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Specific targeting of ultrasound contrast agent (USCA) for diagnostic application: an in vitro feasibility study based on SAW biosensor

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Abstract

The present study described a new strategy to examine the interaction between the targeted ultrasound contrast agent (USCA) and its target under flow conditions with a surface acoustic wave (SAW) transducer. The sensing principle is based on the measurement of the phase change on the sensing element upon the binding of specific biomolecules. Love-wave biosensor array was consisting of sensor elements and reference elements. The sensor elements have been prepared by coating the sensor surface with tumor marker EDB-fibronectin by means of SAM technique and carbodiimide chemistry. Reference elements were left blank or coated with fibronectin and used to eliminate thermal drift, unspecific binding, and turbulence from injection of liquids by calculating the differential phase shift with respect to the sensor elements. The binding of targeted USCA to the sensor surface was constantly recorded by monitoring the phase shift on the sensor element. The binding of targeted USCA generated a high phase shift on the sensor elements, but almost no change on the reference elements. Control experiments using non-targeted and isotype-targeted USCA confirmed the specificity of binding due to anti-EDB-fibronectin scFv-antibody-fragment–EDB-fibronectin antigen interaction. The suitability of the SAW technique to monitor the specific binding behavior of targeted micron-sized USCA in real time has been well established.

Keywords: Surface acoustic wave sensor; Love-wave sensor; Specific targeting; Ultrasound contrast agent; Tumor; EDB-fibronectin

1. Introduction

Molecular imaging is an emerging field in the biomedical area in which ultrasound procedures are a promising approach. The advances in molecular science provide the opportunity to design targeted contrast agents. Diagnostic strategies based on specific receptor–ligand interaction to target cells may find broad utility in tumor diagnosis (Cairns and Sidransky, 1999; Becker et al., 2001). Ligand-modified contrast agents that bind selectively to a target site enhance both quality and content of information of ultrasound diagnostic images. Ultrasound contrast agents (USCA) have proven advantageous over other imaging procedures like magnetic resonance, computed tomography, and optical imaging in the field of molecular imaging. Because of the high spatial resolution of ultrasound contrast agents in the micrometer range and their high sensitivity, single gas-filled microparticles can be imaged and quantified.

Both growth and spreading of cancerous tumors require angiogenesis, the generation of new blood vessels. A reliable marker for tumor neovasculature is the extra-domain B (ED-B) of fibronectin, a sequence of 91 amino acids that can be inserted into the fibronectin molecule by alternative splicing (Catellani et al., 1994; Kosmehl et al., 1995; Zardi et al., 1987; Fattorusso et al., 1999). Antibodies capable of binding

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to new blood vessels, but not to mature vessels can be used to selectively target this angiogenesis region. A single-chain antibody fragment termed “L19” was identified as a suitable binding protein for EDB-fibronectin antigen (Pini et al., 1998).

Here, we report an in vitro study of contrast agents, composed of polymer stabilized, air-filled microparticles, selectively targeted to tumor antigen (EDB-fibronectin) by surface bound single-chain antibody fragment “AP39”, a C-terminus extended variant of L19. A pivotal role in the development of targeted contrast agents plays the study of binding behavior of targeted contrast agents to corresponding receptor surfaces under in vitro conditions. In the past, fluorescence labeled targeted ultrasound contrast agents were studied microscopically with respect to their binding properties towards target antigen carrying cells (Villanueva et al., 1998) or towards immobilized antigen (Klibanov et al., 1999). Microparticles adhered to the target were counted manually, making the procedures very time-consuming, only semi-quantitative, and error-prone. As an improvement, an automated measuring device would allow reproducible measurements with the opportunity to perform a larger number of tests and quality control within reasonable time.

The majority of studies of bioaffinity interactions use fluorescence labeled biomolecules (Neumann et al., 2000; Khan et al., 2003); there is a need for continuous development of sensitive analytical methods for detection of bioaffinity interactions in biological samples without molecular labels. In most cases, label-free detection is performed by using biosensors that consist of signal transducers based on optical or acoustic principles such as surface plasmon resonance (SPR) (Jönsson et al., 1993), and quartz crystal microbalance (QCM) (Luppa et al., 2001).

Acoustic wave biosensors are an attractive alternative to surface plasmon resonance allowing determination of biological interactions with high resolution and precision (Gizeli et al., 2003). Surface plasmon waves are strongly localized to the conducting film. Therefore, depth sensitivity of the surface plasmon is limited by the penetration depth of the evanescent field into the sample medium. The intensity of the plasmon signal is reduced exponentially with penetration depth (Stenberg et al., 1991), which is approximately 200 nm (Jönsson et al., 1993). Thus interactions that occur few hundred nanometers away from the interface remain undetected. This can be considered a major obstacle in detecting particles in the micrometer range.

The quartz crystal microbalance (QCM) is the most widely used acoustic device. Our previous attempts to use both SPR (BIAcore 2000, Freiburg, Germany) and QCM (Institut für Automation und Kommunikation, Barleben, Germany) to develop a functionality assay for target specific air-filled microparticles only led to unsatisfactory results (unpublished data). Binding of microparticles was not detectable by SPR. No correlation was observed between the concentrations of the injected microparticles and the resulting SPR signals. Binding studies using conventional QCM technique led to poor signal-to-noise ratios and poor reproducibility. The measured signal was inconsistent. This might be due to the inappropriate sensor setup with parallel measurement of independently working single flow chamber each with a single sensor element. Therefore interfering effects may not be sufficiently eliminated.

Surface acoustic wave (SAW) sensors (Fig. 1) are more suitable for binding studies, since their higher resonant frequency typically of 100–400 MHz and the localization of energy to the sensor surface lead to considerably increased sensitivities. Love-wave sensors (Harding et al., 1997) exhibit approximately 30-fold higher sensitivity compared to conventional QCMs (Ballantine et al., 1997). A typical value of operating frequency of SAW devices is about 100 MHz, much higher than typical values of QCM devices, which operate at frequencies near 10 MHz. The intensity of the SAW signal is reduced exponentially with penetration depth, which is between 140 nm for 115 MHz and 40 nm for 348 MHz (Weiss et al., 1998).

We have developed an in vitro assay to demonstrate the feasibility of targeting an USCA to a specific antigen by attaching fragments of corresponding antibodies to its surface on the basis of SAW technology. For these studies, a Love-wave sensor was used (Schlensog et al., 2002). The Love-wave biosensor was equipped with a sensitive layer of 11-mercaptoundecanoic acid (MUA) to which EDB-fibronectin was coupled via the carboxylic acid residues. The signal that is primarily detected is the phase shift due to the change in mass loading, which results from interactions between the analytes and the sensitive layer.

2. Experimental

2.1. Materials

Organic compounds and biochemicals were purchased from Sigma, Germany. The antigen EDB-fibronectin and the scFv-antibody-fragment AP39 were manufactured by Schering Research.

2.2. Instrumental setup

The detection system is based on a 20 mm × 20 mm sensor chip with an array of five single sensor elements structured on ST-cut quartz (S-sens analytics; developed by Aptamer Biosensors, Caesar). Details of the fabrication of the Love-wave sensor are described elsewhere (Schlensog et al., 2002, submitted for publication). A flow cell was attached to the sensor via a Viton seal ring to seal the cell to the acoustic device (Fig. 1A and B).

A peristaltic pump (Ismatec Reglo Digital, Glattbrugg-Zürich, Switzerland) and poly(vinyl) chloride (PVC) tubing were used to pump buffer and samples over the sensor

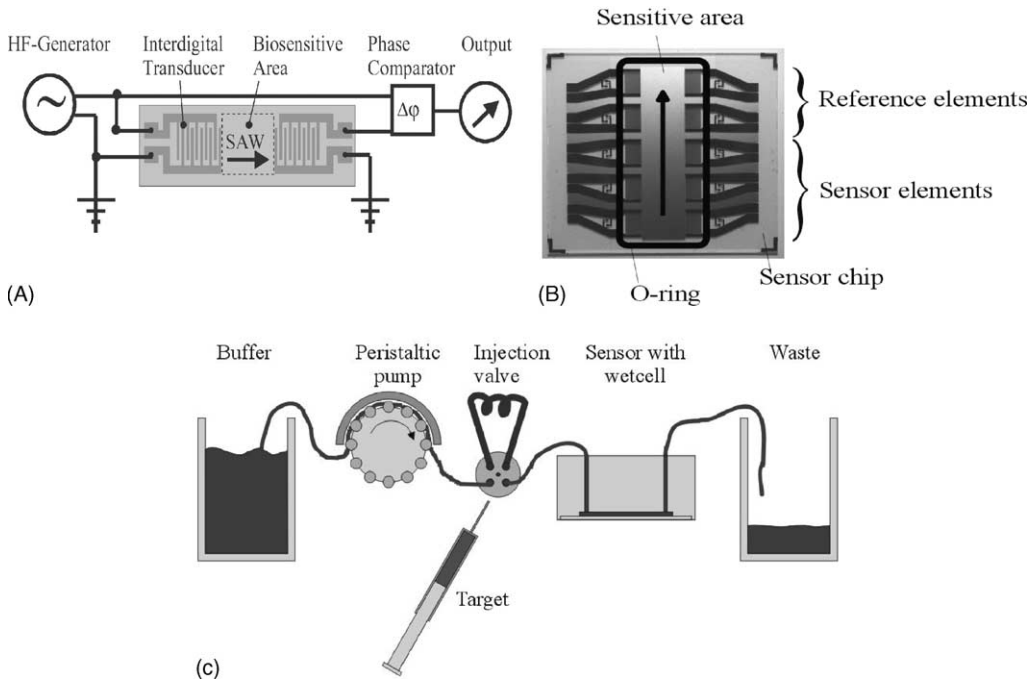

Fig. 1. Scheme of SAW device structure and principle of measurement (A) and design of sensor chip and sealing (B; arrow indicates direction of analyte flow). The sensor chip consists of five sensor elements, each with two interdigital transducers. A high-frequency generator supplied voltage to the interdigital transducers, exciting surface acoustic waves according to the frequency. Mass loading to the sensor surface caused a phase shift between input and output signal, determined by the phase comparator. Two sensors at the inlet of the flow chamber were coated with a specific ligand protein. The two sensors at the outlet were coated with a reference protein. Buffer and analytes were pumped unidirectional over the sensors. (C) Flow diagram of the sensor setup.

surface, under exclusion of the contact pads. An injection valve (Rheodyne 7725) equipped with a syringe needle port was installed between peristaltic pump and flow-through cell for injection of 200 μl of the samples to the cell at a flow rate of 34 μl/min, resulting in a total exchange after about 350 s (Fig. 1C).

During the experiments, phase changes of the single sensor elements were recorded by a network analyzer (Rhode & Schwarz ZVCE, Munich, Germany) giving a signal which is directly proportional to the surface coverage with the air-filled microparticles (e.g. their "mass").

2.3. Preparation of targeted contrast agent

The ultrasound contrast agents were based on air-filled poly(butylcyanoacrylate-co-cyanoacrylic acid) (PBCA-co-CA) microparticles. Streptavidin was coupled to those microparticles using carbodiimide chemistry. Biotinylated anti-EDB-fibronectin AP39 scFv-antibody-fragment was coupled to the surface using biotin/streptavidin interaction (Budde et al., 2002). Hundred microliters of microparticles (1×10^{10} MP/ml) were diluted in 15 ml sodium acetate buffer (10 mM), pH 4.5, and incubated with 7.5 mg of EDC and 300 μg streptavidin for 1 h at RT under stirring and finally incubated over night at 4 °C without stirring. Afterwards, the microparticles were washed two times with HEPES buffer (pH 7.4) containing 0.01% Triton X-100. The presence of streptavidin on their surface was determined by FACS using biotin-FITC as marker. Streptavidin-loaded microparticles (1×10^6) were further incubated with 4 μg biotinylated AP39 in phosphate buffered saline (PBS, pH 7.4) for 30 min at room temperature. To remove free antibody from the system, the mixture was centrifuged for 2 min at 2000 rpm. The supernatant with floating microparticles was collected and resuspended in PBS containing 0.01% Triton. The amount of AP39 coupled to the microparticles was measured by flow cytometry using a conjugate of biotinylated EDB-FN and streptavidin-coupled *R*-phycoerythrin as marker system (data not shown).

2.4. Modification of sensing element

The conditions of the surface chemistry used to functionalize the self-assembling monolayer (SAM) have previously been intensively studied (Schlensog et al., submitted for publication) and applied to the microparticles. The sensor chip surface was incubated in an ethanolic solution of 11-mercaptoundecanoic acid (MUA) for 12 h, forming a self-assembled monolayer with carboxyl groups. The MUA-coated sensor was activated by a solution containing 0.05 M EDC and 0.2 M NHS for 30 min. After washing with distilled water, 10 μl of EDB-fibronectin in PBS buffer (260 μg/ml) was added to the surface of two out of five sensor elements of the chip and incubated at RT for

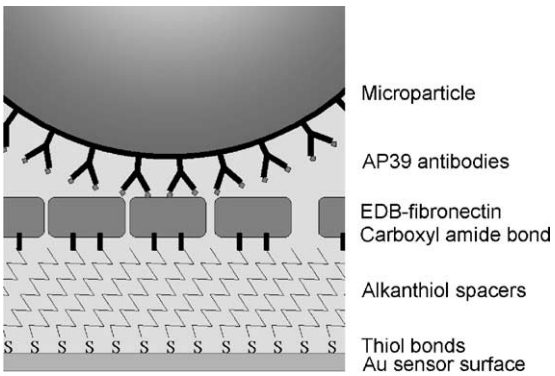

Fig. 2. Binding of microparticles to immobilized EDB-fibronectin via AP39 antibody (not to scale). EDB-fibronectin is coupled to the sensor surface by carboxyl amide bonds provided by a SAM of 11-mercaptoundecanoic acid.

30 min. Remaining active sites on the coated surface were deactivated with 1 M ethanolamine, pH 8.5. After 30 min, the sensors were washed with PBS buffer and distilled water. Two sensor elements of the chip were used as reference elements. Two methods of surface passivation were used:

(1) the activated carboxyl termini were quenched with ethanolamine mixture;
(2) fibronectin without EDB component coupled to the surface via carbodiimide chemistry, as described above.

2.5. Binding assay

The functionalized chip with sensor and reference elements was placed in the flow cell and exposed to a continuous PBS buffer flow of 34 μl/min, until the phase signal was stable. The sample injection volume partly was predetermined by the normally used sample loop. The carrier buffer PBS is used to provide isotonic environment. This ensured high binding of the antibodies to their antigen and will enable transfer of the results to a possible utilization in living organisms. The temperature was kept constant at 25 °C to exclude a temperature effect. For specific binding, microparticles loaded with AP39 were suspended as indicated and injected into the flow system with a 200 μl loop. The phase changes of the sensor and reference elements corresponding to the binding of targeted microparticles were recorded as a function of time. Control experiments were carried out with underivatized microparticles and microparticles loaded with isotype IgG under the same conditions. For the inhibition experiment, 260 μg/ml AP39 were injected into the flow cell followed by an injection of a suspension of 1×10^7 AP39-loaded microparticles/ml, and the change in phase was recorded (Fig. 2). To exclude viscosity effects due to the injection of various concentrations of the microparticles, we calculated the difference of the phase shift before microparticles were injected and after buffer washed away unbound microparticles.

3. Results and discussion

The binding of microparticles to the sensor surface was constantly recorded by monitoring the phase shift of the wave due to mass changes on the sensing surface. In preliminary tests, conditions were optimized, e.g. concentrations of the immobilized proteins to prevent steric hindrance, loading of antibodies to the microparticles, coverage of microparticles with antibodies, or flow rate (data not shown). At higher flow rates, the large microparticles broke off their anchoring, and little phase shift was measured, while at lower flow rates the microparticles were retained in the fluidic system, and accumulated. It was shown that the coverage with antibodies and the stability of binding highly depends on the surface chemistry used to couple antibodies, and the concentration of antibodies in the coupling solution. At lower coverage, binding of the microparticles increased with antibodies coupled to the surface. The amounts used ensure high coverage of the microparticle surface with antibodies to attain high binding.

Fig. 3 shows the phase change observed during the flow of target-specific AP39-loaded microparticles over the sensor or reference elements, respectively, followed by a washing step. The measured phase change of EDB-fibronectin-coated sensor elements is much higher than the phase change of just SAM-coated reference elements. During the first injection, a total phase change of 1.8° for the EDB-fibronectin sensor elements was measured, and 0.5° for the reference elements which were saturated for unspecific binding. This shows the specific binding of the targeted microparticles to the EDB-fibronectin surface. A second injection with the two-fold concentration of loaded microparticles led to a further phase change of about 0.9° by specific binding to the sensor elements. The second phase

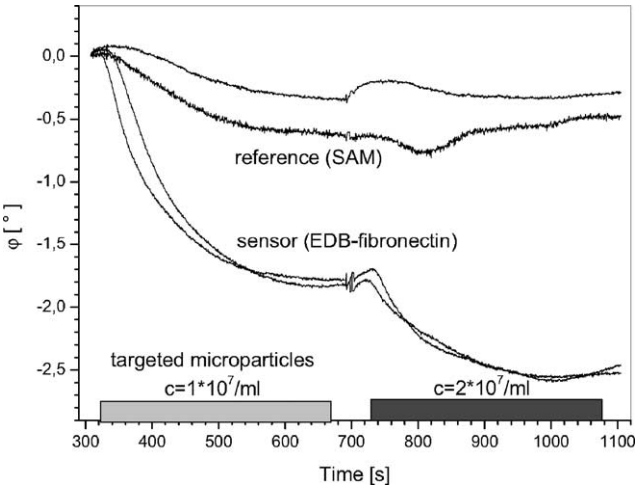

Fig. 3. Non-specific and specific binding of targeted microparticles to the sensor arrays. Response of EDB-fibronectin elements and reference elements during the injection of 200 μl of targeted microparticles, $c = 1 \times 10^7$/ml (light grey bar) followed by $c = 2 \times 10^7$/ml (dark grey bar) with a flow rate of 34 μl/min.

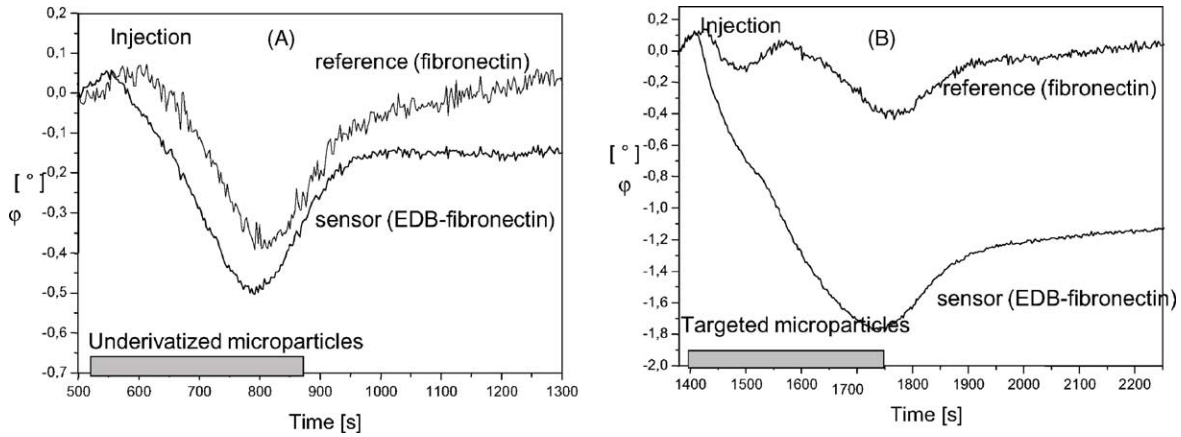

Fig. 4. The response of an EDB-fibronectin sensor element and a reference element during the injection of (A) underivatized microparticles, $c = 1 \times 10^7$/ml, (B) followed by targeted microparticles, $c = 1 \times 10^7$/ml.

change is smaller, presumably due to saturation of binding sites by the first injection, while no further phase changes were detectable on the reference elements. Preliminary experiments showed a correlation between concentration of targeted microparticles and phase shift (results not shown). Further concentration-dependent binding experiments for calibration of the sensor are necessary.

To ensure that the response of the sensor results from specific interaction and does not originate from non-specific adsorption, we performed a set of control experiments. For clarity, the following diagrams show only one reference and one sensor signal. Signal variation was below 5%. During the flow of underivatized microparticles over the sensor chip, a significant phase change was detected on both fibronectin and EDB-fibronectin sensor elements due to viscosity effects. The total phase change was reduced to almost zero when buffer was flowing through the cell (Fig. 4A). After equilibration, a total phase change of about 1.4° was observed for the EDB-fibronectin sensor elements as the result of injection of targeted microparticles (see Fig. 4B).

Injection of isotype IgG-loaded microparticles resulted in a phase change of about 0.3° for both the EDB-fibronectin sensor element and the fibronectin reference element (Fig. 5A). The following injection of targeted microparticles on the same sensor chip resulted in a phase shift of 1.6° for the EDB-fibronectin sensor element and 0.3° for the fibronectin reference element. The measured low response on the reference element is caused by non-specific binding of targeted particles to the fibronectin surface (Fig. 5B). In a second experiment, no difference in the response to IgG isotype antibodies, $c = 260\,\mu$g/ml was obtained, but a second injection of 1×10^7/ml targeted microparticles resulted in a phase shift of 0.9° on the EDB-fibronectin sensor element and 0.1° on the fibronectin reference element, indicating that the signal is specific for the targeted microparticles.

To further confirm specificity, the targeting binding sites were blocked with free AP39 scFv-antibody-fragment. The binding of AP39 generated a high phase shift on the EDB-fibronectin sensor elements, but almost no change on the fibronectin reference elements. This result shows the specific

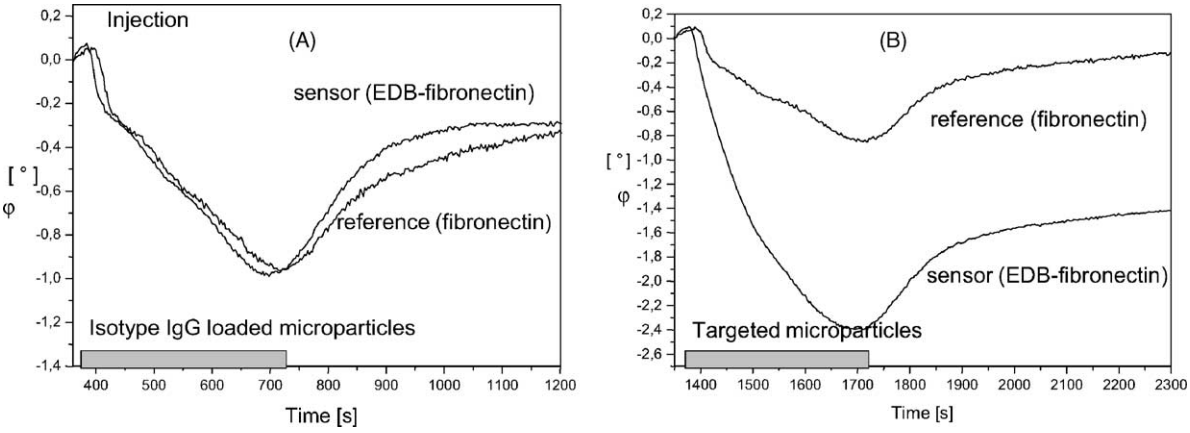

Fig. 5. The response of EDB-fibronectin sensor element (solid line) and fibronectin reference element (dotted line) during the injection of (A) isotype IgG-loaded microparticles, $c = 1 \times 10^7$/ml, (B) followed by targeted microparticles, $c = 1 \times 10^7$/ml.

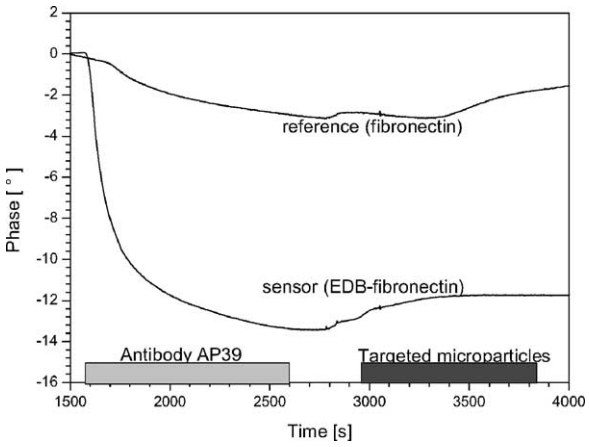

Fig. 6. The response of an EDB-fibronectin sensor element and a fibronectin reference element during injection of AP39, $c = 260$ μg/ml, and AP39-loaded microparticles, $c = 1 \times 10^7$/ml.

interaction of AP39 with EDB-fibronectin-coated sensor elements. Almost no phase change was observed due to the addition of targeted microparticles to the sensor elements blocked with AP39 (Fig. 6).

It can be concluded that the binding sites on the sensor element for specific interaction with AP39-loaded microparticles were saturated. These data provide additional evidence that the binding of targeted microparticles to the EDB-coated surface results from specific interactions.

4. Conclusion

We developed a functionality assay for air-filled target-specific microparticles based on a Love-wave sensor. The experiments suggest the feasibility of SAW technique to study their binding behavior.

It is shown that the SAW technique appears to be the detection system of choice for the characterization of target-specific micron-sized particles. Although published data for penetration depth are in the same order of magnitude for both SPR and SAW, experiments have shown that only with the Love-wave sensor it was possible to detect the particles. However, further experimental work is required to improve the selectivity and sensitivity of the assay.

Key advantages offered by acoustic sensors are an electronic read-out and the measurement of phase shift or resonant frequency, followed with a high degree of precision using conventional electronics. This electronic compatibility and the possibility to automate the process make these sensors more accessible. Furthermore, the cost of the apparatus appears to be rather low. Modifying the sensor arrays with biomolecules using general immobilization techniques offers the opportunity to measure biological interactions in a wide range of applications, thus making the technique versatile even for particulate systems with a density lower than 1 g/ml. In addition, sensor arrays offer the possibility to detect and quantify various analytes with the same system.

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