DDI-μFIA—A Readily Configurable Microarray-Fluorescence Immunoassay Based on DNA-Directed Immobilization of Proteins

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We describe a chip-based immunoassay for multiplex antigen detection, based on the self-assembly of semi-synthetic DNA – protein conjugates to generate an easily configurable protein microarray. The general principle of this microarray-fluorescence immunoassay (μFIA) is similar to that of a two-sided (sandwich) immunoassay. However, covalent single-stranded DNA – streptavidin conjugates are employed for the efficient immobilization of biotinylated capture antibodies through hybridization to complementary surface-bound DNA oligomers. In a model system, we use the DNA-directed immobilization (DDI) of antibodies to generate an antibody microarray for the parallel detection of the tumor marker human carcinoembryonic antigen (CEA), recombinant mistletoe lectin rViscumin (rVis), ceruloplasmin (CEP), and complement-1-inactivator (C1 A) in human blood serum samples. Detection limits down to 400 pg mL^{-1} are reached. In addition, we describe a method for the internal standardization of protein microarray analyses, based on the simultaneous measurement of constant amounts of the blood proteins CEP and C1 A, intrinsically present in human serum, to compensate for interexperimental variations usually occurring in microarray analyses. The standardization leads to a significantly higher data reliability and reproducibility in intra- and interassay measurements. We further demonstrate that the DDI-μFIA can also be carried out in a single step by tagging of the analyte simultaneously with both capture and detection antibody and subsequent immobilization of the immunocomplex formed, on the DNA microarray capture matrix. This protocol significantly reduces handling time and costs of analysis.

Introduction

The development of miniaturized and parallelized immunoassays, based on antigen and antibody microarray technologies, is currently of tremendous interest for a broad range of applications in biomedical diagnostics,[1–3] where several parameters in an individual sample have to be determined simultaneously from a limited amount of material. While the microarray-based analyses of nucleic acids has made large steps towards routine application,[4] the fabrication and employment of protein chip devices is hampered by the intrinsic instability of many proteins. Although protein microarrays have, for instance, been prepared for high-throughput antibody screening,[5] analysis of antibody – antigen interactions[6–8] or identification of the protein targets of small molecules,[9] the stepwise, robotic immobilization of multiple proteins at chemically activated surfaces is often obstructed by the instability of most proteins, which usually reveal a significant tendency towards denaturation, and thus loss of functionality. To circumvent these obstacles, we have developed the method of DNA-directed immobilization (DDI) of proteins,[10] using covalent conjugates synthesized from single-stranded DNA and streptavidin (STV) as molecular adapters for the tagging of biotinylated proteins with single-stranded DNA oligomers.[11, 12] DDI provides a chemically mild process for the site-selective adsorption of delicate proteins onto a solid support, using DNA-functionalized substrates as an immobilization matrix. Because the lateral surface structuring can now be carried out at the level of stable nucleic acid oligomers, the DNA-functionalized substrate can be fabricated and stored almost indefinitely, and then functionalized with proteins of interest by DDI immediately prior to its use in a microscaled fluorescence immunoassay (μFIA). Since both the fabrication of the functional protein conjugates and their immobilization on the solid support are entirely based on self-assembly, the set-up of the DDI-μFIA is readily configurable from the modular reagents employed: that is, covalent STV – DNA conjugates, biotinylated antibodies and a microarray containing complementary DNA capture oligomers. An additional advantage of DDI in immunoassay applications is that binding of the target antigen by antibodies can be carried out in homogeneous solution, instead of in a heterogeneous solid-phase immunosorption. Subsequently, the immunocomplexes formed are captured on the DNA microarray by nucleic acid hybridization.[13]

We report here on the development of DDI-based μFIA for the simultaneous detection and quantification of four different

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protein antigens—the tumor marker human carcinoembryonic antigen (CEA), recombinant mistletoe lectin rViscumin (rVis), ceruloplasmin (CEP), and complement-1-inactivator (C1 A)—in human blood serum samples. The μFIA is carried out either by using three sequential incubation steps (Figure 1 A) or as a single-step capture assay (Figure 1 B). DDI-based μFIAs allowed us to detect low antigen quantities down to 400 pg mL^{-1}, which is comparable to the limits of detection reached in microplate-based enzyme-amplified immunoassays. We used an internal standardization method to reduce the experimental error of the analysis, taking advantage of two proteins, CEP and C1 A, which occur intrinsically in human blood serum. The normalized DDI-μFIA revealed an exceptional reproducibility. Moreover, we demonstrate here that the single-step DDI-μFIA can be applied for the simultaneous detection of antigens with satisfying sensitivity and reproducibility, thereby significantly reducing time and costs of analyses.

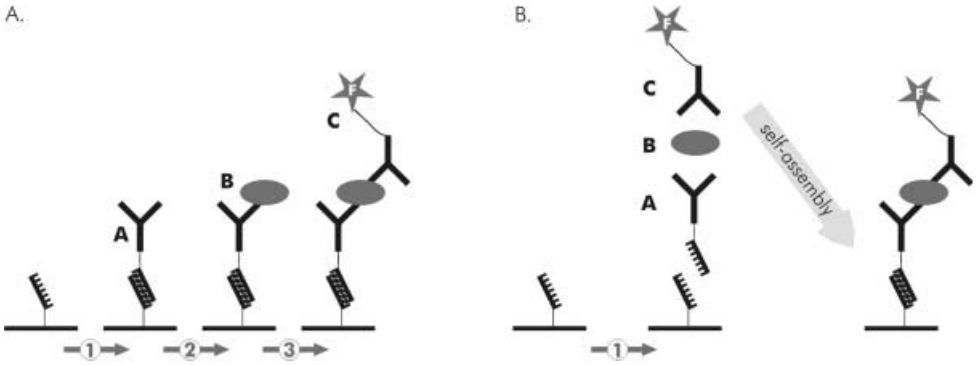

Figure 1. *Schematic drawing of the sandwich immunoassay based on DNA-directed immobilization (DDI), as conducted in this study. A) Sequential incubation of the different components of the assay. B) Single-step capture assay, carried out by simultaneous tagging of the analyte with reagents both for capture and detection.*

Results and Discussion

To facilitate the DNA-directed immobilization (DDI), four covalent conjugates (**HA** – **HD**), containing different base sequences, were synthesized from the corresponding ssDNA oligonucleotides (for sequences see Table 1) and recombinant STV by using the heterobispecific sulfosuccinimidyl maleimido crosslinker, as described previously.[12] The covalent DNA – STV conjugates can be used as versatile molecular adapters, allowing for convenient tagging of biotinylated proteins with a single-stranded oligonucleotide.[11–13] These conjugates, therefore, form the basis of the readily configurable DDI-μFIA. To employ the covalent DNA – STV conjugates for the functionalization of a DNA microarray with capture antibodies, conjugates were produced from **HA** – **HD** and one molar equivalent of the biotinylated antibodies RAC, GAL, SAC, and SCI, with specificity for the tumor marker human carcinoembryonic antigen (CEA), recombinant mistletoe lectin rViscumin (rVis), ceruloplasmin (CEP), and complement-1-inactivator (C1 A), respectively. This coupling leads to the formation of antibody – DNA conjugates capable of hybridizing to surface-bound complementary capture oligonucleotides (Figure 2).

Table 1. *Thiolated oligonucleotide library for the generation of DNA – protein conjugates and the amino-modified complementary capture oligomers. The sequences are shown in the 5′ – 3′ direction.*

Name	Sequence	Modification
tA	TCC TGT GTG AAA TTG TTA TCC GCT	5′ thio link (C6)
tB	ACC TCA AGT GAT CTA CCT ACC TCA G	5′ thio link (C6)
tC	CTC ACA TCC AAC AAT ACA GGT CAC AT	5′ thio link (C6)
tD	TGA GCG TTC GTG GGA TAG T	5′ thio link (C6)
cA	AGC GGA TAA CAA TTT CAC ACA GGA	5′ amino link (C6)
cB	CTG AGG TAG GTA GAT CAC TTG AGG T	5′ amino link (C6)
cC	ATG TGA CCT GTA TTG TTG GAT GTG AG	5′ amino link (C6)
cD	ACT ATC CCA CGA ACG CTC A	5′ amino link (C6)

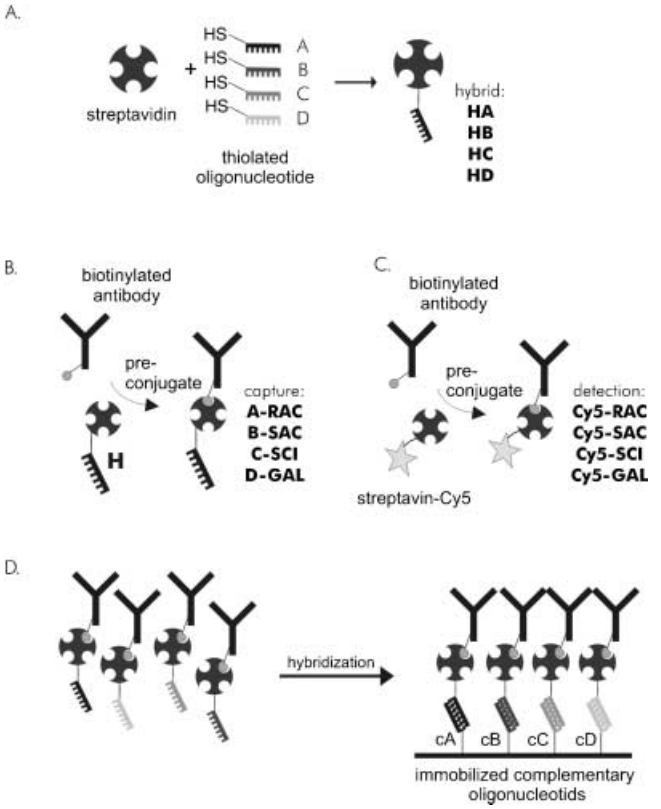

Figure 2. *Schematic representation of the modular preparation of functional conjugates employed in the DDI-μFIA. A) Synthesis of four covalent STV – DNA conjugates (**HA**, **HB**, **HC** and **HD**) from STV and 5′-thiolated oligonucleotides (**A**, **B**, **C** and **D**; for sequences see Table 1). B) Conjugation of **HA** – **HD** with the biotinylated antibodies anti-carcinoembryonic antigen (RAC), anti-ceruloplasmin (SAC), anti-complement-1-inactivator (SCI), and anti-lectin (GAL) to generate capture reagents **A**-RAC, **B**-SAC, **C**-SCI, and **D**-GAL. C) Conjugation of STV-Cy5 with biotinylated RAC, SAC, SCI and GAL to generate fluorophore-labeled detection conjugates. D) Parallel immobilization of the four capture reagents by hybridization with the fully complementary surface-bound oligonucleotides.*

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As an immobilization matrix for the DDI-μFIA, microarrays containing the fully complementary oligomers as capture probes were produced (for sequences see Table 1). Fluorescent signals were generated by using detection conjugates, made up of the corresponding antibodies and Cy5-labelled streptavidin, as illustrated in Figure 2.

In a first set of experiments, the entire set of capture conjugates—**A**-RAC, **B**-SAC, **C**-SCI, and **D**-GAL (25 nM each)—were simultaneously allowed to hybridize with the corresponding complementary capture oligomer on the microarray. Subsequent to hybridization, serial dilutions of samples containing CEA and rVis in standardized human serum (BISEKO) were incubated on the microarray. BISEKO contains 170 μg mL^{-1} of C1 A, a member of the serpin superfamily, and 90 μg mL^{-1} of CEP, a copper-containing dehydrogenase. All four proteins were simultaneously developed through tagging with the antigen-specific Cy5 – antibody conjugates (25 nM each). The fluorescence images obtained are shown in Figure 3. Quantification of the signals (Figure 4 A) revealed a detection limit of about 3 ng mL^{-1} (15 amol μL) for CEA. The recovery rate, determined by using a spiked sample containing 35 ng mL^{-1} (gray line), was found to be about 70%. We had previously investigated the detection of CEA by a DDI-based ELISA in the microtiter plate format. This had revealed a detection limit of about 10 ng mL^{-1} (50 amol μL^{-1}).[13] Thus, the results reported here demonstrate that microplate-ELISA can be converted to the μFIA format, leading to an increase in sensitivity. Moreover, these results nicely demonstrate the robustness of DDI-based assays on various platforms.

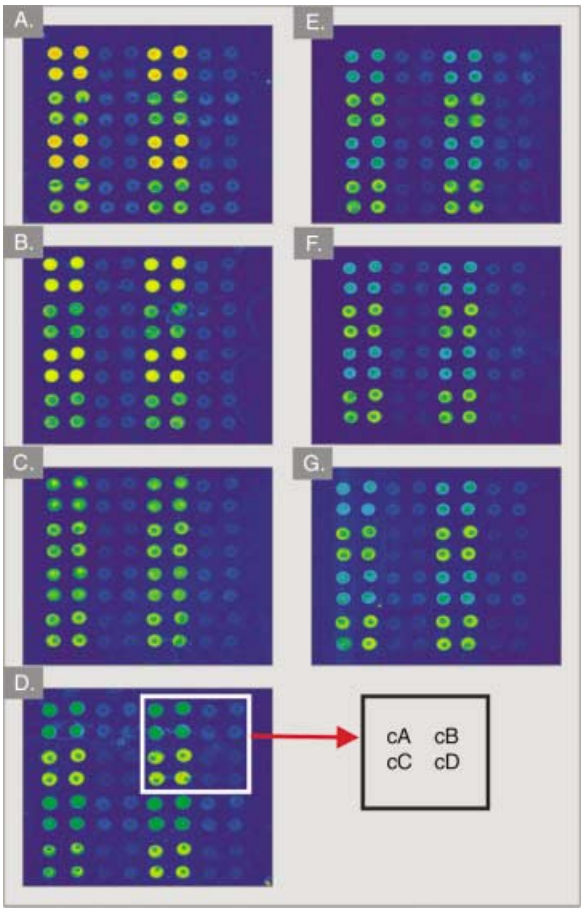

Figure 3. *Fluorescence images of the microarrays obtained from DDI-μFIA experiments. Antibody conjugates* ***A****-RAC,* ***B****-SAC,* ***C****-SCI and* ***D****-GAL (see Figure 2) hybridize with their complementary capture oligomers (****cA****,* ***cB****,* ***cC*** *and* ***cD****), spotted on the microarray in four quadruplets. Serial dilutions of CEA—500 ng mL^{-1} (A), 100 ng mL^{-1} (B), 20 ng mL^{-1} (C), 4 ng mL^{-1} (D), 0.8 ng mL^{-1} (E), 0.16 ng mL^{-1} (F) and 0 ng mL^{-1} (G)—and of rVis—1000 ng mL^{-1} (A), 200 ng mL^{-1} (B), 40 ng mL^{-1} (C), 8 ng mL^{-1} (D), 1.6 ng mL^{-1} (E), 0.32 ng mL^{-1} (F), and 0 ng mL^{-1} (G)—were dissolved in standardized human serum (BISEKO), containing 170 μg mL^{-1} C1 A and 90 μg mL^{-1} CEP, incubated on the array and subsequently developed through the use of STV – Cy5 – antibody conjugates.*

Quantification of rVis by DDI-μFIA, shown in Figure 4 B, revealed a detection limit of about 100 ng mL^{-1} (1.7 fmol μL^{-1}). The alterations in sensitivity reflect individual differences in the kinetic and thermodynamic binding properties of the two antibodies (RAC and GAL) employed in the immunoassay. However, this result shows the extreme specificity of the method, which permits the analysis even of weak signals because of the very low and constant background level (see also Figure 3).

In a second step of microarray data analysis, we normalized the fluorescence signals for the detection of CEA with respect to the values measured for the two blood proteins CEP and C1 A in the μFIA detection. Previous analyses of human blood samples had indicated that these two intrinsically occurring proteins are indeed suitable for standardization of μFIA experiments since they occur in human blood samples in concentrations conveniently traceable by μFIA (data not shown). In this study, the fluctuation of the signal intensities obtained for CEP and C1 A in the various serial dilutions (see signals of cB and cC in Figure 3) point to one of the typical problems of protein microarray analyses: statistical errors resulting from, for instance, variations in the surface properties of the slides.[3] These variations in signal intensity, which are directly proportional to the error in microarray analysis and also assay development, can be compensated for by normalization through the use of internal standard substances. Instead of using non-naturally occurring substances, which need to be spiked to the sample of interest, here we use the intrinsically occurring proteins CEP and C1 A.

For normalization, the fluorescence signal intensities obtained for the serial dilutions of CEA were divided by those obtained for CEP or C1 A, and the resulting values were multiplied with a constant value of 100 for CEP or 1000 for C1 A to generate comparable arbitrary units of signal intensity. The resulting data are shown in Figure 4-C and 4-D. Normalization of the fluorescence signals of CEA by use of the C1 A data, led to an improvement in the detection limit to 400 pg mL^{-1} (2 amol μL^{-1}) of CEA with a spike recovery (35 ng mL^{-1}, grey bar) of approximately 110% for standardization values (Figure 4-C). Similarly, the detection limit and recovery rate for CEA were 500 pg mL^{-1} (2.5 amol μL^{-1}) and ca. 115%, respectively, for normalization by use of the CEP data values (Figure 4 D). The normalization thus improved the detection limit about sevenfold and the recovery rate was improved by about 20%. These results indicate that the correlation of fluorescence signals of a given analyte with signals of internal blood marker proteins is a suitable method for compensation for interassay variations

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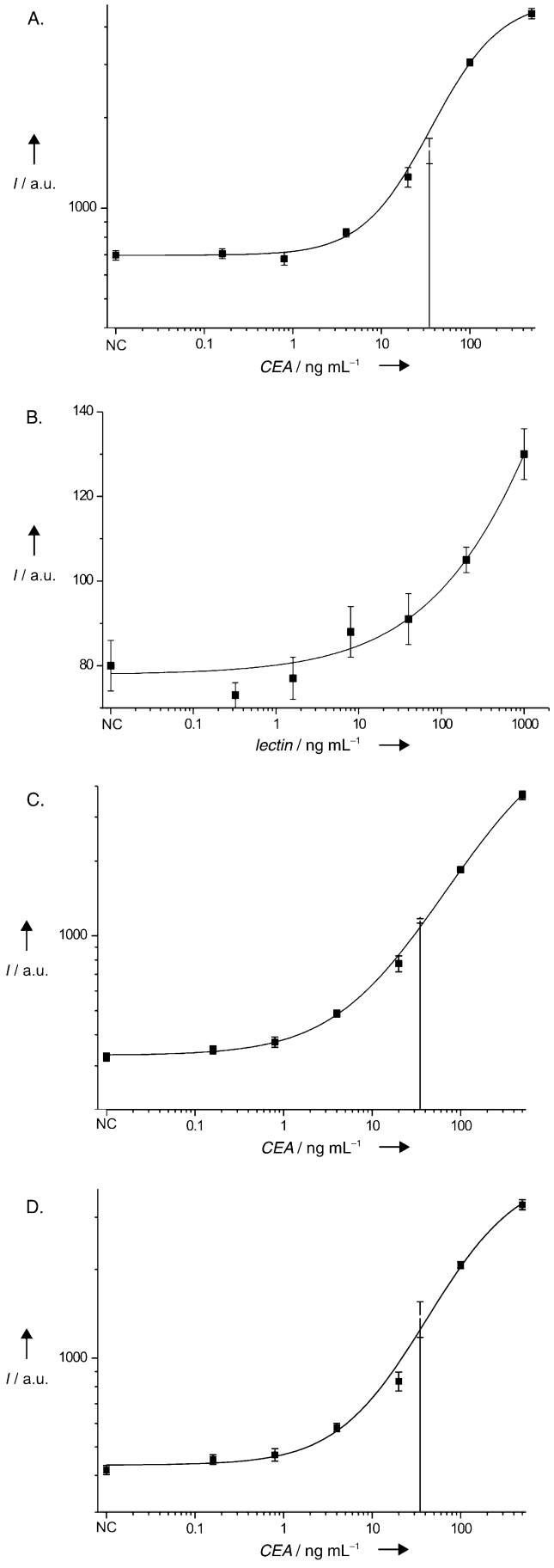

obtained, for instance, in the analysis of patient samples or in the generation of calibration data with various batches of microarray slides.

We further investigated the reproducibility of the DDI-μFIA by carrying out the assay described in Figure 3 in duplicate independent experiments with two DNA microarray slides (gray and dark lines in Figure 5A). Both slides contained each capture oligomer as 12 separated spots, thus allowing for the determination of standard deviations (error bars of either the gray and dark lines in Figure 5A). The average standard deviation obtained on a single slide was found to be about 4%, demonstrating the high reproducibility of the DDI-μFIA, while interassay deviations between the two slides were found to be 10%, thus indicating the problems accompanying microarray-based analyses. After normalization, the average standard deviation between the two slides was significantly improved to about 6% and 5%, by using either C1A (Figure 5B) or of CEP (Figure 5C) signals as an internal standard.

The above results clearly demonstrate the effectiveness of internal standardization for interassay correction. Moreover, the outstanding reproducibility observed here for the DDI-μFIA confirms our earlier studies on the immobilization of antibodies by DNA hybridization, which has already demonstrated the high performance of this self-assembly technique.[11, 13] We reason that the good performance of the DDI-μFIA observed here is due, in addition, to the high quality of the dendrimer-coated microarray slides, which have recently been evaluated in a competitive study by others.[8]

One major advantage of DDI-based immunoassays is the highly specific immobilization, mediated by the base-pairing of complementary nucleic acids. This feature allows simultaneous immobilization of many different DNA-tagged components site-specifically on the DNA microarray. Consequently, DDI-based immunoassays allow for the in-solution binding of antigen targets by DNA-tagged antibodies and subsequent site-specific capture of the immunocomplexes formed on the DNA-microarray. The advantage of this approach lies in the reduction of the number of incubation steps, and thus the minimization of time and costs of analyses. While we have recently demonstrated the feasibility of this capture approach for a single immunocomplex,[13] we have extended this demonstration here to a multiplex DDI-μFIA assay.

To investigate the multiplex DDI-capture assay, CEA quantification was carried out in the presence of C1A or CEP and the absence of rVis, by using the antibody conjugates described in Figure 2. Serial dilutions of CEA in BISEKO were mixed with equal amounts of the four capture conjugates—**A**-RAC, **B**-SAC, **C**-SCI, and **D**-GAL (25 nM each)—as well as the four Cy5-labeled

Figure 4. *Hybridization signals obtained in DDI experiments. Shown are the fluorescence signal intensities obtained from detection of various amounts of CEA and rVIS, described in the legend of Figure 1. A) Raw data obtained from fluorescence quantification of CEA signals. The grey bar represents a spiked sample containing 35 ng mL^{-1} of CEA, measured on a different slide. B) Raw data obtained from fluorescence quantification of rVis signals. C) Signal intensities of CEA after normalization by use of the data obtained from either C1A or CEP (D). Note the improvement in the limits of detection and spike recovery rates.*

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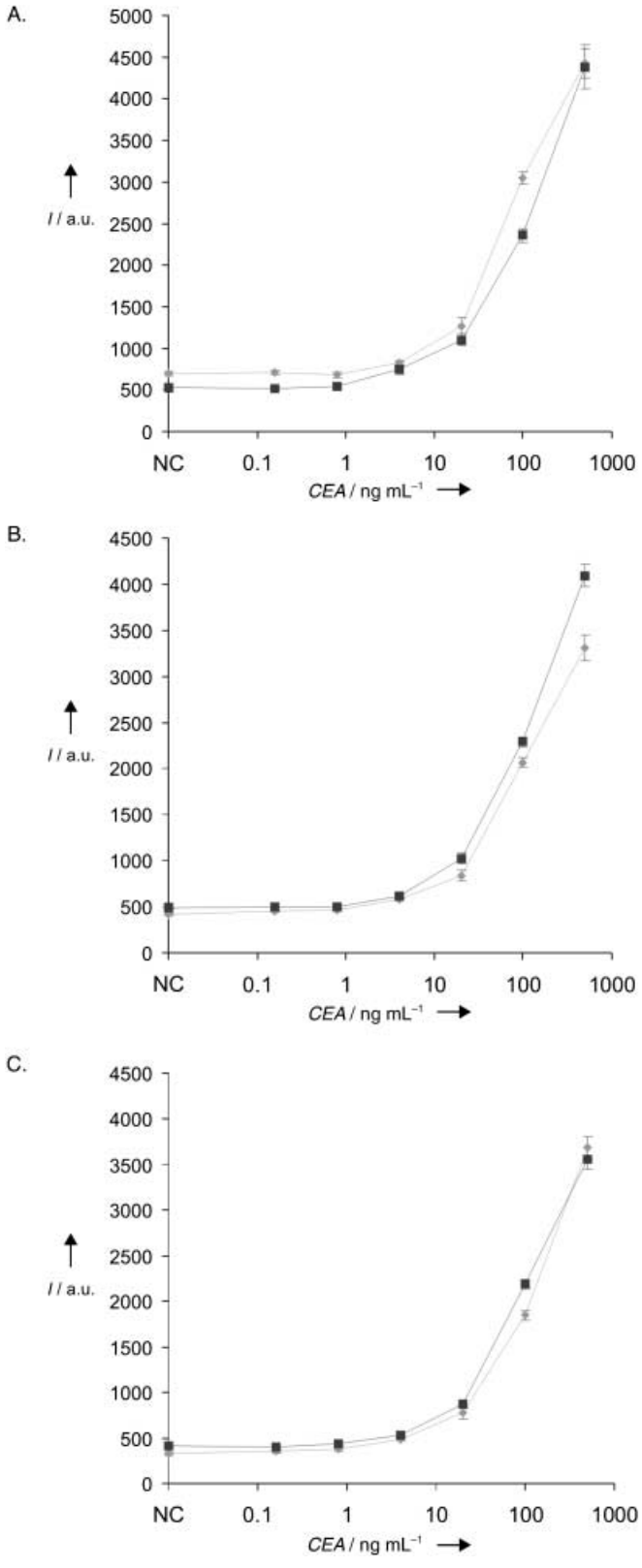

Figure 5. *Hybridization signals obtained in DDI-μFIA experiments for the detection of CEA. Shown are the fluorescence signal intensities of two independent experiments, carried out on different microarray slides. A) Raw data of fluorescence signals, indicating an inter-array deviation of about 10%. B) Signal intensities of CEA after normalization by use of the signals obtained from C1A or CEP (C). Note that the normalization improves the standard deviation observed for the raw data.*

detection conjugates (25 nM each). The mixtures were immediately applied to DNA microarrays, thus allowing for the capture of the immunocomplexes formed in solution. The fluorescence signal intensities obtained are shown in Figure 6 (raw data). The limit of detection for CEA was 10 ng mL^{-1}, as compared to 3 ng mL^{-1}, obtained in the conventional three-step assay (see Figure 4A). This indicates that the DDI-capture assay is less sensitive, but the assay time was reduced from three incubation steps to a single one. Normalization of the CEA signals with those obtained from either C1A or CEP (gray and light gray curves, respectively, in Figure 6) led to a roughly threefold improvement in the limit of detection to about 3 ng mL^{-1} of CEA. To the best of our knowledge, this is the first demonstration of a functional multiplex one-step capture sandwich assay.

Conclusion

We report here on the development of a readily configurable multiplex immunoassay, based on the DNA-directed self-assembly of protein microarrays. Due to the recognition capabilities of covalent single-stranded DNA – STV conjugates, which are used as versatile molecular adapters for the efficient and site-specific DNA-directed immobilization of capture antibodies on DNA-microarrays, this microarray fluorescence immunoassay (μFIA) is readily configurable for a wide variety of applications. We demonstrate here, in a model system, the simultaneous detection and quantification of four different protein antigens—the tumor marker human carcinoembryonic antigen (CEA), recombinant mistletoe lectin rViscumin (rVis), ceruloplasmin (CEP), and complement-1-inactivator (C1A)—in human blood serum samples. The DDI-μFIA allowed us to detect low antigen quantities down to 400 pg mL^{-1}, which is comparable to the sensitivity reached in conventional microplate-based enzyme-amplified immunoassays. Moreover, this assay also revealed an extraordinary robustness and reproducibility, which could even be improved by an internal standardization employing proteins, CEP or C1A, which intrinsically occur in human blood serum. In particular, this normalization allows one to compensate for interassay deviations resulting from the use of various batches of microarray slides. Furthermore, we have also demonstrated that the DDI-μFIA can be carried out in a single step, taking advantage of the specificity of DNA-directed immobilization of immunocomplexes formed in solution, thereby significantly reducing the time and costs of analyses.

Experimental Section

Preparation of DNA – protein conjugates: The synthesis and purification of the four covalent DNA – STV conjugates (**HA** – **HD** in Figure 2) was carried out from the corresponding thiolated oligonucleotides (Table 1, Thermo Electron), as described previously.[12] In brief, STV (10 nmol) was derivatized with maleimido groups by use of a heterobispecific crosslinker (sulfo-SMCC, Pierce), treated with the thiolated oligonucleotide (10 nmol) and subsequently purified by anion-exchange chromatography. The one-to-one molar ratio of oligonucleotide and protein moiety in the conjugate was verified by

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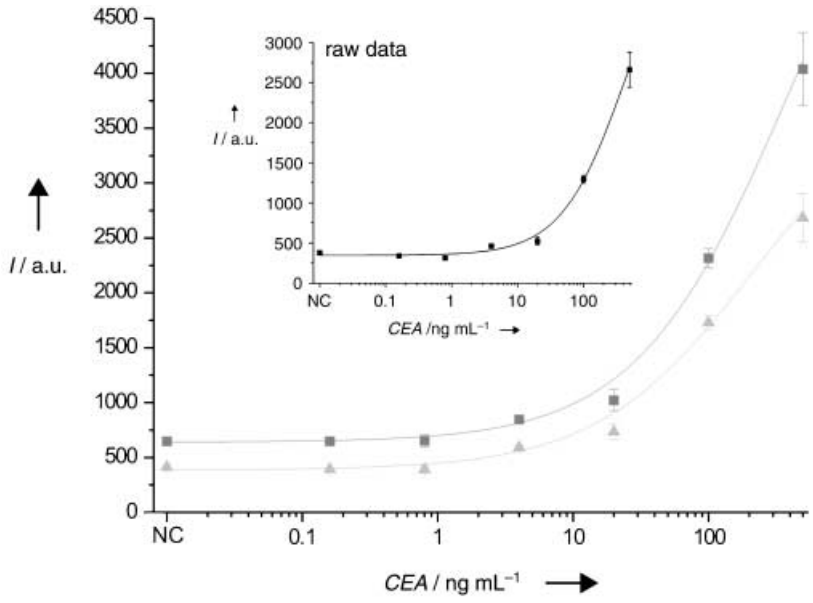

Figure 6. *Hybridization signals obtained in the one-step capture DDI-μFIA, schematically depicted in Figure 1 B. Shown are the fluorescence signal intensities obtained from detection of various amounts of CEA. While the raw data indicate a detection limit of about 10 ng mL^{-1} of CEA, normalization with C1 A (grey squares) or CEP (light gray triangles) improves the detection limit to about 3 ng mL^{-1} CEA.*

gel-electrophoretic and photometric analysis, and its concentration was determined by absorbance measurements.[12]

As shown in Figure 2 B, the adducts of the four conjugates (**HA**, **HB**, **HC** and **HD**) and the biotinylated antibodies were prepared by mixing 0.01 mM stock solutions of the conjugate and equimolar amounts (0.01 mM stock solution) of biotinylated rabbit anti-carcinoembryonic antigen RAC (Dako, biotinylated with NHS-Biotin (Pierce) according to manufacturers' instructions), biotinylated goat anti-lectin GAL (Viscum AG),[14] biotinylated sheep anti-complement-1-inactivator SCI (Biotrend, biotinylated with NHS-Biotin (Pierce)) or biotinylated sheep anti-ceruloplasmin SAC (Biotrend, biotinylated with NHS-Biotin) in buffer A (10 mM Tris buffer, pH 7.5, containing 5 mM EDTA). After incubation for 15 min at room temperature, the mixtures were diluted to 500 nM with buffer B (20 mM Tris-Cl buffer, pH 7.5, 150 mM NaCl, 5 mM EDTA, 0.01 % (w/v) Tween-20, 0.1 mg mL^{-1} reagent grade DNA (Roche), 800 μM D-biotin (Sigma) and 0.5 % milk powder (Oxoid)) and incubated for an additional 10 min. After this, the capture conjugates were mixed together to a final concentration of 25 nM each for the three-step assay, or 125 nM each for the one-step assay.

For fluorescence detection, conjugates were prepared from streptavidin covalently labeled with Cy5 fluorophore (STV – Cy5, Roche) and biotinylated antibodies RAC, GAL, SCI, SAC (Figure 2-C). To this end, 0.01 mM stock solutions of the streptavidin – Cy5 and equimolar amounts (0.01 mM stock solution) of biotinylated antibodies were mixed in buffer A, and after incubation for 15 min at room temperature, the mixtures were diluted to 500 nM with buffer B and incubated for an additional 10 minutes. Afterwards, the four STV – Cy5 – antibody conjugates were combined. The concentration of the detection conjugates was 25 nM each in the three-step and 125 nM each in the one-step assay.

Preparation of DNA microarrays: For the attachment of the capture oligonucleotides complementary to **HA** – **HD** (Table 1), a solution of the 5′-amino-modified oligonucleotides in water (0.3 nL, 10 μM, Thermo Electron) was typically spotted onto 3DProtein Slides (Chimera Biotec, Dortmund) by using a piezo-driven spotting device (GeSiM), and the slides were incubated overnight. Subsequently, the slides were stored at − 20 °C until use.

DDI and μFIA: To reduce non-specific binding of the reagents, the DNA microarray was pretreated for 30 min with blocking solution (Chimera Biotec GmbH) and subsequently dried by centrifugation with the aid of a microscope slide holder (Erie Scientific). Adhesive hybridization chambers (Schleicher & Schuell) were fixed on top of the slide, and the DNA – antibody conjugate solution described above was allowed to hybridize for 120 min at room temperature (Figure 2 D). The slide was washed twice with buffer C (20 mM Tris-Cl buffer, pH 7.5, 150 mM NaCl, 5 mM EDTA, 0.01 % (w/v) Tween-20), and serial dilutions of the antigens were applied. The antigens used were carcinoembryonic antigen (CEA, DAKO), typically ranging from 500 ng mL^{-1} to 0.16 ng mL^{-1}, and recombinant mistletoe lectin rViscumin (rVis, Viscum AG), typically ranging from 1000 ng mL^{-1} to 0.32 ng mL^{-1}, diluted in standardized human serum (BISEKO), containing 170 μg mL^{-1} C1 A and 90 μg mL^{-1} CEP. C1 A and CEP were also used as internal standards. The slide was incubated for 60 minutes at room temperature. After antigen incubation, the slide was washed twice with buffer C and the detection-conjugate mixture was transferred into the hybridization chambers. Binding was carried out at room temperature over 60 min.

For the one-step assay, the DNA – antibody conjugates and the STV – Cy5 – antibody conjugates (final concentrations of 25 nM each) were added directly to the antigen samples containing CEA in serial dilutions. The mixture was incubated for 120 minutes on top of the slide.

After reagent binding, the chips were washed twice for 5 min with buffer C, followed once in double distilled H_2O. The slides were dried by centrifugation, and the fluorescence intensity of the signals was measured with a microarray laser scanning system (Axon) with a 500 photomultiplier and 100 % laser power. Signals were analyzed and quantified with the aid of GenePix pro 4.1 software (Axon).

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Keywords: analytical methods · antibodies · DNA – protein conjugates · microarrays · nucleic acids · supramolecular chemistry

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