Anal. Chem. **2005,** *77,* 3437−3443

An Aptamer-Based Protein Biochip

Karin Stadtherr, Hans Wolf, and Petra Lindner*

Institut für Medizinische Mikrobiologie und Hygiene, Universität Regensburg, Regensburg, Germany

The establishment of an aptamer-based biochip for protein detection is described. Using a model system comprising human IgE as the analyte and single-stranded DNA aptamers specific for IgE or anti-IgE antibodies as immobilized ligands on chips, we could demonstrate that aptamers were equivalent or superior to antibodies in terms of specificity and sensitivity, respectively. Aptamer-based analyte detection on glass slides could clearly be demonstrated at minimum concentrations of 10 ng/mL IgE. In addition, we successfully showed specific analyte recognition in complex protein samples by the aptamer-based biochip system. Using DNA aptamers specific for human thrombin as an additional model receptor/ligand system, dual protein detection on a single slide could be proven. In conclusion, we could show the suitability of nucleic acid aptamers as low molecular weight receptors on biochips for sensitive and specific protein detection, representing an innovative tool for future proteomics.

Recently DNA microarrays became a powerful tool in the investigation of genomewide gene expression and transcription analysis.[1−3] However, mRNA expression profiling is not able to give an account of the actual stage of a cell. Apart from the fact that mRNA levels do not necessarily correlate with the levels of their respective proteins, neither do they provide insights into posttranslational modifications,[4] which exert considerable influence on protein functions.

With the aim of a more detailed analysis of the cellular proteome, several strategies have been evolved. One of the most promising technologies, two-dimensional gel separation combined with mass spectrometric analysis, is currently restricted by high processing times and low sensitivity.[5] Another approach of increasing importance is the identification and quantification of proteins by antibody microarrays, employing the established methodologies of DNA microarrays. Due to their structural instability, the immobilization of antibodies as capture molecules is causing considerable problems. It turned out that immobilization procedures influenced their biochemical activities[6] and even denaturation occurred in some cases.[7,8]

Therefore, we focused on short single-stranded nucleic acids, DNA aptamers, as low molecular weight receptors for protein detection on biochips. Equaling antibodies in binding properties, DNA aptamers are able to replace antibodies in a multitude of common applications, such as ELISA,[9] immunobead assay,[10] western blotting,[11] biosensor applications,[12,13] and, most recently, microarrays.[14] Compared to antibodies, aptamers provide decisive advantages. First, they are more resistant against denaturation and degradation. Second, their binding affinities and specificities can be manipulated easily and improved by rational design or by techniques of molecular evolution.[12] Further, aptamers can be modified with functional groups or tags that allow covalent, directed immobilization on biochips, resulting in highly ordered receptor layers.[12] And finally, since nucleic acid aptamers are produced in vitro or in machina by a method called SELEX (systematical evolution of ligands by exponential enrichment),[15,16] in principle any desired target is accessible for aptamer selection, even nonimmunogenic or toxic proteins.

To investigate the suitability of aptamers as receptor molecules on biochips for protein detection, we chose a well described and by rational design improved DNA aptamer specific for IgE.[12,17] Resulting protein detection characteristics of the aptamers were then compared to that of commercially available monoclonal anti-IgE antibodies. Compared to antibodies, aptamers exhibit similar sensitivities and are even superior in respect to specificity. Using DNA aptamers specific for human thrombin[13,18−21] as an additional

* To whom correspondence should be addressed. E-mail: Petra.Lindner@klinik.uni-regensburg.de. Tel. (+49) 941 59609-616. Fax (+49) 941 59609-608.

(1) Chee, M.; Yang, R.; Hubbell, E.; Berno, A.; Huang, X. C.; Stern, D.; Winkler, J.; Lockhart, D. J.; Morris, M. S.; Fodor, S. P. A. *Science* **1996**, *274*, 610−4.
(2) Ramsay, G. *Nat. Biotechnol.* **1998**, *16*, 40−4.
(3) Shoemaker, D. D.; Linsley, P. S. *Curr. Opin. Microbiol.* **2002**, *5*, 334−7.
(4) Parekh, R. B.; Rohlff, C. *Curr. Opin. Biotechnol.* **1997**, *8*, 718−23.
(5) Gygi, S. P.; Corthals, G. L.; Zhang, Y.; Rochon, Y.; Aebersold, R. *Proc. Natl. Acad. Sci. U.S.A.* **2000**, *97*, 9390−5.
(6) Vijayendran, R. A.; Leckband, D. E. *Anal. Chem.* **2001**, *73*, 471−80.
(7) Metzger, S. W.; Lochhead, M. J.; Grainger, D. W. *IVD Technologies Category: New Technologies*; 2002 (June).
(8) Haynes, C. A.; Norde, W. *Colloids Surf., B* **1994**, *2*, 517−66.
(9) Drolet, D. W.; Moon-McDermott, L.; Romig, T. S. *Nat. Biotechnol.* **1996**, *14*, 1021−5.
(10) Rye, P. D.; Nustad, K. *Biotechniques* **2001**, *30*, 290−5.
(11) Lin, Y.; Nieuwlandt, D.; Magallanez, A.; Feistner, B.; Jayasena, S. D. *Nucl. Acids Res.* **1996**, *24*, 3407−14.
(12) Liss, M.; Petersen, B.; Wolf, H.; Prohaska, E. *Anal. Chem.* **2002**, *74*, 4488−95.
(13) Potyrailo, R. A.; Conrad, R. C.; Ellington, A. D.; Hieftje, G. M. *Anal. Chem.* **1998**, *70*, 3419−25.
(14) Smith, D.; Collins, B. D.; Heil, J.; Koch, T. H. *Mol. Cell. Proteomics* **2003**, *2*, 11−8.
(15) Ellington, A. D.; Szostak, J. W. *Nature* **1990**, *346*, 818−22.
(16) Tuerk, C.; Gold, L. *Science* **1990**, *249*, 505−10.
(17) Wiegand, T. W.; Williams, P. B.; Dreskin, S. C.; Jouvin, M. H.; Kinet, J. P.; Tasset, D. *J. Immunol.* **1996**, *157*, 221−30.
(18) Bock, L. C.; Griffin, L. C.; Latham, J. A.; Vermaas, E. H.; Toole, J. J. *Nature* **1992**, *355*, 564−6.
(19) Macaya, R. F.; Schultze, P.; Smith, F. W.; Roe, J. A.; Feigon, J. *Proc. Natl. Acad. Sci. U.S.A* **1993**, *90*, 3745−9.
(20) Huang, C. C.; Cao, Z.; Chang, H. T.; Tan, W. *Anal. Chem* **2004**, *76*, 6973−81.
(21) Pavlov, V.; Xiao, Y.; Shlyahovsky, B.; Willner, I. *J. Am. Chem. Soc.* **2004**, *126*, 11768−9.

10.1021/ac0483421 CCC: $30.25 © 2005 American Chemical Society
Published on Web 04/30/2005

model receptor/ligand system, dual protein detection on a single slide was proven. We could clearly show that nucleic acid aptamers represent a suitable class of low molecular weight receptors for protein detection on a chip.

EXPERIMENTAL SECTION

Materials. Anti-IgE aptamer DNA D17.4ext (5′-GCG CGG GGC ACG TTT ATC CGT CCC TCC TAG TGG CGT GCC CCG CGC-3′), control aptamer DNA D17.4ext-rev (5′-CGC GCC CCG TGC GGT GAT CCT CCC TGC CTA TTT GCA CGG GGC GCG-3′), and anti-thrombin aptamer DNA (5′-GGTTGGTGTGGTTGG-3′) obtained from Metabion (Munich, Germany), were 3′-modified with amino groups and optionally 5′-modified with Cy5 during solid-phase synthesis. Synthesized products were HPLC-purified by the manufacturer. Monoclonal anti-IgE antibody (IgG2b, mouse) and IgE2b purified from human myeloma were purchased from Quartett (Berlin, Germany), human anti-IgG (rabbit) was bought from DakoCytomation (Hamburg, Germany), and human α-thrombin was purchased from CellSystems (St. Katharinen, Germany).

Radioactive Labeling. ssDNA aptamer was labeled with ^{32}P using γ^{32}P-ATP and T4 polynucleotide kinase in a 20-μL reaction mixture containing 0.5 nmol of aptamer, 5 μCi of γ^{32}P-ATP, and 5.0 units of T4 polynucleotide kinase as recommended by the manufacturer for 30 min at 37 °C. After removal of unincorporated nucleotides (NAP-5 column, Amersham Pharmacia, Freiburg, Germany), the radiolabeled aptamers were eluted in 1 mL of PBS. By addition of unlabeled oligonucleotides, a 20 μM working solution was prepared.

Protein Labeling. IgE was labeled with Texas Red-X (0.4 mg of IgE), Alexa 555 (0.1 mg of IgE), or Alexa 647 (0.1 mg of IgE) as described by the manufacturers (Texas Red-X Protein Labeling Kit or AlexaFluor 555/647 Monoclonal Antibody Labeling Kit, Molecular Probes, MoBiTec, Göttingen, Germany). Labeled proteins were eluted in final volumes of 1 mL (Texas Red) or 140 μL (Alexa 555 or Alexa 647).

As non-anti-IgE aptamer binding controls, either fetal calf serum (2 mg) or meat extract (2 mg) was labeled with Texas Red (Texas Red-X Protein Labeling Kit, Molecular Probes, MoBiTec). Labeled proteins were eluted in final volumes of 310 μL.

After dialysis (PBS buffer) human α-thrombin (0.5 mg) was labeled with Alexa 647 (AlexaFluor 647 Monoclonal Antibody Labeling Kit, Molecular Probes, MoBiTec) as decribed by the manufacturer. Labeled proteins were eluted in a final volume of 800 μL.

Preparation of Activated Glass Slides. Glass slides were cleaned for 1 h in 1 M NaOH at 100 °C, followed by an immersion in HCl (5%) for 5 min. The slides were then rinsed in distilled water and dried at 100 °C. Precleaned microscope slides were immersed in a 90% methanol/water solution containing 2% (3-aminopropyl)trimethoxysilane for 30 min. Slides were washed three times with 90% methanol/water, three times with methanol, and three times with water and finally baked for 45 min at 120 °C. Silanized slides were incubated in a solution containing 2.5% glutaraldehyde, 100 mM Na_2HPO_4, and 150 mM NaCl (pH 7.0) for 1 h. Optional 0.1% $NaBH_3CN$ was added to the reaction mixture. Afterward, the slides were rinsed with PBS^+ (PBS containing 1 mM $MgCl_2$) and centrifuged (1 min, 1200 rpm).

Aptamer/Antibody Coupling on Activated Glass Slides. Various concentrations of 3′-amino-modified ssDNA aptamers (100 μM/2.5 μM D17.4ext or D17.4ext-rev in PBS^+ containing 1 mM $NaBH_3CN$) or antibodies (1 mg/mL anti-IgE or anti-IgG in PBS^+ optionally containing 1 mM $NaBH_3CN$ (glass slides)) were applied as ∼10-nL nanodroplets (MicroCASTer, Schleicher & Schuell Bioscience, Dassel, Germany) onto the activated glass surface or on nitrocellulose-coated slides (FAST slides, Schleicher & Schuell Bioscience) and incubated overnight at 20 °C in a humid chamber. The droplets were allowed to dry, and any noncovalently bound aptamer was removed by washing in boiling water for 30 s. Antibody-spotted slides were washed three times with PBS^+ for 30 min. After rinsing the glass samples twice with PBS^+ (5 min), remaining nonreacted carbonyl groups were blocked with 50 mM ethanolamine, 0.1 M Tris (pH 9.0) for 15 min at 50 °C. Aptamer-spotted glass slides were incubated in 0.1% $NaBH_3CN$ in PBS^+ (1 h, room temperature). Both types of slides were then rinsed in PBS^+ buffer (pH 7.4). Aptamer-coated slides were additionally incubated in 50 mM EDTA (5 min) and afterward washed in PBS^+ (5 min) to allow refolding of the aptamers. Finally, the slides were centrifuged (2 min, 2000 rpm, Falcon tube) and, depending on the following experiment, incubated with fluorescently labeled proteins.

Binding Assay. After blocking for 3 h at 37 °C (0.1% Tween 20, 0.2% I-Block (Applied Biosystems, Darmstadt, Germany) or 1% BSA) in PBS^+ buffer (pH 7.4)), the slides were incubated with fluorescence labeled IgE (2 μg/mL to 1 ng/mL) in a final volume of 800 μL (PBS^+ or PBS^+/BSA) for 16 h at room temperature (FAST Microhybridization Hyb Chamber, S & S BioScience, Dassel, Germany). Optionally binding reactions were supplemented with complex protein samples (250−500 μg/mL). After rinsing the slides with PBS^+ (pH 7.4) containing 0.1% Tween 20 (3 × 5 min), the slides were centrifuged (2 min, 2000 rpm, Falcon tube).

For dual protein detection (IgE and human α-thrombin on the same slide), the aptamer-spotted slides (anti-IgE aptamer, anti-thrombin aptamer) were blocked (see above) and then incubated successively in optimized binding buffers for the respective analytes. For thrombin binding, the slide was incubated in 10 mM Tris-buffer (pH 8.4) containing 5 mM $MgCl_2$, 20 mM KCl, and 1% BSA. Afterward, slides were incubated with 50 mM EDTA (10 min) and equilibrated in PBS^+ (3 × 10 min). IgE binding was performed in PBS^+/BSA.

Nitrocellulose-Coated Slides. Antibodies (anti-IgE antibodies or anti-IgG antibodies were applied as ∼10-nL nanodroplets (MicroCASTer, Schleicher & Schuell Bioscience) onto nitrocellulose-coated slides (FAST slides (Schleicher & Schuell Bioscience) and allowed to dry (1h, room temperature). Slides were then incubated in PBS^+, 0.2% I-Block (Applied Biosystems), 0.1% Tween 20 for blocking of nonspecific binding sites (3h, 37 °C). After rinsing the slides with PBS^+, they were incubated with IgE−Texas Red (2 μg/mL−1 ng/mL) overnight. Subsequently the slides were washed (3 times PBS^+, 0.1% Tween 20) and dried at room temperature.

Signal Detection. Radiolabeled probes were measured on a Phosphoimager (Molecular Imager system, Molecular imaging screen-BI, 800-μm resolution, BioRad, Munich, Germany). Screens were exposed to the radiolabeled probes for 68 h.

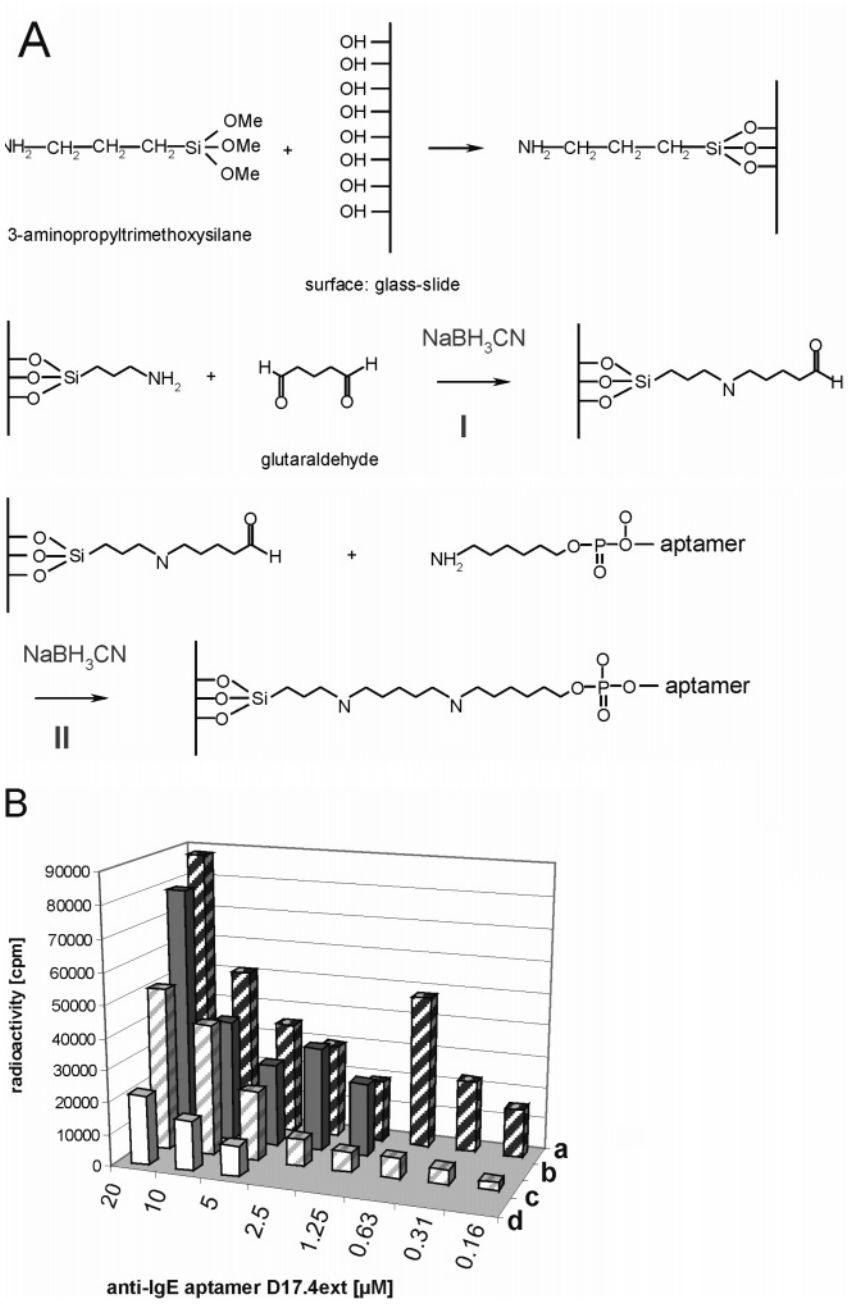

Figure 1. Aptamer coupling on glass slides. (A) Chemical reaction mechanism: 5′-amino-modified single-stranded DNA aptamers were immobilized via glutaraldehyde linkage on amino-silanized glass slides. The supplementation of the reaction buffer with cyanoborohydride ($NaBH_3CN$) in step I and/or step II helps driving the reaction because unstable intermediate products are removed directly after formation by reduction to highly stable secondary amines. (B) Influence of cyanoborohydride on coupling efficiencies: Various concentrations of γ^{32}P-labeled aptamers D17.4ext (20−0.16 μM) were immobilized on glass slides as described in the Experimental Section. After removal of any noncovalently bound aptamers, radioactive signals were measured. The mean ($n = 2$) of measured radioactivity is plotted against the employed aptamer concentrations. The highest signals were obtained if cyanoborohydride was added to the reaction mixtures for both glutaraldehyde linkage (step I) and aptamer coupling (step II). (a) Steps I and II in the presence of (+) cyanoborohydride ($NaBH_3CN$). (b) Step I (−), step II (+) $NaBH_3CN$. (c) Step I (+), step II (−) $NaBH_3CN$. (d) Steps I and II without (−) $NaBH_3CN$.

Fluorescence signals were detected using a microarray laser scanner (LS 200, Tecan Austria GmbH, Salzburg, Austria) in confocal scanning modus: laser excitation wavelength, 543.5 (Alexa555); 632.8 nm (Texas Red, Alexa647, Cy5); emission filter wavelength, 590 (Alexa555), 625 (Texas Red). 670 nm (Alexa647, Cy5).

RESULTS AND DISCUSSION

Optimization of Aptamer Coating and Analyte Binding Assay. Prior to analyzing low molecular weight nucleic acid aptamers as innovative receptors on biochips as compared to antibodies, we optimized the immobilization procedure for aptamers to the glass surface. Afterward the binding assay was improved with respect to obtained signal quality.

First, we developed a strategy for enhancing coupling efficiency of aptamers to the glass surface by using cyanoborohydride ($NaBH_3CN$) as a reducing agent in the reaction mixtures. Therefore, 5′-amino-modified aptamers (D17.4ext) were radiolabeled (^{32}P) and immobilized via glutaraldehyde linkage on amino-

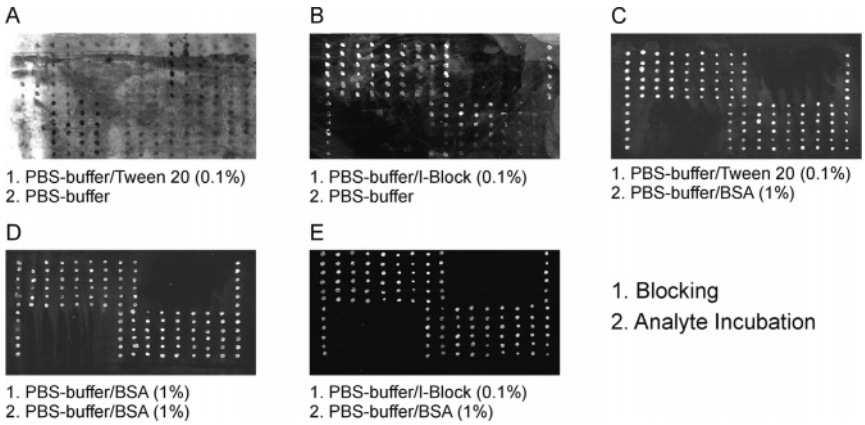

Figure 2. Influence of blocking reagents on signal qualities. Various concentrations of anti-IgE aptamers (D17.4.ext) and negative control oligonucleotides (D17.4.ext-rev) were immobilized on glass slides (see spotting scheme in Table 1 and Experimental Section for coating procedure). Nonspecific binding sites were then blocked using 0.1% Tween 20 (A, C), 0.2% I-Block/0.1% Tween 20 (B, E), or 1% BSA (D) in PBS buffer. Binding of the analyte IgE was then carried out in PBS (A, B) or in PBS supplemented with 1% BSA (C, D, E).

silanized glass slides (Figure 1A). Coupling was carried out in the presence or absence of $NaBH_3CN$ in step I and/or step II (Figure 1A) as described in the Experimental Section, and signals of covalently immobilized aptamers were measured. As shown in Figure 1B, the percentage of coupled aptamer molecules could be substantially increased by the addition of $NaBH_3CN$ to the reaction mixture. $NaBH_3CN$ helps driving the reaction because unstable intermediate products (Schiff bases) are removed directly after formation by reduction to highly stable secondary amines (reductive amination). Coupling efficiency was improved 2.5-fold if $NaBH_3CN$ was present in step I (glutaraldehyde linkage, Figure 1A, Figure 1Bc). Supplementation of the reaction mixture with $NaBH_3CN$ in step II (aptamer immobilization, Figure 1A, Figure 1Bb) resulted in a 2.9-fold increase in coupling rate. We found that the highest coupling efficiencies (~4.8-fold increase) were attained if $NaBH_3CN$ was added to the reaction mixtures during both glutaraldehyde linkage and aptamer coupling (Figure 1Ba). Therefore, all following aptamer coupling procedures were carried out in reaction buffers supplemented with $NaBH_3CN$ in both steps (glutaraldehyde linkage to aminosilane and amino aptamer coupling to glytaraldehyde).

The next step in the development of the aptamer-based protein chip comprised optimization of analyte (IgE) binding to the immobilized aptamers (D17.4ext). All IgE binding experiments were carried out in PBS buffer (pH 7.4) modified to contain 1 mM $MgCl_2$ as this buffer system was employed during the SELEX process.[17] In addition, prior affinity measurements by quartz crystal microbalance[12] or atomic force microscopy[22] demonstrated affine binding of IgE to anti-IgE aptamers in PBS buffer. Various blocking reagents (BSA, Tween 20, I-Block) were tested regarding their generated backgrounds. As shown in Figure 2, the blocking of nonspecific binding sites and the composition of the analyte containing incubation mixture were of critical importance with respect to acquired signal qualities. Any deviations from the optimum procedure, which turned out to be the combination of I-Block for blocking of nonspecific binding sites on the glass slides and a PBS/BSA mixture for analyte incubation, resulted in higher background signals (lower signal-to-noise ratios) or even in a lack of analyte recognition (Figure 2A).

(22) Jiang, Y.; Zhu, C.; Ling, L.; Wan, L.; Fang, X.; Bai, C. *Anal. Chem.* **2003**, *75*, 2112−6.

Aptamer versus Antibody-Coated Chips: Comparison of Sensitivity and Selectivity. After establishing the optimum procedure for aptamer-based analyte detection on the chip formate, we compared aptamer-based protein chips to antibody-based protein chips with respect to achieved sensitivities and selectivities. Therefore, aptamers specific for IgE (D17.4ext) or anti-IgE antibodies were immobilized on glass slides and incubated in solutions containing fluorescently labeled IgE (IgE−Texas Red) in a range from 5 ng/mL to 2 μg/mL (Figure 3). Since antibodies may be susceptible to denaturation on glass surfaces,[7] they were noncovalently attached to nitrocellulose-coated slides (FAST slides) in a parallel approach. Both, antibody- and aptamer-coated slides, showed typical saturation in binding capacities. Aptamer-based slides displayed signal saturation at a concentration of 0.5 μg/mL IgE (Figure 3A). Antibody-coated glass slides performed similarly, whereas antibody-coated FAST slides did not exhibit saturation below a concentration of 1 μg/mL IgE (was applied in the binding assay) (Figure 3A). Although aptamers—due to their smaller size—are likely to be immobilized in a denser arrangement than antibodies, signal saturation did not shift to higher concentrations of IgE. This effect may be caused by steric hindrance between bound analyte molecules. The antibody-coated glass chips generated significantly lower detection signals, possibly caused by partial denaturation of the immobilized antibodies on the glass surface, leading to a decreasing number of correctly folded antibodies being available for specific analyte recognition (Figure 3A).

Concerning obtained sensitivities, aptamers proved to be superior compared to antibodies, even if the antibodies were fixed on nitrocellulose-coated slides, which are shown to be a more appropriate surface for protein immobilization, minimizing unfavorable denaturation effects (Figure 3B). Anti-IgE antibodies were able to specifically detect IgE at a minimum concentration of 50 ng/mL, whereas specific analyte recognition by the aptamer could be observed at least down to a concentration of 10 ng/mL IgE in the binding assay. Taking into account that commercially available ELISA kits provide detection limits of 15 ng/mL IgE (Human IgE ELISA Quantitation Kit, Bethyl Laboratories, Montgomery, TX), aptamer microarrays have every prospect of detecting proteins or any other targets at least as sensitively as other common analytical methods. In a previous approach, anti-IgE antibodies

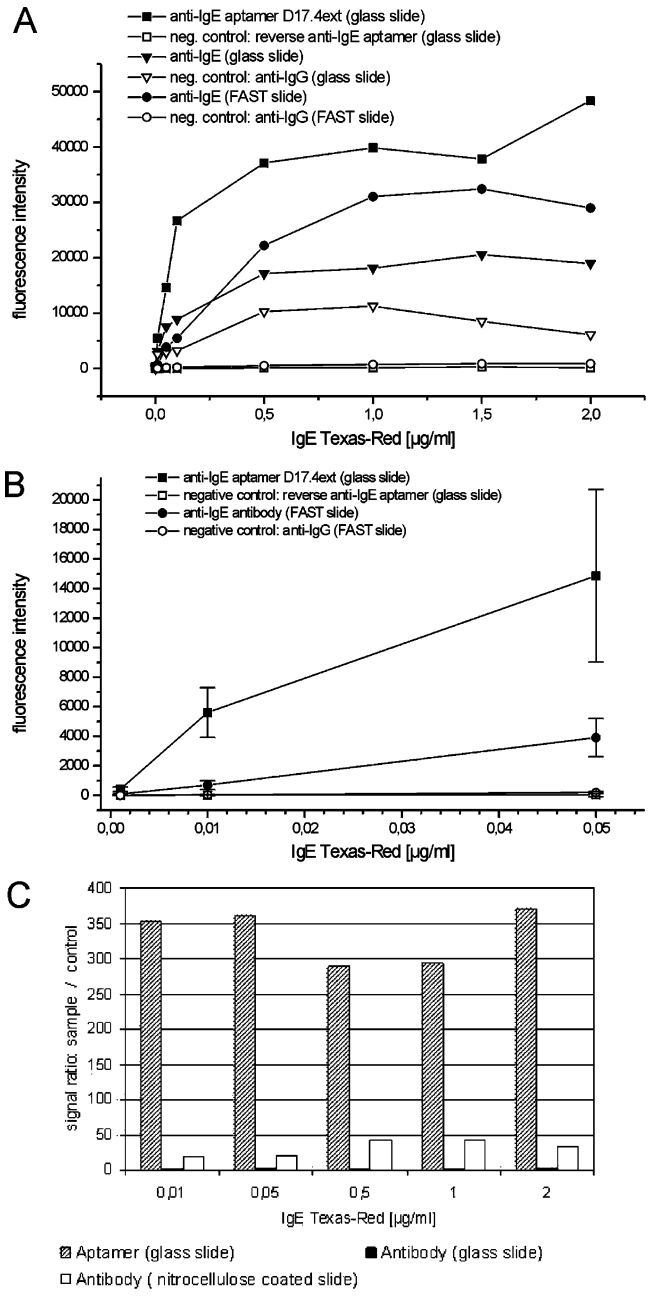

Figure 3. Sensitivity and selectivity: anti IgE aptamers versus anti IgE antibodies. (A) Saturation and signal intensities Anti-IgE aptamers (D17.4.ext) and anti-IgE antibodies were compared regarding their sensitivity and selectivity. Aptamers (40 μM) or antibodies (1 mg/mL) were immobilized on glass slides or, in the case of the antibodies, additionally on nitrocellulose-coated slides (FAST slides). The slides were then incubated with various concentrations of Texas Red-labeled IgE (0.001−2 μg/mL). Fluorescence signals were analyzed using Array-Pro Analyzer (Media Cybernetics, Silver Spring, MD) software. The mean (n = 12) of measured fluorescence signals is plotted against the employed IgE concentrations. As negative controls, either oligonucleotides representing the reverse sequence of the anti-IgE aptamer or anti-IgG antibodies were used. (B) Detection limits. Detailed section of (A) in the range of 0.00−0.05 μg/mL IgE. The 8.3% trimmed mean (n = 12) and error bars (3σ) of the data shown in (A) are plotted. In the case of aptamers (D17.4ext), a detection limit of at least 0.01 μg/mL could be measured. Antibodies (FAST slides) could detect minimum concentrations of 0.05 μg/mL. (C) Selectivity: Signal-to-control ratios of aptamer-coated and antibody-coated slides. Mean values of positive signals (A) were divided by their respective mean values of negative control signals (A).

and aptamers specific for IgE (D17.4ext) were compared as receptor molecules using a quartz crystal microbalance biosensor.[12] Both receptor types detected IgE very specifically at minimum concentrations of 100 ng/mL.[12] Considering repeated regeneration of the receptor layer, which can be performed easily in the case of the aptamers as immobilized ligands, the authors emphasized the superiority of the aptamers compared to antibodies.[12]

One of the greatest challenges designers of protein microarrays are faced with is nonspecific protein binding resulting in high signal backgrounds and therefore decreasing assay sensitivity. In this respect, a comparison between the signal intensities of specific IgE recognition with nonspecific IgE binding to control receptors (sample-to-control ratio, Figure 3C) again emphasized the superiority of aptamers. Anti-IgG served as negative control for the antibody-based slides, and an oligonucleotide constituting the reverse sequence of the IgE specific aptamer (D17.4ext.rev) was used to monitor nonspecific binding on the aptamer-coated chip. The aptamer-based microarrays showed high signal-to-control ratios over the whole range (10 ng/mL−2 μg/mL) (Figure 3C) reflecting only low nonspecific analyte bindings. In contrast, binding assays carried out on antibody-immobilized chips resulted in much higher signal background and therefore lower signal-to-control ratios (Figure 3C). It could be clearly demonstrated that aptamers showed between 7- to 18-fold higher signal-to-control ratios compared to antibodies coated on nitrocellulose, indicating strikingly higher specificities. The differences between signal-to-control ratios of aptamer-coated and antibody-coated slides were even more impressive, when antibodies were fixed on glass slides (about 100−300-fold, Figure 3C).

Aptamer versus Antibody-Coated Microarrays: Comparison of Specificity in Complex Protein Samples. To investigate the capability of aptamers to specifically detect their targets in complex protein samples, we incubated anti-IgE aptamer (D17.4ext)-coated glass slides in IgE-containing protein solutions, constituting a 500−1000-fold excess of non-aptamer-recognizing proteins relative to the IgE content (Table 1, Figure 4A−D). Fetal calf serum (FCS) or meat extract (ME) were used as non-anti-IgE aptamer binding proteins. Binding assays were carried out either with protein mixtures containing fluorescently labeled IgE and non-labeled proteins (FCS, ME) in excess or with IgE combined with fluorescently labeled non-IgE-recognizing proteins. The same experiments were also conducted with antibody-coated slides (Table 2, Figure 4E−H). Neither the aptamer-coated slides nor the antibody-arrays showed nonspecific IgE recognition in complex protein samples containing fluorescently labeled nonanalyte binding proteins (Figure 4B,D,F,H). However, aptamer- and antibody-coated slides that were incubated in solutions of fluorescently labeled IgE in the presence of an excess of nonlabeled FCS or ME (Figure 4A,C,E,G) showed tremendous differences in specificities. Aptamer-based slides using reverse aptamer sequences as negative controls (D17.4ext.rev) did not generate any nonspecific binding signals by incubation in complex protein mixtures containing fluorescently labeled analytes (Figure 4A,C). In contrast, the same experiments carried out with antibody-coated arrays, using anti-IgG antibodies as negative controls, showed a considerably higher rate of nonspecific binding events (Figure 4E,G).

Table 1. Spotting Scheme: Aptamers[a]

100	80	60	40	20	10	5	2.5	40	PBS	100	50	25	12.5	PBS	40
100	80	60	40	20	10	5	2.5	40	PBS	100	50	25	12.5	PBS	40
100	80	60	40	20	10	5	2.5	40	PBS	100	50	25	12.5	PBS	40
100	80	60	40	20	10	5	2.5	40	PBS	100	50	25	12.5	PBS	40
100	80	60	40	20	10	5	2.5	40	PBS	100	50	25	12.5	PBS	40
100	80	60	40	20	10	5	2.5	40	PBS	100	50	25	12.5	PBS	40
40	PBS	100	50	25	12.5	PBS	40	100	80	60	40	20	10	5	2.5
40	PBS	100	50	25	12.5	PBS	40	100	80	60	40	20	10	5	2.5
40	PBS	100	50	25	12.5	PBS	40	100	80	60	40	20	10	5	2.5
40	PBS	100	50	25	12.5	PBS	40	100	80	60	40	20	10	5	2.5
40	PBS	100	50	25	12.5	PBS	40	100	80	60	40	20	10	5	2.5
40	PBS	100	50	25	12.5	PBS	40	100	80	60	40	20	10	5	2.5

[a] Various concentrations (μM) of the respective aptamers were spotted as indicated in the table. Labeled in black, negative control reverse anti-IgE aptamer; labeled in gray, PBS buffer.

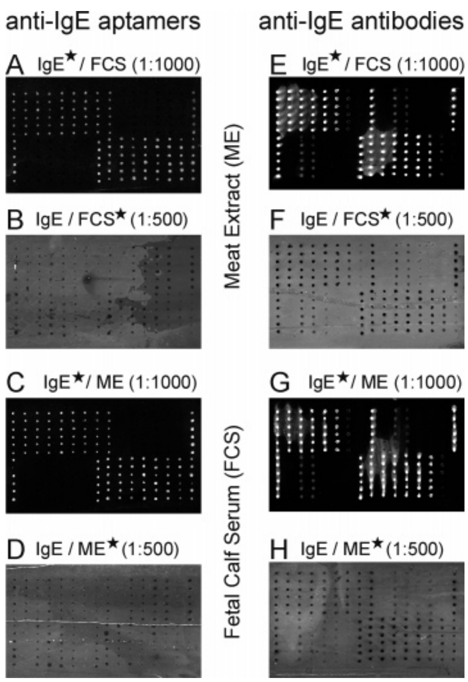

Figure 4. Specificity: anti IgE aptamers versus anti IgE antibodies. Glass slides were coated with various concentrations of either anti-IgE aptamers (D17.4.ext) (A−D) or anti-IgE antibodies (E−H) (see spotting schemes in Tables 1 and 2). Oligonucleotides representing the reverse anti-IgE aptamer sequence or anti-IgG antibodies served as negative controls. For proof of specific analyte recognition in complex protein mixtures, the slides were then incubated with fluorescencently labeled IgE (0.5 μg/mL) in a reaction mixture containing an excess (1:500 (250 μg/mL)−1:1000 (500 μg/mL)) of unlabeled non-anti-IgE aptamer or non-anti-IgE antibody binding proteins (ME (C,G); FCS (A,E)) as indicated in the figure. Controls were carried out using incubation mixtures containing an excess of fluorescence-labeled nonbinders (ME (D,H); FCS (B,F)) and nonlabeled IgE. ★ fluorescence label (Texas Red).

Dual Protein Detection by Aptamers. To demonstrate usefulness of our aptamer-based protein chip for multiple protein detection, we are currently restricted to a limited number of available aptamers and their respective target proteins. Besides, the buffer system applied during the SELEX process is of critical importance for subsequent aptamer−ligand binding experiments. For our approach, it would be desirable that all aptamers were selected in the same buffer and therefore binding experiments (on the slide) can be conducted in a single buffer system. Since no further DNA aptamer plus accompanying target protein selected in PBS^+ buffer (anti-IgE aptamer binding buffer) was available, we chose a DNA aptamer selected against human thrombin[18,19] as a second model aptamer for establishment of dual chip-based protein detection. As the anti-thrombin aptamer was selected in a buffer varying from that employed for the IgE-SELEX, specific analyte recognition had to be carried out successively in two individual buffer systems. Therefore, the anti-IgE aptamer- and anti-thrombin aptamer-spotted slide was first incubated with fluorescently labeled (Alexa 647) human α-thrombin (0.5 μg/mL) in 10 mM Tris buffer (pH 8.4) containing 5 mM $MgCl_2$, 20 mM KCl, and 1% BSA to allow optimal receptor−ligand recognition. Afterward, the same slide was equilibrated and incubated with Alexa 555-labeled IgE (0.5 μg/mL) in PBS buffer modified to contain 1 mM $MgCl_2$ to enable specific IgE recognition. The applied fluorophores for dual protein detection (Alexa 647 and Alexa 555) allow distinct signal discrimination for the two individual targets. As can be shown in Figure 5, the anti-thrombin aptamers specifically detected Alexa 647-labeled human α-thrombin, whereas anti-IgE aptamers were only recognized by fluorescently labeled IgE (Figure 5). Negative controls (reverse sequence of the IgE-specific aptamer, PBS buffer) were detected neither by IgE nor by thrombin (Figure 5).

So far, only few approaches have been described exploring the suitability of aptamers as receptor molecules on solid surfaces for specific protein detection in complex samples. For example, aptamer−analyte binding interactions on a solid phase using fluorescence polarization or anisotropy methods were described.[23] Conventional attachment strategies for immobilization of fluorescently labeled aptamers could be applied but for the readout a special optical system had to be designed.[23] Another attempt focused on using aptamers as receptors on an electronic tongue sensor array.[24] For this approach, aptamers were immobilized on beads that were introduced into micromachined chips on the electronic tongue sensor array and used for the detection and quantitation of proteins.[24] Lee and Walt developed a fiber-optic biosensor using aptamer receptors for the measurement of thrombin.[25] Presently, multiplexed photoaptamer-based arrays represent the most promising strategy for aptamer-based simul-

(23) McCauley, T. G.; Hamaguchi, N.; Stanton, M. *Anal. Biochem.* **2003**, *319*, 244−50.
(24) Kirby, R.; Cho, E. J.; Gehrke, B.; Bayer, T.; Park, Y. S.; Neikirk, D. P.; McDevitt, J. T.; Ellington, A. D. *Anal. Chem.* **2004**, *76*, 4066−75.
(25) Lee, M.; Walt, D. R. *Anal. Biochem.* **2000**, *282*, 142−6.

Table 2. Spotting Scheme: Antibodies[a]

1	0.8	0.6	0.4	0.3	0.2	0.1	0.05	0.4	PBS	1	0.5	0.1	0.05	PBS	0.4
1	0.8	0.6	0.4	0.3	0.2	0.1	0.05	0.4	PBS	1	0.5	0.1	0.05	PBS	0.4
1	0.8	0.6	0.4	0.3	0.2	0.1	0.05	0.4	PBS	1	0.5	0.1	0.05	PBS	0.4
1	0.8	0.6	0.4	0.3	0.2	0.1	0.05	0.4	PBS	1	0.5	0.1	0.05	PBS	0.4
1	0.8	0.6	0.4	0.3	0.2	0.1	0.05	0.4	PBS	1	0.5	0.1	0.05	PBS	0.4
1	0.8	0.6	0.4	0.3	0.2	0.1	0.05	0.4	PBS	1	0.5	0.1	0.05	PBS	0.4
0.4	PBS	1	0.5	0.1	0.05	PBS	0.4	1	0.8	0.6	0.4	0.3	0.2	0.1	0.05
0.4	PBS	1	0.5	0.1	0.05	PBS	0.4	1	0.8	0.6	0.4	0.3	0.2	0.1	0.05
0.4	PBS	1	0.5	0.1	0.05	PBS	0.4	1	0.8	0.6	0.4	0.3	0.2	0.1	0.05
0.4	PBS	1	0.5	0.1	0.05	PBS	0.4	1	0.8	0.6	0.4	0.3	0.2	0.1	0.05
0.4	PBS	1	0.5	0.1	0.05	PBS	0.4	1	0.8	0.6	0.4	0.3	0.2	0.1	0.05
0.4	PBS	1	0.5	0.1	0.05	PBS	0.4	1	0.8	0.6	0.4	0.3	0.2	0.1	0.05

[a] Various concentrations (mg/mL) of the respective antibodies were spotted as indicated in the table. Labeled in black, negative control anti-IgG antibody; labeled in gray, PBS buffer.

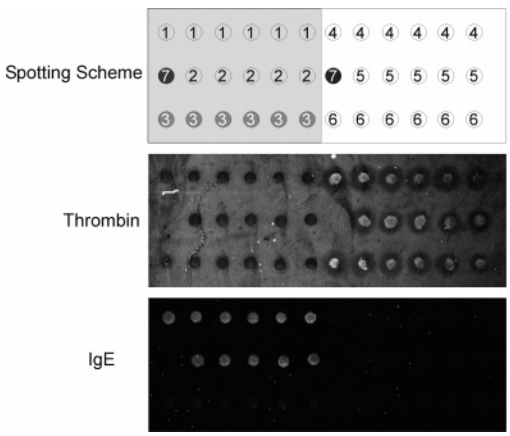

Figure 5. Dual aptamer-based protein detection. Glass slides were coated with various concentrations of anti-IgE aptamers (D17.4.ext) and anti-thrombin aptamers as indicated below. Oligonucleotides representing the reverse anti-IgE aptamer sequence or PBS buffer served as negative controls. For specific dual aptamer-based protein detection, the slide was incubated successively in optimized binding buffers for the respective receptor/ligand recognition (for details, see Experimental Section). For signal detection, binding buffers contained either 0.5 μg/mL Alexa 647-labeled thrombin or 0.5 μg/mL Alexa 555-labeled IgE. (1) Anti-IgE aptamers (100 μM), (2) anti-IgE aptamers (50 μM), (3) negative control (reverse anti-IgE aptamer sequence, 100 μM), (4) anti-thrombin aptamers (100 μM), (5) anti-thrombin aptamers (80 μM), (6) anti-thrombin aptamers (50 μM), and (7) negative control (PBS buffer).

taneous protein detection.[14,26−28] Incorporation of photoreactive 5-bromodeoxyuridine (BrdU) in the photoaptamers provides a means to covalently cross-link the photoaptamer to the bound protein by irradiating with 308-nm light.[26]

Nevertheless, we showed for the first time here that highly specific and sensitive aptamer-based protein detection on glass slides is possible without the use of photoreactive aptamers and covalent cross-linking to the bound protein. With respect to sensitivity and specificity, aptamers appear superior to antibodies as low molecular weight receptors on glass slides. Compared to the above-mentioned approaches of aptamer-based protein detection on solid supports, our microarray formate on glass microscope slides provides the following advantages: First, it represents a low-cost and easy to perform method, allowing the application of well-established DNA microarray techniques. Second, it has the potential to be expanded to high-density multiple protein detection arrays. Third, no special equipment or expensive photoreactive nucleotides are needed.

Our own two aptamers-based chip for protein detection serves as a model system and demonstrates the principal feasibility of our approach. For future aptamer-based multi-protein detection it is desirable that the employed aptamers will be selected in a uniform buffer system.

CONCLUSIONS

Using aptamers specific for IgE (D17.4ext) or anti-IgE antibodies as a model system, we could clearly show that nucleic acid aptamers represent a suitable class of low molecular weight receptors on biochips. Compared to antibodies, aptamer-based analyte recognition was at least as sensitive. Aptamers could detect their analytes (IgE) at minimum concentrations of 10 ng/mL. Regarding specificity, aptamers were even superior compared to antibodies, which exhibited a much higher degree of nonspecific protein binding resulting in higher signal backgrounds. Using DNA aptamers specific for human thrombin[13,19−21] as an additional model receptor/ligand system, dual protein detection on a single slide was unambiguously demonstrated. For the development of a multiple aptamer-based protein chip, we are currently limited by the availability of aptamers and their corresponding target proteins. Since the SELEX protocol has been successfully automated,[27] aptamer arrays on solid surfaces will become readily on-hand. In conclusion, aptamers represent a powerful tool for the establishment of a new generation of protein chips.

ACKNOWLEDGMENT

We thank Dr. Christina Paulus and Dr. Michael Nevels for critically reading the manuscript.This work was supported by the Deutsches Human Genom Projekt (DHGP).

Received for review November 9, 2004. Accepted April 7, 2005.

AC0483421

(26) Bock, C.; Coleman, M.; Collins, B.; Davis, J.; Foulds, G.; Gold, L.; Greef, C.; Heil, J.; Heilig, J. S.; Hicke, B.; Hurst, M. N.; Husar, G. M.; Miller, D.; Ostroff, R.; Petach, H.; Schneider, D.; Vant-Hull, B.; Waugh, S.; Weiss, A.; Wilcox, S. K.; Zichi, D. *Proteomics* **2004**, *4*, 609−18.

(27) Brody, E. N.; Gold, L. *J. Biotechnol.* **2000**, *74*, 5−13.

(28) Brody, E. N.; Willis, M. C.; Smith, J. D.; Jayasena, S.; Zichi, D.; Gold, L. *Mol. Diagn.* **1999**, *4*, 381−8.