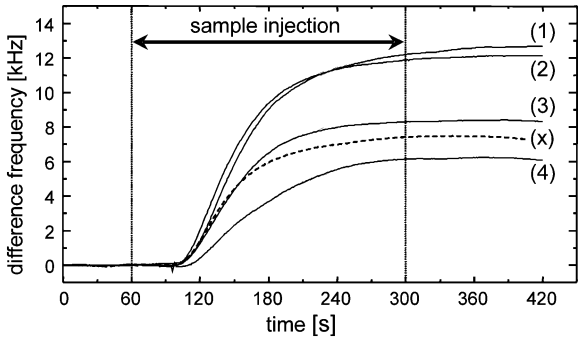

Fig. 1. Injection of biotinylated BSA solution, 10 $\mu g/ml$, in a PBS carrier stream. The SAW biosensor surfaces were modified with parylene C, AMD, and streptavidin. The numbers indicate the GA modification procedure performed prior to streptavidin coupling: (1) GA/H_2O; (2) GA/DMF; (3) GA/melt (30 min); (4) GA/melt (2 h). The dashed line (x) represents an experiment in which the AMD layer was heated 4 h prior to the GA/DMF procedure.

TABLE I
SIGNAL HEIGHTS OBTAINED WITH BIOTINYLATED BSA SAMPLES AND STREPTAVIDIN COATED SAW BIOSENSORS

GA modification procedure prior to streptavidin coupling	Signal height [kHz]
(1) GA/H_2O	12.2 ± 1.4
(2) GA/DMF	11.7 ± 0.6
(3) GA/melt (30 min)	8.7 ± 0.8
(4) GA/melt (2 h)	6.0 ± 0.3

binding was determined by injection of a solution of nonbiotinylated BSA dissolved in PBS, 1 mg/ml, in the PBS carrier stream. If the BSA signal was determined to be negligible, i.e., less than 1 kHz, a solution of biotinylated BSA dissolved in PBS, 10 $\mu g/ml$, was injected in the PBS carrier stream.

III. RESULTS AND DISCUSSION

Fig. 1 shows typical SAW signal responses of five streptavidin coated sensors while treating them with biotinylated BSA. Each sensor was modified using another GA modification procedure. Signal heights were determined by calculating the mean of the signal response curves in the range 330–360 s. Each procedure (1)–(4) was tested in four experiments using a separate sensor for each. Means and standard deviation values of the respective signal heights are given in Table I.

Similar sensor responses were obtained using the experimental procedures based on dissolved GA. The signal responses obtained with molten GA and a reaction time of 30 min, however, were significantly lower than those obtained with the GA solutions. An insufficient reaction time as reason for this behavior could be excluded, because increasing the time to 2 h led to an even further decrease of the signal height. To investigate whether the heat treatment affects the yield of the reaction between amino groups and GA, another experiment based on the GA/DMF procedure was performed, however, in this case the sensor was heated for 4 h prior to the carboxylation step. The signal height obtained with this procedure (see Fig. 1, dashed line) was in the same range as those obtained with the procedures based on molten GA, despite the carboxylation step was not subject to heat treatment. As a result, we think that the increased reaction temperature of 60 °C led to an irreversible change in the physical and structural properties of the intermediary AMD layer. On the one hand, this might decrease the accessibility of binding sites in the subsequent biospecific layer due to steric reasons and reduce the sensor response in this way. On the other hand, when working with SAW biosensors (or other acoustic sensors), it has to be taken into account that it is not only the mass change but also the change of viscoelasticity in the biospecific layer, including layer thickness and penetration depth of the SAW, which contribute to the SAW signal response [5], [6]. Therefore, it has to be considered that the heat treatment may alter the viscoelastic properties of the biospecific layer in a way, that lower sensor responses are obtained, even if the amount of binding events on the surface (and hence the mass change) remains the same.

Consequently, this means that the reaction conditions of the intermediate carboxylation step may play a critical role in the subsequent performance of the biosensor, even if the overall strategy of biomolecule immobilization remains the same. In the presented work, a carboxylation procedure based on dissolved GA, i.e., avoiding heat treatment, should be preferred. However, using intermediary layers which are less affected by heat treatment should minimize the signal reduction obtained with molten GA. This would make the approach still suitable, which might be desirable due to the decreased processing time.

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