

An Aptamer-Based Quartz Crystal Protein Biosensor

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We developed a quartz crystal biosensor designed to detect concentrations and ligand affinity parameters of free unlabeled proteins in real time. Using a model system with human IgE as the analyte and single-stranded DNA aptamers or an anti-IgE antibody as immobilized ligands, we could demonstrate that aptamers were equivalent to antibodies in terms of specificity and sensitivity. Both receptor types selectively detected 0.5 nmol/L of IgE. In addition, the aptamer receptors tolerated repeated affine layer regeneration after ligand binding and recycling of the biosensor with little loss of sensitivity. Because of the small size and nonprotein nature of the aptamers, they were immobilized in a dense, well-oriented manner, thus extending the linear detection range to 10-fold higher concentrations of IgE. In addition to demonstrating for the first time that an aptamer-based biosensor can specifically and quantitatively detect an analyte in various complex protein mixes, the aptamer-ligand proved to be relatively heat resistant and stable over several weeks. Since aptamers consist of nucleic acids, well-established chemistry can be applied to produce optimized affine layers on biosensors that may be developed to specifically detect proteins in solution for analysis of proteomes.

One major postgenomic goal is the analysis of a chosen cellular proteome. While generating gene expression profiles based on nucleic acid detection is hugely informative, there is an increasing need for similarly rapid and sensitive techniques that analyze proteins directly. The conventional ligands for nonnucleic acid targets are antibodies. More recently, however, nucleic acid aptamers that bind to target proteins with high affinity via their three-dimensional structure have been selected from large randomized oligonucleotide libraries.^{1,2} Such aptamers can mimic antibodies in a number of applications, such as ELISA,³ immunobead assays,⁴ cell sorting,⁵ fluorescence microscopy,⁶ Western

blotting,⁷ and affine layers for biosensors or chips^{8–10} (for an overview see ref 11). In comparison to antibodies as capture molecules in biosensors, small aptamer receptors have a number of advantages. Because of their small size, denser receptor layers can be generated, meaning for a given receptor affinity, the sensitivity of these layers can be increased. They are produced in vitro or, indeed, in machina: no animals are needed. This not only allows the production of affine ligands even against nonimmunogenic or toxic proteins and other targets, but also provides much greater flexibility. For example, their binding affinity, specificity, and stability can easily be manipulated and improved by rational design or by techniques of molecular evolution. They can be modified with functional groups or tags to allow covalent, directed or indirect immobilization on biochips, resulting in highly ordered receptor layers. Moreover, although it is difficult to guarantee the same batch-to-batch quality for antibodies, homogeneous qualities of aptamers can be achieved via chemical synthesis. Finally, because of their simple structure, sensor layers based on aptamer receptors can be regenerated more easily than antibody-based layers, are more resistant to denaturation and degradation, and have a much longer shelf life.

To test a model system for rapid detection of various selected proteins, we developed a protein chip based on small capture molecules, here single-stranded DNA aptamers. Compared to unmodified RNA aptamers, DNA aptamers are more stable and easier to synthesize. The receptor molecules were covalently attached to a gold-coated quartz crystal microbalance, thereby avoiding the need to fluorescently label either the analyte or receptor and the subsequent complex photophysics and photochemistry accompanying detection by fluorescence anisotropy.^{8,9} To investigate our biosensor system and at the same time the suitability of aptamers as receptor molecules, we chose a model pair of receptors: a published, well described DNA aptamer specific for IgE (D17.4, M_w 12 kDa¹²) and for comparison, a commercially available monoclonal anti-IgE antibody ($M_w \sim 150$

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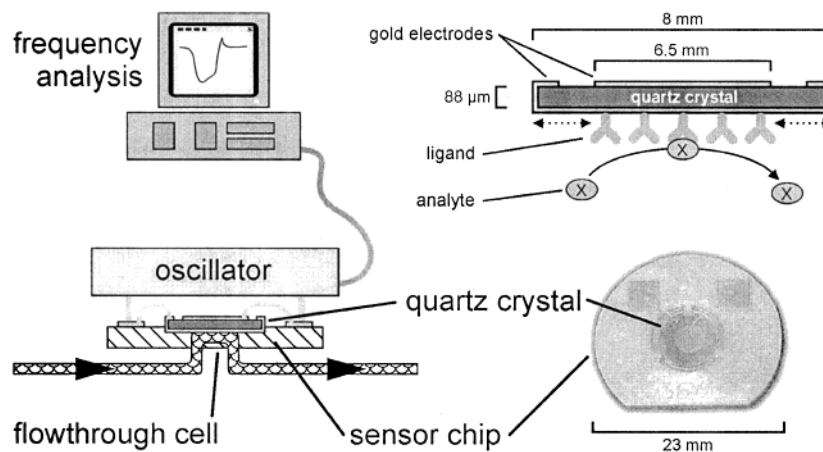


Figure 1. Schematic view of the applied quartz crystal microbalance (QCM) (left) and the quartz crystal (top right). The quartz crystal mounted on the sensor chip is shown bottom right.

kDa). The aptamer was subsequently improved by rational design, and as a result not only displayed equal sensitivity and selectivity compared to the antibody, but also retained the linear measurement range and reusability of the first aptamer.

EXPERIMENTAL SECTION

Materials. Anti-IgE aptamer DNA oligonucleotides D17.4 (5' GGG GCA CGT TTA TCC GTC CCT CCT AGT GGC GTG CCC C 3') and D17.4ext (5' GCG CGG GGC ACG TTT ATC CGT CCC TCC TAG TGG CGT GCC CCG CGC 3') were obtained from Metabion (Munich, Germany) and were either 5' or 3' biotin-labeled during solid-phase synthesis. Synthesis products were purified by HPLC by the manufacturer and showed a single band on a denaturing polyacrylamide gel. Further purification of oligonucleotides was not necessary. Monoclonal anti-IgE antibody (IgG2b, mouse) was purchased from Biogenesis Ltd (Poole, U.K.). The analyte, IgE purified from human myeloma (Biogenesis Ltd, Poole, U.K.), was diluted from a 20 g/L stock in PBS⁺ (PBS containing 1 mM MgCl₂) to concentrations of 10 μg/L to 100 mg/L and stored on ice prior to use on the same day. Bovine serum albumin fraction V (BSA) was obtained from Sigma (Deisenhofen, Germany) and dissolved in PBS⁺ to a final concentration of 0.025%. Negative control analyte DO-1 (anti-p53 IgG2a from mouse) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA) and diluted in PBS⁺ to a final concentration of 5 mg/L. Complex protein solutions included fetal calf serum (FCS) (Gibco BRL), in which total protein concentration was measured using the BioRad Bradford reagent according to the manufacturer's protocol; the broad range protein standard (BioRad), in which the protein concentration was given by the manufacturer; low-fat milk used as a nonfat dry powder dissolved in PBS⁺ and, again, measured for total protein concentration; and meat extract or brain–heart bouillon, both from Merck and both used as dry powder with dilutions based on the dry powder weight.

Biosensor System. Properties of the immobilized receptor aptamer and antibody surface layers were analyzed with a quartz crystal microbalance (QCM) integrated into a flow-through injection analysis system (Figure 1).¹² The core of the biosensor was a quartz crystal with a diameter of 8 mm and a resonance frequency on the order of 20 MHz. The crystal was coated on

both sides with gold layers serving as electrical contacts and ligand support. Receptor molecules (antibodies and aptamers) were immobilized on the gold layer with a surface of 33 mm² facing the flow-through chamber, which has a total volume of 4 μL. Although the quartz crystal oscillates at its resonance frequency under constant flow of buffer through the flow-through chamber, binding of the analyte results in a measurable decrease in the resonance frequency that is in linear relationship to the increase in mass on the receptor layer. Thus, binding and dissociation kinetics can be monitored directly and evaluated quantitatively. The biosensor system was run in a permanent flow of PBS⁺ at 15–30 μL/min. Samples were injected into a 100-μL sample loop using a syringe. Curve fitting of the data obtained was performed using the Origin program (Microcal Software Inc, Northampton, MA).

Immobilization Procedure. Immobilization of antibodies and aptamers on gold-coated quartz crystals was performed as follows: In a first step, gold surfaces were cleaned by two injections with 200 μL of ice-cold piranha solution (one volume 30% H₂O₂, four volumes conc H₂SO₄) followed by an immediate wash with 500 μL of cold H₂O after each injection. The quartz was washed three times with cold H₂O and dried for 1 h at 100 °C. Gold surfaces were activated by injection of 10 μL of a 4 mg/mL solution of 3,3'-dithiodipropionic acid di(*N*-succinimidyl ester) (DSP) (Fluka, Neu-Ulm, Germany) in water-free *N,N*-dimethylacetamide (DMA) (Sigma, Deisenhofen, Germany), sealed with adhesive tape, and incubated at room temperature for 15 min (Figure 2).¹³ The surfaces were then washed three times with 150 μL of PBS⁺, followed by the immediate injection of 10 μL of a protein dilution in PBS⁺ (anti-IgE antibody, 2 mg/mL or streptavidin, see below), sealed with tape, and incubated at 4 °C overnight. After protein immobilization, the flow-through chambers were rinsed five times with 100 μL of PBS⁺, followed by a 1-h incubation at room temperature with 10 μL of a 0.025% BSA solution to saturate unreacted binding groups on the gold surface. In the case of antibody-coated surfaces, chips could then be used immediately or covered with a 0.02% sodium azide solution in PBS⁺ and stored at 4 °C for <1 week.

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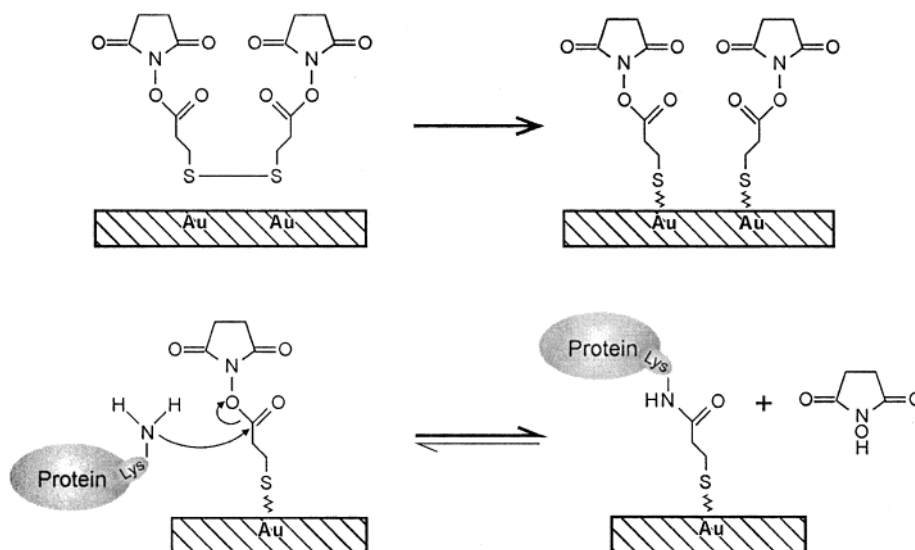


Figure 2. Immobilization of proteins to a gold surface using 3,3'-dithiodipropionic acid-di(*N*-succinimidylester) (DSP).

Quartz crystals intended for aptamer immobilization were coated with streptavidin (1 g/L) (Sigma, Deisenhofen, Germany) as described above for direct antibody immobilization. 5'- or 3'-biotin-labeled aptamer solutions of a concentration of 0.2–2 pmol/ μ L in PBS⁺ were heated to 95 °C for 3 min and chilled on ice to ensure correct intramolecular folding. A 10- μ L portion of aptamer solution was injected into the quartz crystal chamber, sealed, and incubated at room temperature for 1 h. Aptamer-coated chips were either used immediately or covered with a 0.02% sodium azide solution in PBS⁺ and stored at 4 °C for <1 month.

RESULTS AND DISCUSSION

Biosensor System. The detection system for the comparison of immobilized aptamer and antibody affine surface layers is based on the mass dependency of the resonance frequency of a quartz crystal microbalance (QCM) integrated into a flow-through injection analysis system (Figure 1).¹² This system allows real-time quantification of analyte concentrations present in the test solution as well as the determination of affinity parameters of the ligand molecule.¹⁴ Since the change in quartz resonance frequency is in direct linear correlation with its mass, the measurable increase of its mass due to the binding of an analyte can be calculated from the frequency change according to the formula of Sauerbrey,¹⁵

$$\Delta f_r = -c_f \frac{\Delta m}{A} \quad \text{with} \quad c_f = \frac{2f_r^2}{\rho_q v_q}$$

where Δf_r (Hz) is the change of the resonance frequency, Δm (ng) is the change in mass on the quartz surface, A (cm²) is the size of the coated quartz surface, and c_f is the sensitivity constant. The constant c_f is calculated from f_r , the original resonance frequency of the quartz (here, 20 MHz); ρ , the quartz density (2648 kg m⁻³); and v_q , the velocity of sound in the quartz crystal (3340 m s⁻¹). Thus, the sensitivity constant c_f is ~ 0.9 cm² Hz ng⁻¹. With

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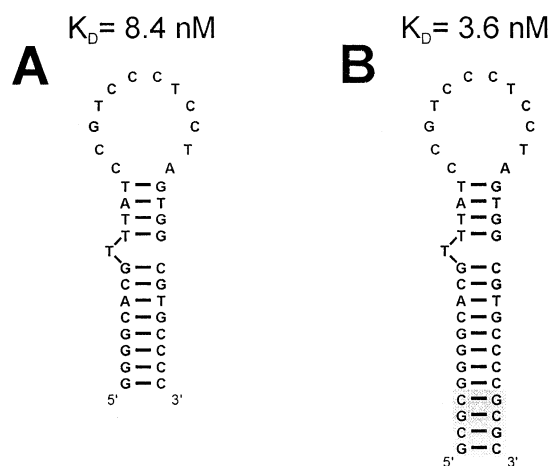


Figure 3. Estimated secondary structure of the published anti-IgE aptamer D17.4 (A) and the engineered aptamer D17.4ext, which now has a stem extended by four base pairs (B).

a sensor surface of 0.33 cm², this results in a frequency change of -27 Hz/10 ng.

Proteins (antibodies) and nucleic acids (aptamers) must first be immobilized on the gold surface of the quartz crystal. In principle, proteins can be fixed noncovalently onto gold by physical adsorption. However, these surfaces are not stable during prolonged buffer rinses, especially if regeneration procedures are carried out. To sterically assist analyte binding, it is advantageous to couple the receptors to the gold surface via a linker molecule. Therefore, we chose the homobifunctional cross-linking reagent DSP (Figure 2),¹³ which binds to gold by a cleavable disulfide bridge and forms a peptide bond to free amino groups of lysine residues. We immobilized antibodies on the biochip using this mechanism (Figure 2).

Initially, aptamers were coupled essentially in the same way to the sensor surface: 5'- and 3'-amino-modified oligonucleotides obtained from the manufacturer were coupled to DSP activated surfaces. Although these receptor layers showed specific IgE binding, they were not as sensitive as the antibody-based sensors because of the rather fast dissociation rate of IgE molecules (0.0034 s⁻¹), in contrast to antibody and biotinylated aptamer

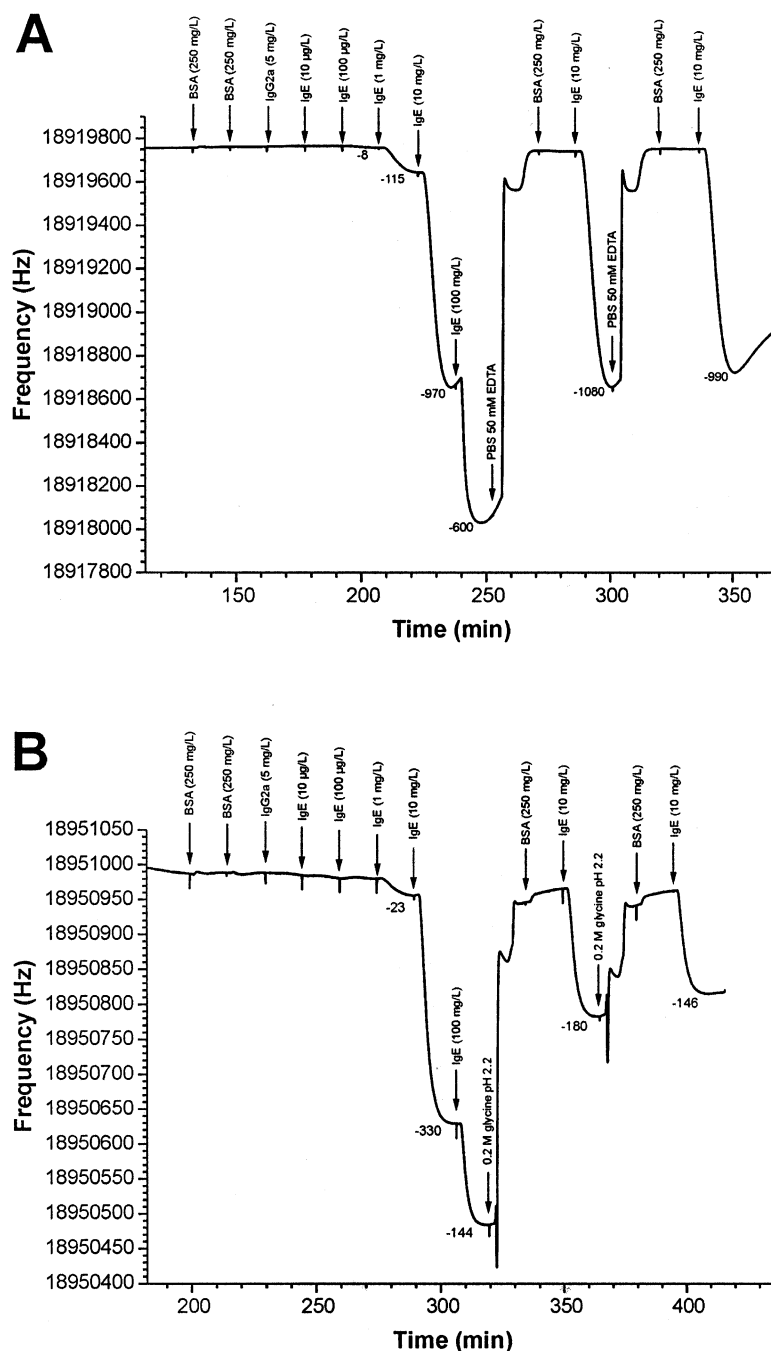


Figure 4. Sensograms using a quartz crystal coated with the anti-IgE aptamer D17.4ext (A) and the anti-IgE monoclonal antibody (B). BSA (250 mg/mL) was injected first to block signals due to unspecific binding. Subsequent injection of a negative control (IgG2a, 5 mg/L) generated no signal, whereas IgE could be detected with high sensitivity and selectivity.

surfaces, which exhibited dissociation rates around 0.0005 s^{-1} (data not shown, and see below).

We, therefore, used biotinylated aptamers and immobilized them via DSP-bound streptavidin. Both 5'- and 3'-biotinylated aptamers showed specific and sensitive binding behavior with far better analyte binding characteristics than the directly coated amino-modified aptamers. The data presented in this report were thus exclusively performed with 5' biotinylated oligonucleotides.

By direct injection of 5'-biotin-labeled aptamer D17.4ext into the biosensor and monitoring the binding to a streptavidin-coated quartz crystal, we measured a total frequency change of -360 Hz . This is calculated to be 133 ng or 9.5 pmol of bound aptamer

($M_w = 14 \text{ kDa}$). Thus, we estimate the density of aptamer molecules on the gold surface to be 4 ng/mm^2 , which corresponds to 286 fmol/mm^2 or $171\,600 \text{ molecules}/\mu\text{m}^2$ (data not shown). The maximum frequency change of a D17.4ext-coated chip saturated with IgE was -1685 Hz , which equals an increase in total weight of 624 ng or 3 pmol ($M_w=200 \text{ kDa}$). Thus, the highest achievable aptamer/analyte ratio was 3:1.

Post-SELEX Improvement of the Aptamer. Quartz crystals coated with monoclonal anti-IgE antibody or aptamer D17.4 were inserted into the sensor, and the analyte, IgE, was injected. Using curve-fit algorithms,¹⁶ we could calculate dissociation constants of 1.9 nM for the immobilized antibody and 8.4 nM for the

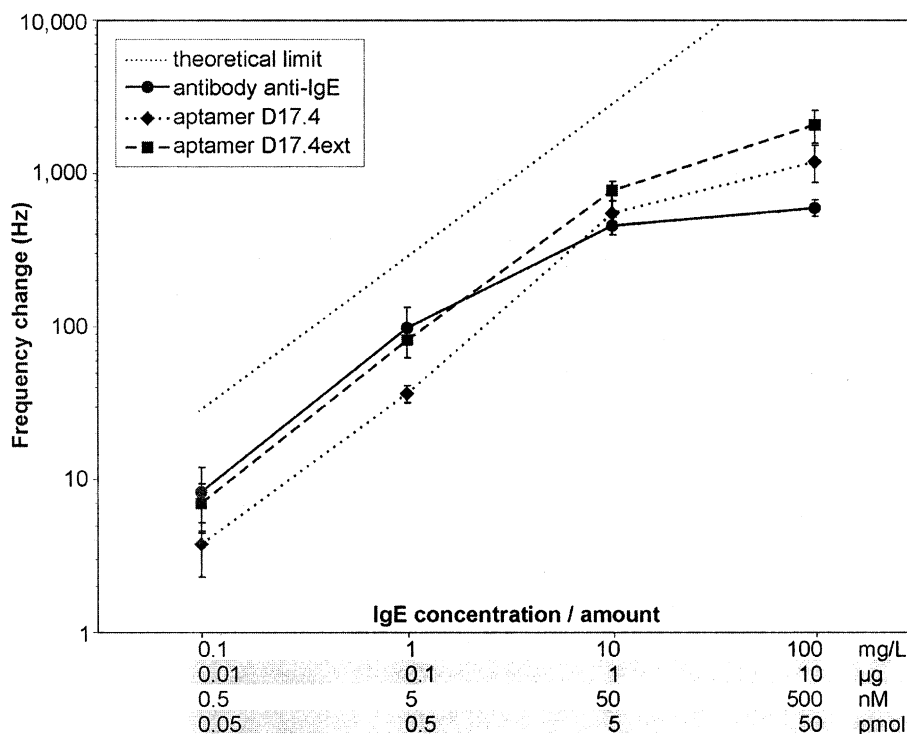


Figure 5. Concentration dependence of analyte detected by receptor layers comprising anti-IgE monoclonal antibody, anti-IgE aptamer D17.4 and extended anti-IgE aptamer D17.4ext. For each receptor type the mean values and standard deviations of four independent measurements are shown. All receptors detect a minimum IgE concentration of 100 $\mu\text{g/L}$. The antibody-based receptor layer has an upper limit to the linear range of detection that is lower than either aptamer-based receptor layer, leveling off above an IgE concentration of 1 mg/L. The black dotted line represents the theoretical maximal signal calculated according to Sauerbrey (1958), assuming that all molecules of the injected 100- μL sample are bound to the biochip.

immobilized aptamer D17.4. The published value for this aptamer in solution is 10 nM.¹² Rapid dissociation sets the detection limit for the analyte and is incompatible with the extensive washing steps necessary for specific detection of analytes in complex samples. We reasoned that the fast dissociation might result from instability of the three-dimensional structure of the aptamer, which spontaneously unfolds and releases the analyte, even under binding buffer conditions (Figure 3). To stabilize the structure of the aptamer, we extended both the 5' and 3' ends of D17.4 by adding 5' GCGC 3' sequences, allowing formation of a longer stem and, consequently, a more stable tertiary structure. This strategy reduced the dissociation constant of the aptamer to 3.6 nM, thus improving its binding characteristics. The engineered aptamer was called D17.4ext (extended).

Comparison of Selectivity, Sensitivity, and Detection Range. Antibody and aptamers were characterized with respect to their selectivity to IgE, their sensitivity, and their detection range (Figures 4 and 5). Both receptor types detected IgE very specifically at minimum concentrations of 100 $\mu\text{g/L}$. The respective frequency changes at this analyte concentration were 8 ± 4 (antibody), 4 ± 1 (D17.4), and 7 ± 2 Hz (D17.4ext), where recent studies with this biosensor have shown that a change of resonance frequency of at least 3 Hz can be considered statistically significant.¹³ As predicted, the sensitivity of the engineered aptamer is higher than that of the published D17.4. Injection of a negative control protein (IgG2a) generated no signal. The linear range of measurement of both aptamers, D17.4 and D17.4ext, was extended

compared to that of the antibody, since at analyte concentrations of > 1 mg/L the antibody-coated quartz suffered saturation effects, whereas the aptamer chips had a linear measurement range up to 10 mg/L (Figure 5). This extended linear range most likely reflects the dense and highly ordered nature of the aptamer receptor layer.

Surface Regeneration. The regeneration of binding surfaces is important for reusable biosensors but is often difficult to achieve.¹⁷ We tried to regenerate the biosensor surface after binding IgE (Figures 6 and 7) by rinsing the antibody-coated chips with acid buffer (0.2 M glycine-HCl, pH 2.2), a common procedure to dissociate antibody-antigen complexes. This resulted in a complete release of the analyte. However, all subsequent injections of IgE led only to reduced frequency changes. Apparently, the receptor layer was either irreversibly damaged or the antibodies were unfolded, and refolding was too inefficient to be effective between subsequent injections of the analyte.

In contrast, binding of IgE to the aptamers is an almost completely reversible process. Rinsing the surfaces with 50 mM EDTA leads to dissociation of the analyte, the receptor layer being completely reconstituted simply by returning to original buffer conditions (PBS⁺). This is because most aptamers need bivalent metal ions to assemble into their three-dimensional structure and unfold in the presence of EDTA. They refold very rapidly when EDTA is withdrawn and replaced by Mg^{2+} -containing buffer.

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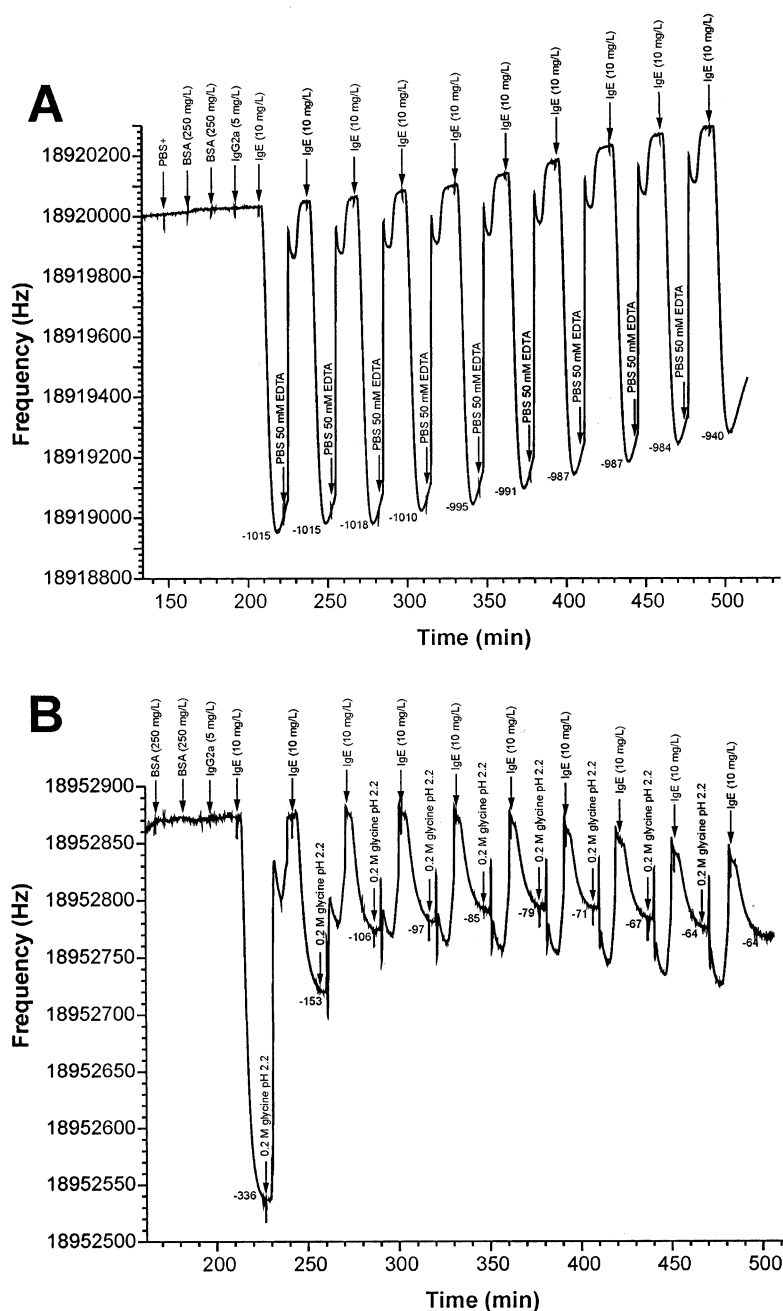


Figure 6. Sensograms of repetitive measurements and regenerations of the receptor layer: (A) aptamer D17.4ext-coated chip, regeneration with 50 mM EDTA; (B) anti-IgE antibody-coated chip, regeneration with 0.2 M glycine-HCl, pH 2.2.

This effective regeneration procedure can be applied to all aptamers requiring Mg^{2+} for their tertiary structure. Alternatively, we found we could also regenerate aptamer receptor layers with acidic (0.2 M glycine-HCl, pH 2.2) or chaotropic (6 M urea) buffers with no significant loss of sensitivity (data not shown).

Surface Stability. In contrast to vulnerable antibody surfaces, affine surface layers based on aptamers can be stored for several weeks without loss of sensitivity. Furthermore, aptamer surfaces are heat-stable. Even after 30 min of incubation at 100 °C, aptamer-coated quartz sensors retained 25% of their sensitivity on average, whereas their antibody counterparts lost activity completely.

Sensitivity and Specificity in Complex Protein Solutions.

To demonstrate the capability of aptamer-coated biosensors to specifically detect IgE in a complex protein sample, we combined defined amounts of IgE (10 mg/L) with other protein mixes of defined protein amounts. These were broad range protein standard (10-fold total protein excess), FCS (100-fold total protein excess), low-fat milk (100-fold total protein excess), meat extract (100- and 1000-fold total protein excess) and brain-heart bouillon (100- and 1000-fold total protein excess). These samples were used to test aptamer sensitivity and selectivity, although it also proved necessary to modify the running buffer of our system. Although the measurement of pure IgE was performed in PBS⁺, the addition of 0.1% BSA to the running buffer was necessary and sufficient to

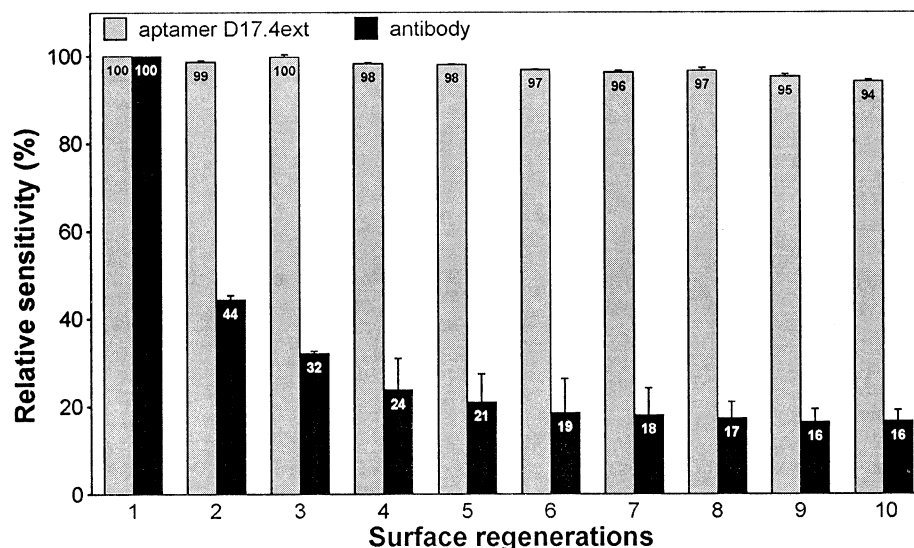


Figure 7. Comparison of 10 cycles of measurement and regeneration of aptamer-based biochips (grey) and antibody-based biochips (black). Following regeneration, only the aptamer layers retain their full binding capacity. Mean values and standard deviations of three independent measurements are shown.

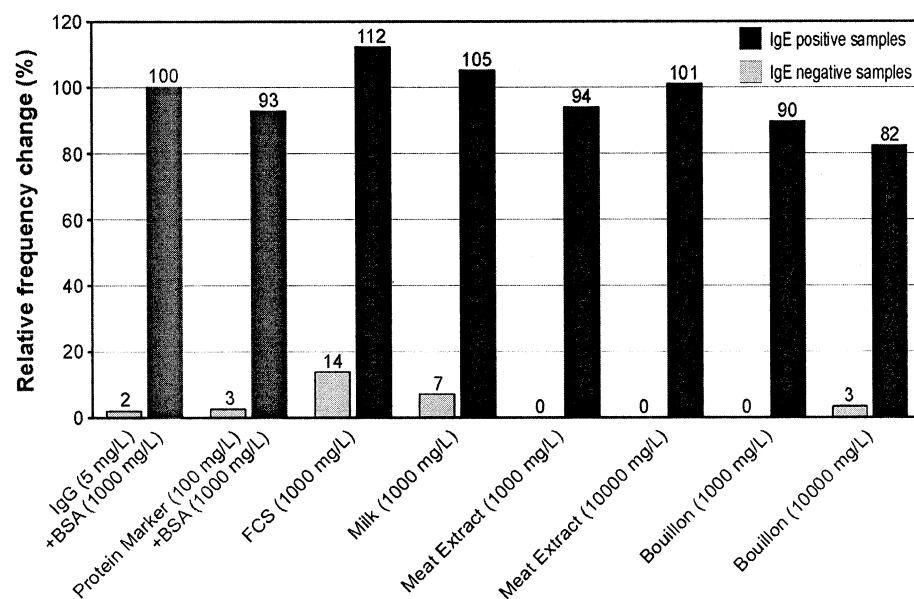


Figure 8. Relative signal intensities generated by different analyte samples (complex protein mixtures with and without 10 mg/L IgE) are shown in relation to the signal generated by IgE + BSA alone (100%).

prevent unspecific detection of bulk protein in the complex mixes.

In these samples, measured signals were specific for the presence of IgE and correlated almost quantitatively ($\pm 18\%$) with the amount of injected analyte present in the samples (Figure 8). These data are the first demonstration that specific detection and quantification of proteins from complex protein samples is also possible with an aptamer-based biosensor.

CONCLUSIONS

Our model biosensor was designed to directly assess important parameters of an immobilized ligand. Affine selectivity, sensitivity, affinity, detection range, regeneration properties, and stability need to be optimized and calibrated before considering alternative biosensor or chip designs. The quartz crystal microbalance provided real-time, quantitative measurements and could be

generally applied as both a selective biosensor and comprehensive test system with any receptor ligand and a variety of target proteins in solution.¹⁸

Here we assessed a DNA-aptamer as an affine ligand, and it compared favorably with its antibody counterpart. Aptamers offer a wide range of features and capabilities that make them attractive ligands for a variety of detection methods. Moreover, automation of SELEX will provide the tools for rapid and cheap isolation of new aptamers soon.¹⁸ Since many details of the highly elaborate (genome-based) DNA microarray technologies are easily transferred to aptamer chips, these low-molecular-weight receptors could become the perfect complement for proteome-based analyses.

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