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Label-free detection of breast cancer marker HER-2/neu with an acoustic biosensor

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Abstract

Surface acoustic wave (SAW) biosensors based on horizontally polarized surface shear waves enable label-free, sensitive and cost-effective detection of biomolecules in real time. Binding reactions on the sensor surface are detected by determining changes in surface wave velocity caused mainly by mass loading in the sensing layer. Typically, SAW devices are coated with biochemically sensitive layers including analyte-specific capture molecules (e.g., antibodies) or ligands. The covalent binding of antibodies to intermediate hydrogel layers (e.g., dextran or polyethylene glycol) tends to result in undirected orientation of capture molecules and leading to a lower signal response in a subsequent analyte binding experiment. Therefore, a coupling procedure was developed using two linkers, neutravidin and biotinylated protein A, allowing directed orientation of capture antibodies. This assembly enables label-free and direct detection of the breast cancer marker HER-2/*neu* at a concentration of 10 ng/ml (threshold: 13-20 ng/ml).

Keywords: biosensors; label-free; immobilization; breast cancer; HER-2/neu

1. Introduction

HER-2/*neu* (human epidermal growth factor receptor 2), also known as ErbB2, is a protein marker for breast cancer, which is currently used to predict response to trastuzumab (Herceptin®)-based treatment and to prognosticate course of disease [1]. There are two possibilities to measure the molecular state of HER-2/*neu* in patients: immunohistochemistry (IHC) staining of the whole cell membrane receptor in tissue sections obtained from biopsy or detection of the concentration of the extracellular domain (ECD) of HER-2/*neu* (HER-2/neu-ECD), which is shed in serum, via ELISA featuring the advantage of a biopsy-free analysis. However it exists a commercial available ELISA from Bayer [2], there is still a need for a direct, label-free, rapid and cost effective method for the detection

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of HER-2/*neu*-ECD in serum. This can be met by SAW biosensors, which already have been applied successfully to detect proteins, DNA and bacteria [3]. We developed a SAW biosensor system consisting of a SAW device embedded in a flow cell integrated in a flow injection analysis (FIA) system. This set-up was used successfully in several affinity binding experiments [3-5]. The surface modification was adapted for the detection of low concentrations of clinical relevant protein marker HER-2/*neu*-ECD.

2. Experimental

2.1. SAW device and fluidic setup

A shear horizontal SAW resonator based on a small (4 x 4 mm²) 36°YX-$LiTaO_3$ device with gold transducers and a frequency of operation of 428.5 MHz was used. SAW measurements were performed in an oscillator circuit developed in-house with the SAW resonator as frequency-determining element. Details of the set-up have been described earlier [6]. A flow cell was designed in which the SAW device was mounted upside down onto isolated contact pads on the electronic board and coupled capacitively [6;7]. A flow channel between the contact pads allowed the fluid to pass the SAW sensor.

2.2. Sensor surface modification

Chemicals were obtained from Merck, Germany and Sigma-Aldrich, Germany, unless otherwise noted. The antibody anti-HER-2/*neu* (monoclonal mouse IgG; R&D Systems, Germany) was used as capture molecule. Neutravidin (Thermo Scientific Fisher, Germany) was used as first linker; biotinylated protein A (Thermo Scientific Fisher, Germany) was used as second linker.

2.2.1. Covalent binding of intermediate hydrogel layer

All SAW devices used in the following experiments were first coated with 0.1 µm parylene C to obtain a chemically homogeneous surface [8]. The parylene C layer was activated by oxidation via plasma treatment and subsequent silanization with (3-glycidyloxypropyl)trimethoxysilane. After rinsing with acetone the sensors were immediately used for hydrogel coupling [5; 9].

Dicarboxy polyethylene glycol (DC-PEG), M_r 2,000 (Rapp Polymere, Germany) was immobilized as follows: 10 µl of a solution of DC-PEG in dichloromethane, c = 2 mg/ml, was applied on the activated sensor surfaces. After evaporation of the solvent, the sensors were heated to 70 °C for 20 h, rinsed with bidistilled water and dried.

Amino-biotin polyethylene glycol (AB-PEG), M_r 3,000 (Rapp Polymere, Germany) was immobilized as follows: 10 µl of an aqueous solution AB-PEG, c = 3 mg/ml, was applied on the activated sensor surfaces. After reaction for 20 h at room temperature, the sensors were rinsed with bidistilled water and dried.

2.2.2. Covalent immobilization procedure on carboxylated surfaces

After each modification step the sensors were rinsed with phosphate buffered saline (PBS). First the carboxylated surfaces (DC-PEG) were activated with an aqueous solution of 0.05 M N-hydroxy succinimide and 0.2 M N-(3-dimethylaminopropyl)-N`-ethylcarbodiimide hydrochloride for 10 min. Second the surface was incubated with 1 mg/ml anti-HER-2 or 50 µg/ml neutravidin in acetate buffer, pH 5, for 30 min. Third the remaining active groups on the surface were deactivated by treatment with a solution of 1 M ethanolamine, pH 8.5, for 10 min. After that, sensors with immobilized antibodies were immediately used for the subsequent assays. Sensors with immobilized neutravidin were treated with biotinylated protein A, c = 10 µg/ml, for 30 min. Then the sensors were treated with anti-HER-2/*neu*, c = 20 µg/ml, for 30 min. After that sensors were immediately used for the binding assay.

2.2.3. Non-covalent immobilization procedure on biotinylated surfaces

After each modification step the sensors were rinsed with PBS. First the non-covalent coupling of neutravidin, c = 50 µg/ml, to the biotin groups of AB-PEG was performed for 30 min. Then the sensors were treated with biotinylated protein A, c = 10 µg/ml, for 30 min and treated with anti-HER-2, c = 5 µg/ml, for 30 min. After that the sensors were immediately used for the binding assay.

2.3. SAW biosensor measurements

Recombinant human (rh) HER-2/*neu* (R&D Systems, Germany) was used as analyte. Experiments were performed with the FIA system described elsewhere [6]. PBS was used as carrier stream, the flow rate was set to 0.05 ml/min. Samples were loaded in the sample loop, injected into the carrier stream via the injection valve, and transported to the sensor. The injection interval was set to 60-300 s. After each sample injection the sensor was rinsed in the carrier stream for 5 min. Shielding against unspecific binding was determined by injection of a solution of BSA (Serva, Germany), c = 1 mg/ml, in the PBS carrier stream. If the BSA signal was determined to be negligible, samples containing c = 0 (0.001; 0.01; 0.1) µg/ml HER-2/*neu* in PBS were injected subsequently in the carrier stream. Signal heights were calculated from the SAW signal response curves as difference between the SAW signals after and before the injection interval.

3. Results and discussion

For the detection of HER-2/*neu*-ECD, the sensor surface was modified with a corresponding monoclonal antibody (anti-HER-2). Unspecific binding was prevented by coupling the binding molecules via an intermediate polyethylene glycol (PEG) hydrogel layer on the sensor surface. Our “standard” coupling procedure of the antibody anti-HER-2/*neu* via carbodiimid chemistry on DC-PEG did not enable the detection of HER-2/*neu*-ECD up to 100 ng/ml (figure 1, blue).

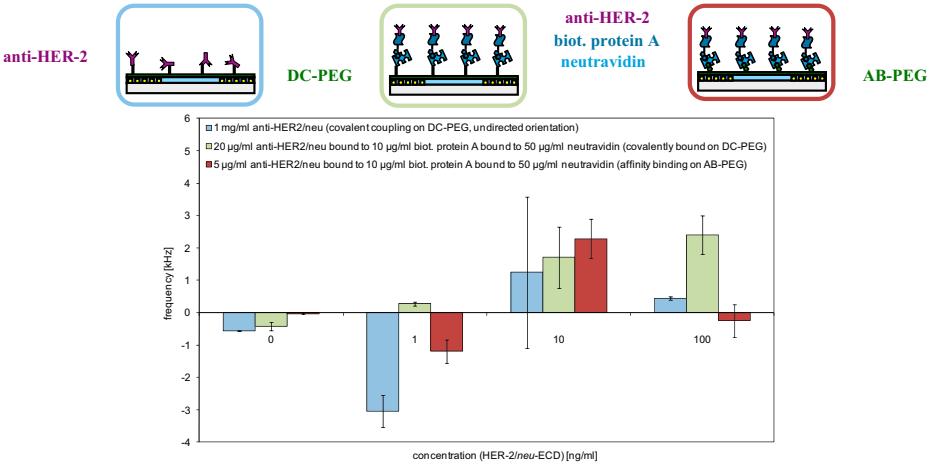

Fig. 1: Detection of HER-2/neu-ECD. Comparison of signal response (see columns) based on different immobilization methods (see schemes with corresponding colors of frames). Undirected orientation with covalent immobilization of anti-HER-2/neu on DC-PEG (blue) and directed orientation of anti-HER-2/neu via covalent (green) and affinity (brown) coupling of neutravidin on DC-PEG and AB-PEG, respectively, prior to binding of biotinylated protein A. Samples containing 1 (10; 100) ng/ml HER-2/neu-ECD were applied subsequently on each sensor. Columns represent means , error bars represent standard abbreviation values out of three measurements.

Therefore, we developed an immobilization method of anti-HER-2/*neu* via two linkers, neutravidin and biotinylated protein A. We compared the effects of covalent coupling and affinity coupling of the first linker, neutravidin, in the subsequent HER-2/*neu*-ECD assay. Affinity coupling of neutravidin, via AB-PEG, prior to binding of biotinylated protein A and anti-HER-2/*neu* (figure 2, brown), led to higher signal responses in this assay than covalent coupling of neutravidin to DC-PEG prior to binding of biotinylated protein A and anti-HER-2/*neu* (figure 2, green). The optimized surface modification procedure enabled the detection of 10 ng/ml HER-2/*neu*-ECD.

4. Conclusions

In this work the effect of different strategies of antibody immobilization on the signal response of SAW biosensors in a subsequent assay was investigated. An efficient coupling procedure was developed for the affinity system anti-HER-2/*neu* and HER-2/*neu*-ECD, where the latter is a potential biomarker for breast cancer. The optimized protocol based on site-directed antibody coupling via two linkers, neutravidin and biotinylated protein A, where neutravidin was coupled via affinity binding to AB-PEG. Based on this procedure, 10 ng/ml HER-2/*neu-ECD* could be detected. The cut-off value for diagnostic applications is 13-20 ng/ml. The next step would be the testing of this surface modification procedure with real samples, such as serum. As antibodies are widely used as biospecific capture molecules in binding assays, our findings should also be valid for other biosensor principles and applications in clinical diagnostic.

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